

DEVELOPMENT OF METHODS FOR
ELECTROSTATIC IMMOBILIZATION AND
COUPLING OF DIFFERENT MICROBIAL
CELLS

Iaroslav Rybkin

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Asst. Prof. Dr. Aleš Lapanje, Jožef Stefan Institute and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia

Co-Supervisor: Asst. Prof. Dr. Tomaž Rijavec, Jožef Stefan Institute and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia

Evaluation Board:

Prof. Dr. Milena Horvat, Chair, Jožef Stefan Institute and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia

Asst. Prof. Dr. Rinke J. van Tatenhove-Pel, Member, Delft University of Technology, Delft, the Netherlands

Prof. Dr. Cornelius Fischer, Member, Helmholtz-Zentrum Dresden-Rossendorf, Leipzig, Germany

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



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RAZVOJ METOD ZA ELEKTROSTATSKO
IMOBILIZACIJO IN POVEZOVANJE RAZLIČNIH
MIKROBNIH CELIC
Doktorska disertacija

Supervisor: Asst. Prof. Dr. Aleš Lapanje

Co-Supervisor: Asst. Prof. Dr. Tomaž Rijavec

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To my family

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Abstract

Naturally formed microbial communities are present in the form of cellular aggregates that create a set of diverse biochemical pathways through cross-feeding between the members of the microbial community. One of the most important factors so that some biochemical reactions can occur is the appropriate spatial placement of a particular bacterium with its metabolic capability, which results in the formation of closed biogeochemical cycles that increase metabolic efficiency and robustness of the community. Even though many bacteria have a set of optimized metabolic traits, the absence of the initial conditions, which promote the attachment of compatible bacteria, prevents them from forming closed biochemical cycles. If we provide the conditions to first, couple the cells together, and second, make them compatible, we can assemble different bacteria in flocs, similarly as it occurs in nature. Since bacteria are charged, it allows us to alter their cell charge using electrostatic deposition of oppositely charged polyelectrolyte (PE). As a result, oppositely charged bacterial cells can be attached to each other or to the surface of a material merely by electrostatic interaction. Therefore, to be able to simulate the naturally aggregated cellular structures, we aimed to develop a method to modify the bacterial cell surface using the strategy of PE deposition. We used it to study the biological responses caused by the changes in the properties of the cell surface and the physical confinement of cells. Knowledge of biological responses can be further used to our advantage. The construction of artificial aggregated structures allows us to carry out specific biogeochemical cycles in applicative setups for bioremediation, biotechnology or biomedicine.

The main goal in our study was to prepare the approach for the deposition of tailor-made PE coatings on the surface of a bacterial cell, which altered the cell surface charge, controlling the time of the first cellular division and the formation of cell aggregation. The thickness of the coating determined the delay of cell division and the increase in cell size and the production of Green Fluorescent Protein (GFP) up to 2 and 5 times, respectively. In some cases, the introduction of the PE coating led to a decrease in cell viability, therefore, we selected two strains, based on their resistance to the PEs, to assess the effect of PEs on cell viability and to find the conditions that would not reduce it. The toxicity of PEs was found to be dependent firstly, on the strain, and secondly, on the conditions of exposure to the PEs, which was manifested by different outcomes in viability. In order to improve cell viability, we used two approaches: (i) using a decreased PE concentration and (ii) using acetylation to reduce the positive PE, which both enabled us to safely use the PEs for various bacterial strains. We also extended the applicative potential of our method by combining it with graphene and paramagnetic nanoparticles (NPs).

In conclusion, we prepared a versatile method for the preparation of artificial aggregates based on the electrostatic method, which allows us to study the physiology of bacterial cells and to produce artificial cellular structures with potential for biotechnological, biomedical and environmental solutions.

Povzetek

Naravno oblikovane mikrobne združbe so prisotne v obliki celičnih agregatov, ki z navzkrižnim izmenjevanjem metabolitov med člani združbe ustvarjajo niz različnih biokemičnih poti. Eden najpomembnejših dejavnikov, da se določene biokemične poti med bakterijami vzpostavijo, je ustrezna prostorska umestitev določene bakterije z njeno presnovno sposobnostjo znotraj bakterijske združbe. Nastanek zaprtih biogeokemičnih ciklov poveča presovno učinkovitost in robustnost celotne združbe. Čeprav imajo številne bakterije niz optimiziranih presovnih lastnosti, odsotnost začetnih pogojev, ki spodbujajo vezavo kompatibilnih bakterij, preprečuje, da bi te tvorile zaprte biokemične cikle. Če zagotovimo primerne pogoje na način, da najprej omogočimo združevanje celic med seboj in nato združimo skupaj najbolj kompatibilne, lahko sestavimo različne bakterije v skupke, podobno kot se to dogaja v naravi. Ker so bakterijske celice na površini nabite, lahko ta naboj spreminjamo z elektrostatičnim nanašanjem nasprotno nabitega polielektrolita (PE). Posledično se lahko nasprotno nabite bakterijske celice pritrdijo med seboj ali na površino materiala zgolj z elektrostatično interakcijo. Za simulacijo naravno združenih celičnih struktur smo želeli razviti metodo za spreminjanje površine bakterijske celice s pomočjo strategije odlaganja PE. Z njim smo preučevali biološke odzive, ki jih povzročajo spremembe lastnosti celične površine in fizična ujetost celic. Znanje o bioloških odzivih lahko tako uporabimo v svojo korist. Konstrukcija umetnih agregatnih struktur nam omogoča izvajanje posebnih biogeokemičnih ciklov v aplikativnih rešitvah na področjih bioremediacije, biotehnologije ali biomedicine.

Z našo raziskavo smo razvili pristop za nanašanje PE na površino bakterijske celice, s čimer smo spremenili naboj celične površine, nadzorovali čas prve celične delitve in vplivali na nastanek celične agregacije. Debelina prevleke je določila zakasnitev delitve celic in povečanje velikosti celic ter proizvodnjo zelenih fluorescentnih beljakovin (GFP) do 2 -krat oziroma 5 -krat. V nekaterih primerih je uvedba premaza PE povzročila zmanjšanje sposobnosti preživetja celic, zato smo na podlagi njihove odpornosti proti PE izbrali dva seva, da bi ocenili učinek PE na sposobnost preživetja celic in našli pogoje, ki niso zmanjšali preživetja. Ugotovili smo, da je toksičnost PE vezana na bakterijski sev in na pogoje izpostavljenosti PE, kar se je pokazalo z različnimi sposobnostmi preživetja bakterij. Za izboljšanje sposobnosti preživetja celic smo uporabili dva pristopa: (i) uporabo zmanjšane koncentracije PE ali (ii) uporabo acetilacije za zmanjšanje pozitivnega naboja PE, kar nam je omogočilo varno uporabo PE za vse bakterijske seve. Razširili smo tudi uporabni potencial naše metode, tako da smo jo združili z nanašanjem grafenskih in paramagnetnih nanodelcev (NP).

Metoda, ki smo jo razvili z raziskavo deluje na osnovi elektrostatične interakcije med delci, je vsestranska za pripravo umetnih skupkov, ki imajo različne presovne sposobnosti in nam omogoča preučevanje fiziologije bakterijskih celic in izdelavo umetnih celičnih struktur s potencialom za biotehnološke, biomedicinske in okoljske rešitve.

Contents

List of Figures	xv
Abbreviations	xvii
1 Introduction	1
1.1 Conventional Approaches for Confinement of Cellular Biomass.....	4
1.1.1 Matrix entrapment	4
1.1.2 Limitations of nutrient transport and difficulties in precise orientation of cells during biomass entrapment	4
1.1.3 The effect of confinement on physiology of cells	5
1.2 Confinement of Individual Cells Based on Electrostatic Interaction.....	6
1.2.1 Application of positively charged polyelectrolytes to bacterial cells shows different outcomes in terms of viability.....	8
1.2.2 Differences in the Structure of the Cell Membrane Might Affect Toxicity of Polyelectrolytes	9
1.2.3 Challenges in preparation of Layer-by-Layer assembled capsules on the surface of individual living cells.....	10
1.3 Biological Traits Needed for Preparation of Multispecies Bacterial Entities	11
1.4 Incorporation of Nanoparticles into Polyelectrolyte Layers	14
1.4.1 Application of graphene nanoparticles.....	14
1.4.2 Application of magnetic nanoparticles for guiding bacterial cells.....	15
2 Aims and Hypotheses	17
2.1 Introduction to Hypotheses.....	17
2.2 Research Aims	19
2.3 Hypotheses.....	20
3 Scientific Publications	23
3.1 Thickness of Polyelectrolyte Layers of Separately Confined Bacteria Alters Key Physiological Parameters on a Single Cell Level.....	24
3.1.1 Method for synthesis of protein in culture of bacterial cells.....	39
3.2 Strain-dependent Selective Antimicrobial Action of Cationic Polyelectrolytes on Gram-negative Bacteria	47
3.3 When Beneficial Biofilm on Materials Is Needed: Electrostatic Attachment of Living Bacterial Cells Induces Biofilm Formation	81
3.4 Medium-Dependent Antibacterial Properties and Bacterial Filtration Ability of Reduced Graphene Oxide	97
3.5 Effect of GO on Bacterial Cells: Role of the Medium Type and Electrostatic Interactions	111
3.6 <i>In vivo</i> Capture of Bacterial Cells by Remote Guiding.....	119
4 Discussion and Summary	145

5	Conclusions	151
	References	153
	Bibliography	167
	Biography	171

List of Figures

Figure 1.1: Preparation of cell aggregates.....	3
Figure 1.2: Key limitations and advantages for preparing coated cells.....	8

Abbreviations

CFU	...	Colony Forming Units
EPS	...	Extracellular Polymeric Substances
GMO	...	Genetically Modified Organisms
GO	...	Graphene Oxide
GFP	...	Green Fluorescent Protein
ppGpp	...	Guanosine Pentaphosphate
LbL	...	Layer-by-Layer
LPS	...	Lipopolysaccharide
MIC	...	Minimal Inhibitory Test
NPs	...	Nanoparticles
OD	...	Optical Density
PAH	...	Poly(allylamine) hydrochloride
PEI	...	Poly(ethylenimine)
PE	...	Polyelectrolyte
rGO	...	Reduced Graphene Oxide
SEM	...	Scanning Electron Microscopy
STED	...	Stimulated Emission Depletion Microscopy
TLCM	...	Time Lapse Laser Confocal Microscopy

Chapter 1

Introduction

There is a growing demand for using environmentally friendly and sustainable technologies that should decrease the impact on the environment. Currently, the most promising cases to reach sustainability are based on the use of indigenous or synthetic consortia of bacteria in setups of oil bioremediation (Brune & Bayer, 2012; Shankar et al., 2014), pesticide degradation (Jariyal, Jindal, Mandal, Gupta, & Singh, 2018), wastewater treatment (Hassan et al., 2018) and production of biochemicals (Bhatia et al., 2018). The use of consortia is also beneficial for microbes that are part of consortia as it increases metabolic stability and robustness. The naturally occurring microbes are environmentally safe and active against specific contaminants, originating from industrial plants. However, such solutions can be even more efficient and long-lasting if we structure the consortia in the desired manner and increase its stability, which is currently problematic (Bhatia et al., 2018).

In order to increase the efficiency of the cell consortia, the introduction of genetic elements could be used. However, for many microbial metabolic processes that occur in nature, the use of Genetically Modified Organisms (GMO) is not suitable as genetic elements have associated antibiotic resistant genes, which can be easily transferred by other organisms, spreading the genes for drug resistance in the environment (Hoque & Fritscher, 2019). In addition, the use of GMOs also presents several drawbacks, such as (i) the choice of metabolic reactions is limited by the plasmid size, which usually does not exceed 1-2% of the total genome (Norman et al., 2014; Shintani, Sanchez, & Kimbara, 2015), (ii) the stability of genetic elements is problematic in many cases since the gene source and acceptor might be very different, which leads to lower compatibility of the inserts, and (iii) it is hard to keep selection pressure on plasmids as metabolic pathways of plasmids are not important for survivability of modified strains, which makes the plasmid not energetically favorable for the replication.

Rather than using GMO, we envision a solution that would enable to form a united structured community composed of different species through: (i) the appropriate spatial arrangement of cells for closing biogeochemical cycles and minimizing their energy, (ii) the confinement of cells for keeping the obtained consortia ordered, and (iii) the use of different bacteria to intensify and balance compatibility. In addition, our idea can help to solve GMO limitations, as the genes are used according to the local environment and close distance, meaning it would be possible to maximize the use and replication of these inserts if we initially order cells in consortia.

Bacterial communities in nature form a variety of diverse metabolic pathways through the interaction of their members, which leads to the development of sustainable biological cycles. Such joint communities are present in the environment as multispecies aggregates of flocs, biofilms, mats or granules. Within the biofilms bacteria are arranged to be

compatible with other microbes, which makes them energy effective and metabolically robust (Freilich et al., 2011; Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016). Their coexisting results in the formation of specific niches by exposing various receptors to promote surface interaction, modulating ecological niches (by changing pH or oxygen), or providing metabolites to selectively choose cells that enter the biofilm (Bucci, Nadell, & Xavier, 2011; Jakubovics, 2015; Stadler, Wei, Fürbeth, Grooters, & Kukliński, 2010). Usually bacteria are entrapped in polysaccharide matrices that protect the cells from the external influence and facilitate nutrient and signal exchange within these aggregates (Dragoš & Kovács, 2017). In such a matrix, the cells are cross-feeding each other, living on “waste” products of the metabolic partners. We can envision that such compact and isolated entities composed of different bacterial cells already have a wide range of chemical reactions, which are well optimized for the particular biological pathway. Probably, by a simple rearrangement and positioning of cells, it would allow us to create unique metabolic pathways that can be re-designed for each specific task. Additionally, if we specifically arrange the cells, we would be able to control various conditions as distribution of aerobic and anaerobic cells, their ability to attach and to form a biofilm. Therefore, if we want to make a viable system, which is based on combining different microbial cells with specific metabolic traits, we would need to provide them with such conditions as they occur in nature.

However, most cells do not tend to interact very well since (i) they might compete (Nadell, Drescher, & Foster, 2016), (ii) they cannot attach on the surface of other cells (Rendueles & Ghigo, 2015), or (iii) perhaps they need very defined conditions to promote the formation of multispecies structures, which they do not have. Therefore, it is expected that the solution to force cells to form multicellular structures should require using strategies for surface modification, which would physically enable the attachment of one cell to another. The closest strategies to above mentioned criteria for coupling cells together are based on confinement of cells in different matrices. Although physical attachment should be relatively simple since cells can be merely embedded in matrices, it requires careful management of bacteria in order to keep the activity of culture and to build a compatible system for preparation of cell-to-cell couples (Balkundi, Veerabadrán, Eby, Johnson, & Lvov, 2009). Since physical matrices have lowered diffusion due to gel-like structure (Tanaka, Matsumura, & Veliky, 1984) and do not enable constant spatial distribution (Allan-Wojtas, Hansen, & Paulson, 2008), they might not be appropriate for precise physical positioning of cells. In contrast, the most suitable approach of confinement would be when a cell is attached to another cell with some sort of glue, which would not limit diffusion of metabolites, but would firmly keep cells together. In relation to that, the strategy that enables merely electrostatic interaction and as a result, simple physical coupling of cells, can be a Layer-by-Layer (LbL) approach based on altering cell surface charge by deposition of the charged PEs (Hillberg & Tabrizian, 2006). The concept of the preparation of such aggregates is presented in Figure 1.1.

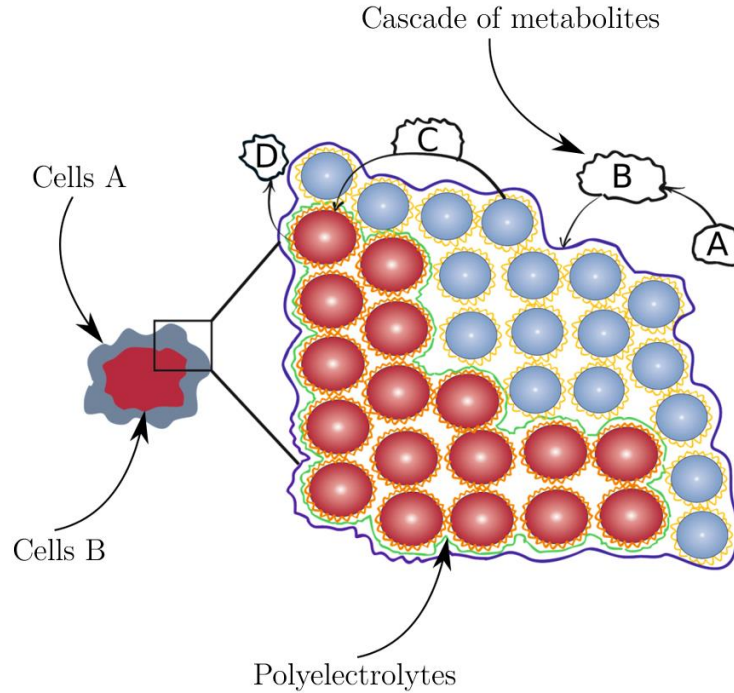


Figure 1.1: Preparation of cell aggregates.

However, any sort of confinement and manipulations with cells based on electrostatic interaction are expected to affect the physiology of bacteria, as it inevitably alters surface charge and creates a physical barrier. Therefore, it can affect the attachment, the growth or the nutrient transport. Since the approach is based on using electrostatically attractive charges, we can expect that sometimes it can lead to a change in viability, since not all bacteria can tolerate positively charged molecules on the membrane. Moreover, it would be hard to specifically coat a single cell, preventing other bacteria from random co-aggregation, keeping a high precision of the consortia.

It is thus needed to summarize available findings and carefully redesign or develop the optimal solution to uniformly coat the bacteria and to provide them the most suitable growth conditions.

Therefore, the main purpose of this work was to elaborate approaches that would allow bacteria to be manipulated in such a way that they can be arranged in the form of single cells or precise aggregates, not reducing viability. To achieve this purpose, we aimed to study different aspects of the cell physiology and physicochemical characteristics of such systems. We also aimed to show several proofs of concepts that the use of confinement of bacteria might be extremely beneficial for biotechnological as well as medical applications.

Since the method for preparing the aggregated cells based on electrostatics would be a multi-step process, it is also needed to evaluate the developed solutions and to specify the problems that arise from various steps. We would also need to consider potential benefits or limitations of the conventional methods as it can help us either to use the most promising parts or to avoid the most problematic ones for preparing our solution.

1.1 Conventional Approaches for Confinement of Cellular Biomass

1.1.1 Matrix entrapment

The application of confinement matrices is an integral part of biotechnological processes since, on the one hand, it allows to protect embedded cells from physical influence, and on the other hand, it facilitates the product separation from the bacterial culture (Bayat, Hassanshahian, & Cappello, 2015). In some cases, the immobilization of cells can increase metabolic stability and reduce competition between bacteria (Lu et al., 2020). The immobilization approach found wide application in development of sustainable agriculture (Ajeng et al., 2020), bioremediation (Gentili, Cubitto, Ferrero, & Rodríguez, 2006) and wastewater treatment (Covarrubias, de-Bashan, Moreno, & Bashan, 2012; Horemans et al., 2017).

The confinement requires the use of matrices that can keep biomass attached to the surface of the matrix. Matrices can be composed of various biocompatible and biodegradable components as natural polysaccharides, proteins, polypeptides, lipids or synthetic polymers (Martín, Lara-Villoslada, Ruiz, & Morales, 2015; Vemmer & Patel, 2013). The application of matrices increases mechanical stability and, in the meantime, protects bacteria from colonization by other microbes (Covarrubias et al., 2012). Moreover, confined cells are protected from the external abiotic influence, surviving even at a very low pH (Ding & Shah, 2009) or tolerating changes in temperature or mechanical stress (Abbaszadeh, Gandomi, Misaghi, Bokaei, & Noori, 2014; Vemmer & Patel, 2013). When conditions become suitable for growth, bacteria can be safely released from the confinement matrix, keeping their metabolic activity (Mirtič et al., 2018). To increase mechanical stability, bacteria can also be absorbed onto various natural porous non-degradable carriers that are later entrapped in the matrix. Carriers are especially needed when prepared setups are used in environmental conditions for bioremediation or wastewater processing as they might reduce shear stress, grazing of bacteria by protozoa (Horemans et al., 2017) as well as flushing cells out of the system (Bayat et al., 2015). However, in many cases, bacteria can be immobilized in a matrix and fabricated without any solid support, which is still sufficient to preserve activity of the culture (Kailasapathy, 2002). Therefore, the choice of matrices for the cell immobilization is designed according to conditions where the setup is going to be applied.

1.1.2 Limitations of nutrient transport and difficulties in precise orientation of cells during biomass entrapment

Current approaches for the fabrication of confined cells are based on various techniques as encapsulation, extrusions, emulsions and microfluidics, cell immobilization, electrospinning and others (Alkayyali, Cameron, Haltli, Kerr, & Ahmadi, 2019; Kailasapathy, 2002; Vidhyalakshmi, Bhakayaraj, & Subhasree, 2009). By using a combination of different approaches for immobilization and selecting different matrices with carriers, it allows to design specific properties of the matrices and to prepare confined cells in the optimal way (Martín et al., 2015).

However, despite the wide use of such approaches they face several limitations when bacteria are entrapped in the matrices as (i) limited diffusion of the nutrients, (ii) low accuracy of the spatial organization, (iii) lowered viability of cells, (iv) degradation of matrices, (v) low strength of matrices, and (vi) cells cannot be attached directly onto the surfaces. Since the applied matrices have a gel-like structure, the nutrient flow depends on

the thickness and concentration of the matrix material, its charge and size of pores (Idris & Suzana, 2006). Although compounds with a low molecular weight (up to $2 \cdot 10^4$ Da) have been shown to freely pass through the matrix pores, diffusion of compounds with a high molecular weight ($6,9 \cdot 10^4$ Da and $1,5 - 3,4 \cdot 10^4$ Da) is restricted (Tanaka et al., 1984), which decreases metabolic choice of products or nutrients for this system.

Several studies have shown that cells are randomly distributed within the beads, making it difficult to precisely arrange bacteria in spatial structures (Gbassi, Vandamme, Ennahar, & Marchioni, 2009; Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011; Zohar-Perez, Chet, & Nussinovitch, 2004). Since it is hard to disperse cells in a very precise and separate manner, we expect the formation of cellular aggregates, which might deplete oxygen locally. A decrease in the oxygen inward within the matrices can lead to a significant reduction in the viability of aggregated bacteria (Orive, Hernandez, Gascon, Igartua, & Pedraz, 2003). Viability can be improved either by entrapment of bacteria in smaller beads to facilitate diffusion of oxygen and nutrients or cells have to be well dispersed to prevent aggregation. However, dispersion of cells is clearly not the case, especially when aggregates need to be prepared (e.g. formation of biofilms or flocs).

Since matrices also have elastic properties, depending on the matrix composition, the Young's modulus of the beads ranges between of 5-1000 kPa, while tensile strength is between 55 to 1900 kPa (Chan et al., 2011; Distler et al., 2021; Ramdhan, Ching, Prakash, & Bhandari, 2020). Although, the values of the Young's modulus can be significantly increased up to 77 MPa (Ramdhan et al., 2020), an increase from 5 kPa to 10 kPa can lead to an approximately twofold decrease in the diffusion of 1 kDa size compounds (Capone et al., 2013). In some cases, confinement with low diffusion was shown to artificially induce quorum sensing at the single cell level since such matrix does not enable proper diffusion of metabolites from cells (Carnes et al., 2010).

1.1.3 The effect of confinement on physiology of cells

In fact, all cells are experiencing a situation of confinement within natural extracellular matrices. Bacteria get confined when they accumulate at interfaces to form polymicrobial aggregates such as films, mats, flocs, sludge, granules or 'biofilms'. In addition, most bacteria in soil and bedrock live in pores of 6 μ m size or smaller (Ranjard & Richaume, 2001), and during host infection the bacteria are constrained in the confined space of soft tissues and bones or inside artificially implanted medical devices.

It is very obvious that the confinement matrix surrounding cells represents a barrier for diffusion of substances in both directions, either towards or from the cells. This diffusion barrier can be beneficial, on the one hand, for bacteria when exposed to antimicrobial acting substances (Tseng et al., 2013), or, on the other hand, disadvantageous since it reduces the diffusion rate of nutrients and gasses (McLean et al., 2008; Stewart et al., 2016). As a result, conditions of limited diffusion or nutrient sink caused by cells both enable the formation of various niches with specific physicochemical conditions within biofilms.

Another important property of the confinement matrix is that it provides a mechanical barrier. The matrix can prevent bacteria to get detached from the surface under very strong flow conditions (Abe, Polyakov, Skali-Lami, & Francius, 2011), or mechanically disable the invasion of bacteria from outside into biofilms (Nadell, Drescher, Wingreen, & Bassler, 2015) and also protects cells from protozoan and macrophage grazing (Matz & Kjelleberg, 2005). Substantial evidence is shown that the same mechanical force that is protecting the cells can also prevent entrapped cells from escaping from the matrix. In such conditions within the matrix and narrow constrictions bacterial cells adapt to mechanical strengths by changing their shape (Amir, Babaeipour, McIntosh, Nelson, & Jun, 2014) and radius as

they generate forces by growth and division that compel themselves through the constrictions and pores (Männik, Driessen, Galajda, Keymer, & Dekker, 2009).

From another perspective, one can hypothesize that the force that the cells need to struggle during growth should counteract the growth of the cells and reduce their growth speed and division. However, due to the super-positioning of mechanical effects of confinement with the effects of restrictions of the diffusion, it is currently not possible to study these two effects separately. For example, if we study mechanical effects on cell growth by exposing bacterial cells to elevated hydrostatic pressure, it will increase the solubility of gasses (Ishii, Sato, Wachi, Nagai, & Kato, 2004; Kato, Sato, & Horikoshi, 1995). If we use a microfluidic chamber, it will decrease the availability of nutrients between different cells either by the restriction of diffusion through the chamber walls or through the uptake of nutrients by the surrounding cells (Burmeister et al., 2019; Stewart et al., 2016).

In contrast, the confinement of bacteria in PE matrices should not reduce diffusion properties in contrast to entrapment in natural or artificial gel matrices. Cells coated in a permeable PE mesh will only experience a physical barrier that opposes cell division but keeps the nutrient flow. Since the Young’s modulus of coatings can be very high (up to 300 MPa), it should create sufficient force, which can even oppose cell division (pressure above 50 MPa can already stall division (Bartlett, 2002)). Thus, we can observe an extreme case, where the coated cells are alive, but mechanically restricted from division.

Although the LbL procedure has been proven to be used in encapsulation of different eukaryotic cells and bacteria (Fakhrullin & Lvov, 2012; Franz et al., 2010; Hillberg & Tabrizian, 2006), LbL layers have not been shown to affect growth and division only by mechanical force. In several studies, researchers have shown a delay in growth of the yeast cells (Kozlowskaya et al., 2011), the cells of *Staphylococcus epidermidis* (Jonas et al., 2018) and *Bacillus subtilis* strain (Balkundi et al., 2009). This delay could be attributed to the toxicity of the procedure of PE deposition, which can lead to viability of a small fraction of cells, resulting in a false prolonged lag phase observed in the measurement of optical densities (OD). Another factor that can cause a delay is the formation of aggregates, resulting in lower diffusion rates of cells kept inside the aggregates and suboptimal growth detected by the measurements of OD.

If we optimize the procedure and prepare a non-toxic coating on a single cell, we should observe the pure mechanical effect of such confinement. We assume cells will pass some stages of biological adaptation to compensate the presence of the coatings. In this case, confinement might change the cell morphology (Ishii et al., 2004; Männik et al., 2009; Yayanos & Pollard, 1969), leading either to expected change in metabolic profiles or to an effect on the population level when cells are forming different microcolonies and biofilms (You, Pearce, & Giomi, 2021). Therefore, we will study the changes in physiology of the cells confined in PE capsules and analyze to which extent the bacteria can oppose the mechanical force of PE capsule in Section 3.1.

1.2 Confinement of Individual Cells Based on Electrostatic Interaction

From a physical point of view, we could avoid problems with diffusion if the matrix represents a porous mesh rather than a gel. For the preparation of such a mesh, we can apply a simple LbL method of consequent PE adsorption. Since most surfaces are charged, in physical experiments inanimate templates were used for deposition of the oppositely charged PEs by electrostatic interaction (Decher, Eckle, Schmitt, & Struth, 1998). The mesh of the PEs can be fabricated either in the form of a film (Decher et al., 1998), or in

the form of microcapsules (Sukhorukov et al., 1998), depending on the type of the template. PEs might be promising gluing materials that could couple and firmly hold different bacteria together. Each pair of PEs contributes only 2-3 nm to the thickness, allowing the preparation of ultra-thin tailor-made coatings (Sukhorukov et al., 1998). Since PE capsules form a porous mesh, 4 kDa molecules (or even up to 70 kDa) can pass unobstructed through the pores under physiological condition (Tong, Dong, Gao, & Möhwald, 2005), maintaining appropriate rates of nutrient and gas exchange in the cells. In contrast to the diffusive properties of gel matrices as alginate or hydrogels, an increase in the thickness of the capsule due to the deposition of more nano-thick layers of PEs does not significantly affect the diffusion of molecules through the coatings (Georgieva, Moya, Donath, & Bäumlér, 2004; Kozlovskaya et al., 2011). Moreover, using the capsule properties, it could help protect bacteria from biotic or abiotic factors by merely physical (e.g. light, magnetic field, ultrasound) or chemical methods (e.g. pH, ionic strength), (Delcea, Möhwald, & Skirtach, 2011; J. H. Park et al., 2014). By selecting the most appropriate PEs, the formed capsules can facilitate the attachment of bacteria onto various surfaces as the surface of the gut epithelia or increase the viability of probiotic bacteria in harsh bile conditions (Anselmo, McHugh, Webster, Langer, & Jaklenec, 2016; Priya, Vijayalakshmi, & Raichur, 2011). In addition, the surface of the shells can even be additionally ornamented using enzymes or NPs that can facilitate degradation of various types of substrate (T. Liu et al., 2019; J. H. Park et al., 2014). Since PE capsules are extremely durable, tolerating high pressures up to 300 MPa (Vinogradova, 2004), it will also allow studying physiology of cells present under physical constraint being not dependent on diffusion and viability. Due to the high values of Young's modulus, we can expect to regulate cell growth as it would take a certain amount of time for cells to resist the force of the capsules.

In simple terms, bacterial cells can be considered as negatively charged surfaces that can be used as templates for the PE deposition, similar to inanimate surfaces. In physical experiments on surface modification, it has been demonstrated that the bacterial surface is suitable for consequent adsorption of oppositely charged PEs, resulting in preparation of uniform PE coatings on the cell surface (Franz, Balkundi, Dahl, Lvov, & Prange, 2010). However, the development of an efficient and extremely accurate strategy for confinement of cells based on electrostatics will require careful management of bacteria in order to keep the activity of the culture and to enable the formation of cell-to-cell couples. It has been clearly shown that such surface modification leads to high aggregation (Franz et al., 2010) and sometimes to a decrease in viability (Drachuk et al., 2015; Fakhrullin et al., 2009; Jonas et al., 2018; Kozlovskaya et al., 2011), which limits either the application of the procedure or the number of strains suitable for use.

Most importantly, since biological properties of the cells were not considered in the demonstrated cases, it could result in lowered viability, low spatial organization of cells and uncontrollable aggregation. Therefore, compared to an inanimate surface, the safe and accurate deposition of PEs on bacteria can be very problematic due to the specific properties of cells: (i) bacteria can divide within 20 minutes, (ii) the cell membrane is more fluid and mechanically softer than in eukaryotes, (iii) the bacterial size is very small, (iv) some strains are sensitive to positively charged molecules, (v) the membrane can have specific appendages as flagella or pili. In addition, being entrapped in a strong PE mesh, living bacteria must somehow resist the coating, adapting their metabolism. Key limitations and expected advantages for preparing coating procedure are summarized in Figure 1.2.

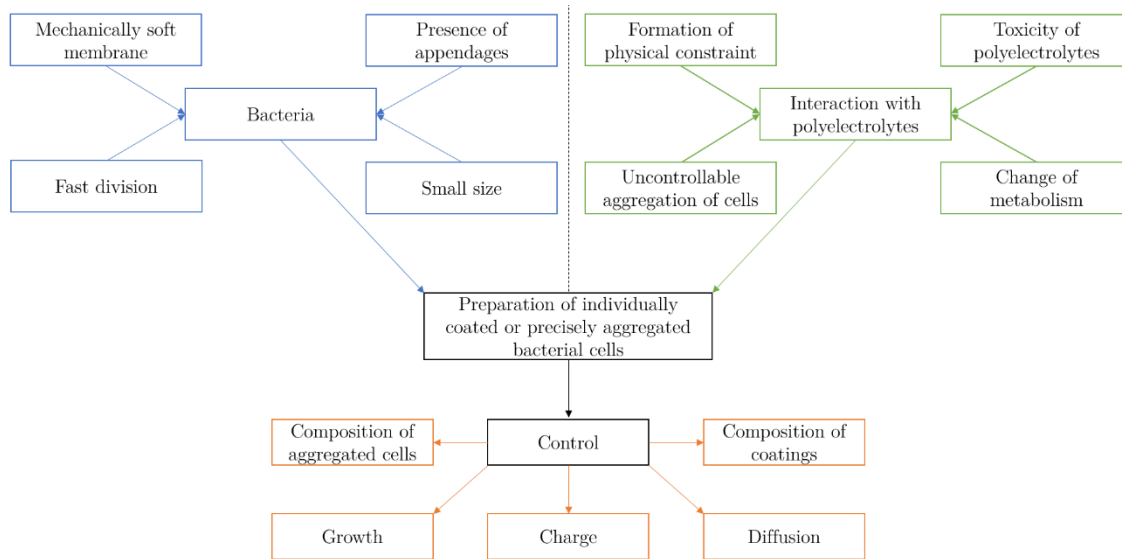


Figure 1.2: Key limitations and advantages for preparing coated cells.

In summary, the preparation of optimal and safe solution would require to solve the following problems: (i) the toxicity of the positively charged PEs and their coatings, (ii) the unknown effects of confinement on the metabolism of the cells, (iii) the control of colloidal stability, which prevents or precisely controls the aggregation or attachment of cells. Therefore, the present work will be focused on the development of methods that allow to safely confine bacterial cells in PE matrices, study the physiological changes of confined bacteria and analyze toxicity aspects when different PEs are introduced to cells.

1.2.1 Application of positively charged polyelectrolytes to bacterial cells shows different outcomes in terms of viability

Although PEs are used to prepare capsules on the cell surface, they have also been clearly shown to be toxic to bacterial cells and the reasons of the ambiguities in viability have not been elucidated. There have been many studies demonstrating the general application of bactericidal PEs to inhibit bacterial growth by preparing bactericidal surfaces or solutions (Broghden, 2005; Carmona-Ribeiro & de Melo Carrasco, 2013; Epanand & Vogel, 1999; Fjell, Hiss, Hancock, & Schneider, 2012; Jain et al., 2014; E.-R. Kenawy, Worley, & Broughton, 2007). In cases when PE capsules or cationic peptides were toxic (Jonas et al., 2018; Kozlovskaya et al., 2011), they induced the formation of pores in the bacterial membrane, causing cell lysis (Carmona-Ribeiro & de Melo Carrasco, 2013; Gee et al., 2013). However, we observed that the levels of toxicity are not the same between bacteria, since either Gram-negative bacteria (Gibney et al., 2012; E.-R. Kenawy et al., 2007; Thoma, Boles, & Kuroda, 2014) or Gram-positive bacteria were found to be more resistant to PEs (Jain et al., 2014; Y. Li, Xiang, Zhang, Huang, & Su, 2012).

Since the same PEs were used either for the bactericidal surface or entrapment, the introduction of PEs by the LbL method should also be toxic. However, unlike the aforementioned cases of toxicities, in the coating of yeasts (Fakhrullin et al., 2009), bacterial cells (Franz et al., 2010; Hillberg & Tabrizian, 2006; Priya et al., 2011) or bacterial spores (Balkundi et al., 2009), the authors did not determine a decrease in viability caused by PEs or just observed prolonged time of the lag phase. Moreover, several studies reported that positively charged PE coatings are not toxic even when they are introduced in several layers (Drachuk et al., 2012; Fakhrullin et al., 2009; Franz et al., 2010; Priya et al., 2011).

In these cases, the lack of toxicity can be caused by several reasons: (i) the strains used for coatings were resistant to PEs, (ii) the cells were aggregated, which reduced the exposure to PEs, thus less lowering viability, (iii) only a small amount of cells survived at the population level, resulting in the prolonged lag phase, or (iv) since a certain number of cells can survive exposure to a bactericidal surface, bacteria exposed to the coated surface might have very different conditions than bacteria coated in PE layers. In our preliminary experiments, we observed a strain-dependent toxicity of LbL coatings even within Gram-negative groups of bacteria. We also preliminarily determined that a strain, which is resistant to LbL coating, might be sensitive to the exposure to a bactericidal surface or a bactericidal solution, consisting of the same PEs. Based on these findings, we expect that toxicity may be in some way related to specific differences between bacterial strains and exposure conditions.

Therefore, such ambiguities clearly call for development of a new systematic approach to comprehensively evaluate the toxic effect and define the conditions of such effect.

1.2.2 Differences in the Structure of the Cell Membrane Might Affect Toxicity of Polyelectrolytes

In our preliminary experiments, we found that the level of toxicity depends on the particular pair of bacteria-PE, while the general conclusions about the sensitivity of bacteria to PE seem very ambiguous from one PE to another. If we assume that the toxicity only depends on the chemical structure of molecules (e.g. presence of amino groups, hydrophobic chains, counter ions), we should expect similarly close effects of one molecule on various bacterial strains. However, it is clearly not the case since many studies demonstrated that Gram-negative bacteria are more resistant to the effect of PEs or peptides (Gibney et al., 2012; E.-R. Kenawy et al., 2007; Thoma et al., 2014), while in other cases, Gram-positive bacteria were more resistant (Jain et al., 2014; Y. Li et al., 2012) or both groups were equally sensitive to molecules (Carmona-Ribeiro & de Melo Carrasco, 2013; Gordon, Romanowski, & McDermott, 2005; E.-R. Kenawy et al., 2007; Séon, Lavalle, Schaaf, & Boulmedais, 2015). Moreover, excluding specific aspects of architecture of cell wall, the evidence for differences in susceptibility within one Gram group is unclear (Carmona-Ribeiro & de Melo Carrasco, 2013; E. Kenawy, Abdel-Hay, El-Shanshoury, & El-Newehy, 2002). Thus, we believe that the toxicity phenomenon cannot be studied without taking into consideration the structure of the cell wall.

Since in our preliminary tests we found differences in the viability of various Gram-negative cells exposed to PEs, we hypothesized that the structure of the cell membrane might somehow influence the interaction with PEs. In support of our hypothesis, it was shown that the electrokinetic potential of Gram-negative bacteria has a higher negative charge and a less soft surface than Gram-positive bacteria (Sonohara et al., 1995). Therefore, the degree to which PE interacts with the cell envelope can depend on the composition of the membrane, which determines the electrokinetic properties. Moreover, we can expect an even greater contribution of the membrane and its specific parts on the process of the PE deposition since the toxicity was shown to vary even within species with the same Gram architecture (E. Kenawy et al., 2002).

The cell envelope of Gram-negative bacteria is composed of a phospholipid membrane, an intermediate layer of peptidoglycan and a Lipopolysaccharide layer (LPS) bound to a phospholipid layer (Beveridge, 1999). Both the peptidoglycan and the LPS layer act as a barrier preventing the penetration of charged molecules (Ebbensgaard, Mordhorst, Aarestrup, & Hansen, 2018). In contrast, Gram-positive bacteria lack an outer membrane composed of a phospholipid bilayer and LPS. Instead, they have a much thicker layer of peptidoglycan than Gram-negative bacteria (Rajagopal & Walker, 2015). Since positively

charged molecules are naturally present as peptides, both types of cells have developed specific mechanisms to prevent peptides from binding to the membrane. Accordingly, Gram-negative bacteria can reduce binding to positively charged peptides by modifying the outer membrane. The modification occurs by reducing the fluidity of the membrane by increasing the density of hydrophobic tails or introducing more positively charged molecules (phosphoethanolamine, 4-amino-4-deoxy-L-arabinose), which both decrease the total charge of cells and thus binding with positively charged peptides (Gunn, 2001; Gunn et al., 1998; Trent, Ribeiro, Lin, Cotter, & Raetz, 2001). With Gram-positive bacteria, the decrease in peptide binding is controlled by the insertion of neutral or positively charged molecules such as D-alanine or L-lysine, which reduce the total surface charge of the bacteria (Brogden, 2005). Thus, we can also expect similar mechanisms to protect the membrane that prevent binding with positively charged PEs.

Although Gram-negative bacteria are believed to be more resistant to the action of positively charged PEs because of a double membrane and LPS layer (Gibney et al., 2012; E.-R. Kenawy et al., 2007; Thoma et al., 2014), it is not necessarily the case as the results are contradictory (Jain et al., 2014; Y. Li et al., 2012). Since the results are very ambiguous, it is important to determine if there is a clear relation between the toxicity of PEs and the structure of Gram cell wall. For this purpose, we chose two bacterial strains with the same Gram architecture to study the difference in the viability of the cells exposed to the PEs in Section 3.2.

1.2.3 Challenges in preparation of Layer-by-Layer assembled capsules on the surface of individual living cells

In contrast to the cases of harmless LbL coatings, several studies observed that the same type of PEs notably reduced the viability of the coated bacteria and yeasts (Drachuk et al., 2015; Fakhrullin & Lvov, 2012; Jonas et al., 2018; Kozlovskaya et al., 2011; M. B. Oliveira, Hatami, & Mano, 2016). We expect that the ambiguous reports on the toxicity of PEs used for LbL encapsulation could be related to several reasons: (i) the aggregation of cells, which reduces the diffusion of oxygen and nutrients, (ii) the formation of physical constraint, stalling division of cells, (iii) damage of the cell envelope, and (iv) structure of PEs, which can contribute to the toxicity between different strains. In addition, although attempts have been made to optimize the procedure of PE deposition by selecting the most promising PEs for each bacterium, the selected pairs of strains and PEs were not the same across studies and conditions, resulting in a lack of standardization of the use of a specific PE for a particular strain.

Application of multiple PE layers has been shown to induce intense aggregation of cells (Drachuk et al., 2015; Franz et al., 2010; Hillberg & Tabrizian, 2006; Rivera-Betancourt, Sheppard, Krause, & Dluhy, 2014). We hypothesize that aggregation of bacteria occurs when centrifugal force is applied to an extent, which opposes the coated bacteria from repelling and makes the Van der Waals forces more prominent. Also, the differences in the cell structure, i.e. membrane, proteins, flagella, pili and etc., may promote aggregation as the membrane can be more electrostatically attractive. In particular, if cells produce Extracellular Polymeric Substances (EPS), it results in suppression of electro-repulsive forces and the development of attachment (Tsuneda, Aikawa, Hayashi, & Hirata, 2004), which can lead to better interactions with other cells. In the opposite case, when EPS are absent, increased electrostatic interaction suppresses attachment (Tsuneda, Aikawa, Hayashi, Yuasa, & Hirata, 2003) and makes cells more repulsive from each other. Therefore, one would expect the formation of either of well-coated repulsive cells or partially coated cells that aggregate. To assess the contribution of the appendages, we can apply Oshima's soft particle theory, which views bacterial cells as colloidal particles entrapped within

weakly charged PE layers (Ohshima & Kondo, 1991). By analyzing the electrophoretic mobility of cells, it allows us to estimate linear charge density and softness parameters, i.e. the depth of ion penetration within the cell membrane and surface potential (Sonohara, Muramatsu, Ohshima, & Kondo, 1995; Tsuneda et al., 2004). As a result, we can predict the most suitable time for coating, when the cells should resemble more electrostatically “rigid” particles and have the highest charge density, which ensures a good distribution of the coating. In addition, the surface properties of bacterial cells change during growth due to the production of different proteins or polysaccharides that are specific for each growth phase. These changes of surface can affect electrokinetic potential of the cells (e.g. the charge becomes more neutral) and thus deposition of PEs. Therefore, preparing the best possible coating on the cells would require to predict the most appropriate time and conditions for the PE deposition for each selected strain. In return, it should allow us to coat each cell, avoiding the formation of appendages that may shield the charge or reduce binding to PE. For this purpose, it is needed to prepare a method, which allows to individually coat bacterial cells, which we will prepare in Section 3.1.

Once PEs are deposited on the cell surface, the cells should experience a situation of a strong but permeable confinement. Since PE capsules can be very resistant to tearing (Vinogradova, 2004), bacteria would need a certain time to leave the capsule, which is expected to slow the rate of fission. It has been shown, but not further investigated, that PE coatings create a barrier that inhibits cell growth (Franz et al., 2010), not reducing viability. Thus, we cannot attribute the observed effects of the delayed growth to the conventional idea of toxicity and we further need to investigate the existence of microbes under physical constraint of the PE capsules, which we will study in Section 3.1.

The bactericidal action of positively charged PEs is believed to be driven mainly by electrostatic interaction between the positively charged groups of the PE and the negatively charged membrane (Andreu & Rivas, 1998; Brogden, 2005; Epand & Vogel, 1999; Gibney et al., 2012), resulting in the formation of membrane pores (Bechinger, 1999; E.-R. Kenawy et al., 2007; X. Li et al., 2010) that release intracellular compounds (E. Kenawy & Mahmoud, 2003). Structural factors of PE that can contribute to toxicity include the positive charge, the molecular weight of the PEs and the structural characteristics of the molecule, i.e. the distance between functional groups, the nature of counterions and the ratio of hydrophobic to hydrophilic parts (Jain et al., 2014; E.-R. Kenawy et al., 2007; Palermo & Kuroda, 2009; Timofeeva & Kleshcheva, 2011).

To reduce toxic effects, it would require to coat the cells with specific PEs that do not harm the bacterium. Since the general toxic effect of PEs is related to the excess of the positive charge (Huang et al., 2016; Kügler, Bouloussa, & Rondelez, 2005), we hypothesize that toxicity can be reduced by either decreasing the linear charge density of PE (Forrest, Meister, Koerber, & Pack, 2004) or the number of the deposited PEs in each particular case. Therefore, we will study the differences in viability after exposure of bacteria to various PEs under different conditions and develop cases for toxicity reduction in Sections 3.2 and 3.3.

1.3 Biological Traits Needed for Preparation of Multispecies Bacterial Entities

If there is a possibility to change the surface charge of bacteria and to keep them alive, then it will also be possible to attach cells to each other due to the differences in charges. Since we expect to control the growth and division of individual bacteria using PE coatings, it should allow us to build a well-balanced structure, which can be designed and fabricated for the desired metabolic process (e.g. oil degradation, salt removing, entrapment of specific

metals, pesticide degradation and etc.). Moreover, structuring the community additionally solves several problems as (i) many microbes will be physically forced to interact although they do not tend to interact well in nature, (ii) the initial conditions (oxygen gradient, metabolic compatibility of cells, growth speed) can be adjusted without energy consumption for the establishment of these processes, and (iii) as a result, it is possible to fully design the composition of the community, independent only of natural conditions, which will contribute to the addition of only specific microbes.

From a biological point of view, microbes often require attaching to a solid support in order to form a biofilm. The surface provides more beneficial conditions as it becomes an interface for the collection of soluble organic nutrients that are deposited from liquid surrounding (Tuson & Weibel, 2013). It also provides more favorable conditions for protection, as bacteria might be well isolated in small pores against predators (Vargas & Hattori, 1986).

Therefore, if we want to enable multispecies aggregation, it is necessary to investigate the basic concepts of the formation of bacterial entities and to provide such initial conditions for bacteria, i.e. attachment and nutrient exchange as they have in nature. Usually cells are present in a biofilm, which has protective functions as (i) resistance to colonization, (ii) production of toxins or resistance from them, (iii) providing appropriate conditions for the establishment of metabolically sustainable pathways and attachment of cells to the surface for further colonization (Burmølle et al., 2006; Epstein, Hong, Kim, & Aizenberg, 2013; X. He, McLean, Guo, Lux, & Shi, 2014; Matz & Kjelleberg, 2005). Biofilm formation has found important technological applications in fermentation processes (Cheng, Demirci, & Catchmark, 2010) and wastewater treatment plants (Chaali, Naghdi, Brar, & Avalos-Ramirez, 2018; Nicolella, Van Loosdrecht, & Heijnen, 2000), because microbes are protected from physical influence and biomass can be used a longer time.

In contrast to the beneficial presence, in many cases biofilm formation is a serious health threat due to contamination of clean surfaces (Veerachamy, Yarlagaadda, Manivasagam, & Yarlagaadda, 2014) (Veerachamy et al., 2014) or their biofouling and corrosion (Beech, Sunner, & Hiraoka, 2005). Additionally, the presence and activity of first colonizing microbes can promote the growth of pathogenic bacteria and the development of the correlated disease (Kho & Lal, 2018; Whittaker, Klier, & Kolenbrander, 1996). Since diffusive processes are decreased within bacterial films, and bacteria exchange some genes for antibiotic resistance, it makes them inaccessible to the action of the drug (Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010).

However, if we can establish the initial conditions by artificial placing beneficial bacteria, it can help in either of the two cases of the presence of biofilm on the surface. Accordingly, we can significantly promote attachment of bacteria in cases of bioremediation, or populate the surface with beneficial cells that inhibit the attachment and development of pathogenic ones (Rijavec, Zrimec, van Spanning, & Lapanje, 2019).

Biofilm formation is a sequential process, which is initiated by the attachment of microbes to a surface, where the first step is to overcome a strong repulsive barrier of the surface. From a physical point of view, repulsion is the result of several factors, including electrostatic repulsion, steric repulsion, and ion concentration. Since bacteria and surfaces are both negatively charged, it opposes the attachment of microbes due to a strong electrostatic repulsion (Dan, 2003). Bacteria can also be sterically repulsed if the affinity of the molecules of bacteria and surface that come into contact is low (Rijnaarts, Norde, Lyklema, & Zehnder, 1999). Media with low ionic strength would promote repulsion due to the low electrostatic shielding of negatively charged ions of bacteria and surface (Morisaki & Tabuchi, 2009). Additionally, a low-ionic-strength environment makes the appendages on the bacterial surface less rigid and less compressed, resulting in lower adhesion and higher steric repulsion (Abu-Lail & Camesano, 2003). Thus, under such

conditions, only certain microbes can firmly attach and further populate the surface. Therefore, many bacterial strains with very useful metabolic traits might not be applicable, or to become not applicable if they lose the ability to form a biofilm over a certain time (TerAvest & Ajo-Franklin, 2016).

Attachment requires a close proximity of cells to the surface for a certain time to experience the effect of short-acting forces such as the Van der Waals forces (C. G. Kumar & Anand, 1998; Palmer, Flint, & Brooks, 2007). For attachment bacteria engage various surface appendages i.e. pili, flagella, receptors and produce extracellular polysaccharides (Tuson & Weibel, 2013), which enable to overcome repulsion and irreversibly attach within a certain time (~20 min). Thus, if a cell loses the production of such elements, it significantly reduces the likelihood of attachment, as electrostatic repulsion becomes more prominent with decreasing distance (Adair, Suvaci, & Sindel, 2001; Tsuneda et al., 2004). The ability of cells to irreversibly attach to a surface will determine the opportunity for further colonization of this surface and, therefore, biofilm formation (Hori & Matsumoto, 2010).

The bacteria attached to the surface further adapt the microenvironment for introducing new microbes using physicochemical and biological mechanisms. The physicochemical mechanisms are based on (i) the exposure of surface receptors that allow secondary colonizers to physically attach (Castonguay et al., 2006; W. Liu et al., 2017; Marsh, Moter, & Devine, 2011) and (ii) altering surface charge, hydrophobicity and microtopography (Katsikogianni & Missirlis, 2004; Palmer et al., 2007). Biological factors are based on: (i) adjusting the microenvironment by changing pH or oxygen (Jakubovics, 2015; Zuo, 2007) and (ii) providing metabolites or nutrients to promote or inhibit the growth of neighboring cells (Bucci et al., 2011; Rendueles & Ghigo, 2015). Therefore, in environmental systems, biofilm composition strictly depends on the colonizing microbes, environmental conditions, and local conditions that are regulated by these microbes.

When a new cell becomes a member of a biofilm, it diversifies the metabolite production, allows the exchange of genes and metabolites (Ponomarova & Patil, 2015), reduces energy burdens and reduces competition between cells. To exchange metabolites, bacteria can use contact-dependent or contact-independent pathways. In both cases, the transfer of metabolites has specific traits. Accordingly, metabolites diffuse freely in contact-independent pathways. However, their distribution in biofilms can be significantly restricted due to uptake, altering, degradation, or consumption by other bacteria. In contrast, contact-dependent pathways avoid nutrient loss. However, it requires additional energetics to establish surface-to-surface contact with a suitable partner (D'Souza et al., 2018). Moreover, the metabolite-dependent bacteria must increase proximity with the metabolite-producer cells in order to maximize nutrient uptake in the microenvironment, thereby reducing competition with other bacteria for this type of nutrient (van Tatenhove-Pel et al., 2020).

As a result, the process of biofilm formation is driven by the most crucial steps of attachment of cells together and their metabolite exchange (N. M. Oliveira et al., 2015). The preparation of artificial aggregates would require the attachment and placement of cells in close distance to reach the optimal conditions for the growth. It can be expected that if we introduce positively charged PEs as a single layer, it would lower the energy barrier between the coated cells and the surface to initiate attachment. As a result, it can increase the probability of biofilm formation even if biofilm non-forming strains are used, since the cells will present beyond the repulsive barrier. It is also expected that surface-modified bacteria will be in very close contact with each other after they are aggregated. According to Rinke J. van Tatenhove-Pel et al., close distance should also significantly improve the nutrient flow within such artificial entities, since the losses caused by diffusion would be minimal (van Tatenhove-Pel et al., 2020). In addition, if bacterial cells are

artificially placed in aggregates, the composition of the community should not be problematic.

1.4 Incorporation of Nanoparticles into Polyelectrolyte Layers

Since we can alter the surface charge of bacterial cells by PE deposition, our solution for preparing coatings will allow us to (i) control cell growth, (ii) control the position and distribution of microbes and (iii) label the coatings.

In order to demonstrate how to additionally modify bacteria, we will develop an approach to deposit NPs on the cell surface. Such manipulations will allow us to add new modalities to bacteria by introducing various sources of NPs that can catalyze specific reactions, make cells more responsive for remote signals or modify the physical properties of coatings (thermal conductivity, electrical conductivity, mechanical strength).

1.4.1 Application of graphene nanoparticles

The coated bacterial cells might be arranged in different aggregates or artificial biofilms and applied in a microbial fuel cell for generating electric current from organic waste (Santoro, Arbizzani, Erable, & Ieropoulos, 2017), for preparing specific biosensors or setups for bioremediation (R. Kumar, Singh, Zularisam, & Hai, 2018). However, in many cases, the energy transfer is problematic for some biological reasons: (i) the biofilm is poorly formed or ordered (Palanisamy et al., 2019), (ii) the matrix is not well conductive (R. Kumar et al., 2018), which forces only the closest bacteria to transfer electrons to the electrodes and (iii) membrane biofouling (Gude, 2016). If the biofouling and structuring of the biofilm could be arranged using controllable growth of bacteria in PE, energy transfer is still problematic.

To prepare such a setup, we need to introduce a conductive material into the PE coatings on the bacterial cell surface. To improve conductivity, we chose graphene NPs, which have outstanding conductive, mechanical and thermal properties (Pei & Cheng, 2012). Graphene materials can become even more beneficial if the surface is additionally functionalized with oxygen groups (Shen et al., 2009), which can enable a better interaction with hydrophilic surfaces via hydrogen bonds. Although the properties of both Graphene Oxide (GO) and its reduced form Graphene Oxide (rGO) should be similar, we expect that the presence of oxygen groups in GO may be more preferable for hydrogen bonding, but it also can induce unwanted toxicity caused by oxidation of membrane. Therefore, we specifically selected these two forms of graphene in order to find the optimal material for introduction onto the surface bacterial cells. In addition, since PEs might be toxic, GO or rGO can potentially be applied as a spacer material that separates the toxic positively charged PEs and the cell membrane when graphene is attached to the cell surface.

However, the preparation of a harmless coating for modifying a bacterial surface using GO or rGO requires ensuring the safety and non-toxic effect of the chosen NPs for bacteria. The application of GO and rGO has a diametrically opposite effect on viability since in one case it is used for preparing bactericidal surfaces, while in others it has no toxicity on cells. In particular, the toxic effect of these materials on cell growth has shown for both GO and rGO (Lukowiak, Kedziora, & Streck, 2016; Tegou et al., 2016). However, there are no coherent conclusions in toxicities of graphene NPs for bacteria, since in some cases Gram-negative bacteria (Akhavan & Ghaderi, 2010; Musico, Santos, Dalida, & Rodrigues, 2014; Pulingam et al., 2019) were demonstrated to be more resistant, while in other cases Gram-positive bacteria (Nanda, An, & Yi, 2015; Pham et al., 2015; Romero-Vargas

Castrillón, Perreault, De Faria, & Elimelech, 2015a; Tang et al., 2013) or both types of cells were susceptible (Yousefi et al., 2017). Moreover, the determined toxicities vary even within the same species (Hegab et al., 2016). The toxicity of graphene materials is supposed to be mediated via several mechanisms, which include (i) membrane damage, (ii) oxidative stress and (iii) decrease of nutrient flow. Since bacteria interact with graphene materials, the latter can cause membrane damage or pore formation (Lukowiak et al., 2016; Pham et al., 2015). However, pore formation is not obvious for all bacteria, since only Gram-negative bacteria were shown to have pore formation (Tang et al., 2013), or both groups were prone to lysis (Lukowiak et al., 2016; Pham et al., 2015). Another mechanism that can cause toxicity is based on an oxidative stress of the bacterial membrane (Hegab et al., 2016). Since graphene materials have oxygen groups on the surface, it is assumed that they produce reactive oxygen species, oxidizing membrane lipids and proteins (Shaobin Liu et al., 2011; Nanda et al., 2015). We need to consider that charge densities, which repel bacteria and GO particles, can make the oxidative stress more prominent (Romero-Vargas Castrillón, Perreault, De Faria, & Elimelech, 2015b) than direct physical damage to the membrane. However, high level of oxygen groups can promote formation of hydrogen bonds, decreasing the distance between cells and particles, which can lead to physical damage. Another aspect that affects the viability is based on wrapping of cells into graphene sheets. Since graphene materials have an extremely high surface area, being introduced to bacteria, they can decrease cell growth by isolating cells from the nutrient environment (Akhavan, Ghaderi, & Esfandiar, 2011; Hegab et al., 2016). However, the contribution of each mechanism in a complex effect still needs further study.

In contrast to the cases when graphene materials are toxic, there have been several studies showing no toxicity to microorganisms (Barbolina et al., 2016; Y. Luo et al., 2016; Mu, Zhou, Zhao, Liu, & Hu, 2019; Ruiz et al., 2011; Wang, Wang, Zhang, Xu, & Yang, 2013). In these cases, graphene materials acted as a support that promoted cellular growth and induced the production of extracellular compounds and proteins (Y. Luo et al., 2016). Since the toxicity of graphene may be related to impurities during preparation (Wong et al., 2014), it was demonstrated that purified GO has no impact on viability and cell integrity (Barbolina et al., 2016). Moreover, graphene materials can be introduced by the LbL method not reducing viability, but introducing new modalities as increased mechanical properties or cryoprotection (L. He et al., 2021; H. Luo et al., 2018; Yang et al., 2012). Summing up, we suppose that the introduction of GO and rGO can be very promising for technological applications. However, if we want to efficiently and safely modify the bacterial surface by introducing graphene materials directly to cells or by the LbL method, it is needed to consider all aspects leading to toxicity. Since there are also no coherent conclusions on toxicity, we will perform additional tests on toxicity and evaluate the interaction of bacteria with GO or rGO particles in Sections 3.4 and 3.5.

1.4.2 Application of magnetic nanoparticles for guiding bacterial cells

It has been shown that mixing exact ratios of positively and negatively charged bacteria results in the formation of very precise aggregates (van Tatenhove-Pel et al., 2020), with a maximum yield not exceeding 3 %. To increase the number of bacterial aggregates per unit volume, we propose to develop a method for modifying the cell surface with paramagnetic NPs. Such coating would enable to separate and maximize the enrichment of magnetic sensitive aggregates in a magnetic field, thereby facilitating their preparation.

Since we wanted to observe how bacterial cells are acting when we combine all above aimed-to-develop methods into one complex solution, we chose the case of preparing cells for remote guiding in a living organism. Therefore, by a modification of the bacterial surface, we aimed to functionalize the cell surface with paramagnetic NPs first, to enrich

the cell biomass, and second, to test the idea of guiding, detecting and monitoring the distribution of bacteria within the mammal body in order to make bacterial therapy safer.

The ideal solution for disease treatment would be to use tiny, programmable “robots” that specifically target tumors and selectively destroy them (Forbes, 2010). Such units should also be easily detectable, responsive to external “commands” and self-propelled through the system by sensing the local environment. According to all the requirements, bacteria might be excellent candidates that share most of the properties required for an ideal cancer therapy. Many different bacterial strains were shown to accumulate in tumor regions, i.e. *Salmonella* (Pawelek, Low, & Bermudes, 1997), *Escherichia* (Yong et al., 2004), *Clostridium* (Parker, Plummer, Siebenmann, & Chapman, 1947), *Bifidobacterium* (Kohwi, Imai, Tamura, & Hashimoto, 1978), *Listeria* (Pan, Weiskirch, & Paterson, 1999) and *Streptococcus* (Maletzki, Linnebacher, Kreikemeyer, & Emmrich, 2008). Using bacteria is expected to induce a strong immune response since their surface is highly immunogenic. Since cells can be self-propulsive and guided by a chemotaxis, they can be very promising for reaching inaccessible for passive therapies places and allow the local application of more toxic compounds, reducing the toxic effects on the host (Sai Liu et al., 2014). Bacterial cells can be even more promising by engineering the system for production of cytokines (Barbé et al., 2005), cytotoxic agents (Jiang et al., 2010) or modified for targeting (Nguyen et al., 2010).

Although reports of anticancer bacterial therapy are very encouraging, the current state is far from ideal cancer therapy since (i) the bacterial surface is highly immunogenic, (ii) it is hard to target bacteria and to prevent dissemination of cells, which can additionally increase the risk of sepsis, and (iii) many promising bacteria lack the ability to target, which calls for the development of alternative approaches for helping cells to reach tumors.

Here, we hypothesize that a solution to improve the introduction of bacteria into living organisms can be prepared by the modification of the bacterial cell surface with PE multilayers. Each PE layer can be modified with fluorescent labels and some specific molecules, i.e. proteins, enzymes, antibodies, to allow detection of PE shells and to help target the tumors (Ai, Jones, & Lvov, 2003; Campàs & O’Sullivan, 2003; Johnston, Cortez, Angelatos, & Caruso, 2006). To improve guiding, we can use paramagnetic NPs, which are very responsive to the external magnetic field (Fakhrullin et al., 2009; B.-W. Park, Zhuang, Yasa, & Sitti, 2017).

Since this approach should allow us to form and enrich aggregates using only magnetic fields, we will prepare the case to apply such a system to remotely control bacterial cells in the mammal body. Specifically, we aimed to: (i) develop a method of the cell surface modification and (ii) to modify the surface in such a way that cells can be detected and targeted at a desirable place of organism, which we will prepare in Section 3.6.

Summarizing, the present work will focus on the development of methods for electrostatic immobilization of individual bacterial cells or precise aggregates, determination of changes in cell growth and attachment due to electrostatic confinement, determination of conditions leading to a decrease in viability, and the development of methods for its improvement. To increase the versatility of the method and to increase the yield of the coated cells, we will modify the cell surface by introducing various NPs, and test the prepared solution in a living organism, combining all the developed stages.

Chapter 2

Aims and Hypotheses

2.1 Introduction to Hypotheses

In nature, many biological pathways are connected to each other, and bacterial cells are cross-feeding each other, living on “waste” products of metabolic partners. In particular, there are various examples of the cross-feeding interactions as gut microbiota with host cells, plant associated microbes, bacteria associated with corrinoid auxotrophic algae or associated bacterial entities in soil. This enables the formation of specific ecological niches and diversifies processes, making such a system very robust. By artificial placing bacteria in structures such as biofilms or flocs, we can create a set of metabolic pathways that can be coupled according to the needs to produce or to degrade a particular compound. For micro arranging cells in a metabolic cascade, it is necessary to closely arrange bacteria and initially set the community for its further development. Although there are different strategies such as encapsulation, extrusions, emulsions, cell immobilization into various matrices (e.g. polysaccharides, proteins, polypeptides, lipids matrices) that initially set the community, they do not allow to precisely arrange bacteria together and to minimize the distance between cells for increasing nutrient transfer. Matrices can also limit diffusion and consequently lower viability. In addition, the growth of each member of natural microbial entities is controlled by the availability of nutrients and external factors that inhibit or promote growth. However, if we combine microbes with significantly different growth rates, it may cause overgrowth of one specific bacteria over others. Based on these objectives, we propose a method for modifying the bacterial surface of an individual cell through PE deposition that allows to electrostatically combine cells together with maximum proximity and control the growth rate of each cell by strengthening PE coatings.

When the charge of the cells changes, it allows us to manipulate the cells for attachment. This can then enable the formation of a variety of aggregates composed of very diverse cells, by coupling multiple metabolic pathways together. These aggregates in artificial films increase the variability of metabolites and can lead to the formation of very specific products. By coupling different cells, it can also enable turning multi stage processes for biotechnology (fermentation of different products) or bioremediation (entrapment of metals) into a single step stage (e.g. aerobic and anaerobic conditions present within one aggregate). Anaerobic conditions can be achieved since the external side of the aggregate interface will be exposed to atmospheric conditions, while the internal side will be depleted of oxygen. Micro-geographic placement can also change the local environment to anaerobic, low nutrient, or high pH. In return, it makes very specific niches that promote or inhibit cell growth. However, it is not known how to keep cells viable or regulate physiology of bacteria using this approach. Therefore, the development of a method for multispecies aggregation of bacteria based on electrostatics will require a clear

understanding of the basic concepts of functioning and growth of bacteria in a confined space, and solving the problem of PE toxicity.

In different experiments, which apply LbL coatings, it has been found that bacteria can be suitable templates for the PE deposition as similar as any kind of inanimate templates. However, the deposition of PEs results in uncontrollable excessive aggregation, which is not preferable when cells should be studied separately. From this point of view, only an individually coated cell will allow us to assess the growth dynamics of confined bacteria and the effect of toxicity not interfering with population level, where many other interactions or consequences may take place. In addition, aggregation can reduce the nutrient diffusion within the aggregates when the cells are coated in a matrix. We hypothesize that aggregation can occur because of a physical factor (e.g. excessive centrifugation) and biological properties of the surface structures of bacterial cells, which were not taken into consideration during the LbL coating experiments.

In this regard, we will develop an approach for cell coating using the LbL strategy, which should allow us to keep the lowest possible aggregation for different cells using mild centrifugation. We will test if bacterial cells at different growth stages would affect the efficiency of the coating. For this purpose, we will apply Oshima's theory to determine the contribution of appendages on the cell surface during different growth stages on the deposition of the PEs. It will enable us to determine the softness parameters of the cell surface, which should define the most appropriate time for coating during cell growth, and if this leads to a decrease in aggregation.

By combining all the steps, we plan to elaborate an innovative method to apply to various bacterial cells. Since our confinement based on electrostatic interaction does not obstruct the nutrient flow but makes a strong capsule, it will enable us to study the physiological dynamics of the confined bacteria. It is known that the metabolic parts of bacteria can withstand an increase in pressure up to 200 MPa, which leads to the cessation of metabolic functions (growth, division, DNA replication and others). Since PE coatings can be resistant to the fracture up to 300 MPa, a gradual increase in the thickness on the cell surface can lead to metabolic suppression of some parts of the coated cell. It is also known that the cell size of bacteria is regulated, depending on nutrient availability. Taking into consideration these facts, we suppose that the entrapment of cells into permeable PE coatings should constantly engage metabolism by regulating cell size. We hypothesize to observe a new paradox, where cells get nutrients but cannot divide due to a mechanical restriction, which suppresses the division part. For this purpose, we will show the effect of PE confinement on cell division, depending on how strong the PE capsules are. We will also try to relate the effect of confinement to metabolic changes occurring in cells. In return, it can be beneficial for biotechnological applications, since bacterial growth can be more controlled. To demonstrate this case, we will test if there is a suitable window for the thickness of PE coatings, which just prolongs the lag phase not stalling division. We will also find a correlation between the strength and the timing of the lag phase.

Also, in physical experiments, researchers ignored the viability of LbL-coated cells. This can be a special case, since PEs are strongly attached to the cell and can damage the membrane, therefore, methods for evaluating toxicity need to be developed. Here, we also foresee that the surface should be both positively charged and non-toxic. Since we assume that toxicity is related to the charge density in the molecule, we will reduce toxicity by either using chemical binding of positively charged amino groups with acetic groups or decreasing PE concentration. As a result, we should be able to get a positively charged PE coating, which is not toxic to bacteria. A review of toxicity studies did not demonstrate obvious reasons for preferential choice of one sort of PEs over others for one bacterium and the opposite choice for another. Moreover, some cells were found to be more suitable for deposition of PEs than others. We attribute these ambiguities in susceptibility to

differences in structure of cell envelopes and cell metabolism or the choice of PEs that are used to coat a specific strain. Therefore, we will study the contribution of both biological and physio-chemical aspects to determine whether some bacteria are resistant or some PEs are less toxic.

Since PE coatings can be made non-toxic to bacteria, the approach of PE deposition can be a versatile tool for functionalizing the surface of various bacteria. To expand the repertoire of strains that can be used in biotechnology, we will consider the case of the artificial attachment of bacteria via PE deposition. Many technologically relevant strains cannot populate surface due to the lack of special receptors or due to the electrostatically repulsive barrier, which occurs due to the negative charge of bacteria and the surface. From a physical point of view, there is a characteristic distance from the surface that these electrostatic forces are not effecting cells anymore. Breaching this distance would immediately engage Van-der Waals and short-range forces that promote attachment. Therefore, the deposition of positively charged PEs will cause a decrease in distance, which will give the cell more time for irreversible attachment (approximately 20 minutes).

It has also been shown that the maximal yield of precisely assembled aggregates does not exceed 3%. To facilitate the production of aggregates, we aimed to develop a method that allows to deposit paramagnetic NPs on the cell surface, which can help spatially arrange or separate bacteria merely using a magnetic field. Since we are able to individually coat and label cells, to remotely control their division rate and position, we aimed to develop a solution, which not only allows for the production of aggregates but also helps to improve the guiding of cell biomass in the organism.

In addition, we have also extended the applicative potential of our method by combining it with graphene NPs to potentially improve energy transfer from bacteria for possible application in a microbial fuel cell. However, in many cases, rGO and GO were shown to be toxic and the toxicity varied between different bacteria. Therefore, our first aim was to study the toxic effect of these particles on bacterial cells and assess the interaction of NPs with the cell surface.

2.2 Research Aims

We followed the main goal of the present dissertation, which was to develop a method of the PE deposition on bacteria for the preparation of individually coated or precisely aggregated bacterial cells and to study the biological responses caused by changing the cell surface properties at a single cell and at population levels:

To achieve the main goal, we also aimed five specific goals:

- i. Since the solutions that are used for the deposition of PEs on bacterial cell surfaces exhibited uncontrollable aggregation, we aimed to develop a method for consequent PE deposition, which avoids excessive aggregation and toxic effect on cells and to study a single cell or population dynamics of coated bacteria.
- ii. From published research reports, it was found that PE deposition in some cases does not have a toxic effect. However, it creates a mechanical confinement, which prolongs the lag phase of the coated cells. Therefore, we will study the effects of physical constraint of PE coatings on the growth, division and constitutive expression of proteins in bacteria.
- iii. Although in some cases the introduction of either PEs or NPs did not demonstrate toxicity, many studies reported toxic effects on bacteria when they were introduced to cells. Hence, it is needed to determine how the composition

and conditions of PEs and NPs affect bacterial toxicity in combination with the type of exposure and possible solutions to reduce toxic effects. Based on our preliminary studies, we observed that some cells are more resistant to coating, while others are extremely sensitive to it and the viability of such cells is notably reduced, even though the cells have the same Gram architecture. Therefore, we will determine the difference in susceptibility to coatings between different species, consisting of the same architecture of the cell wall.

- iv. The introduction of PEs was demonstrated to alter the charge either of bacteria or inanimate surfaces. In return, it could allow bacteria to have a better chance of attachment. Hence, we will study the effect of the cell surface modification by the PEs on the longevity of cell attachment and the formation of microcolonies.
- v. Since the prepared method for the surface modification allows adding novel properties to bacteria, which cannot be achieved only through genetic manipulation, we will develop a method that makes cells responsive like particles to magnetic fields as well as detect in the far red spectrum in order to expand their intrinsic biological capabilities.

2.3 Hypotheses

Although bacterial cells were used in physical experiments where PE deposition was applied, the biological aspects and effects of such manipulation on bacterial cells have not been described yet. In order to determine and distinguish these different physical and biological aspects, occurring after the deposition of PEs, we proposed the following hypotheses:

- i. Since PE capsules can form very strong mechanical obstacles sustaining tension in the range of a few hundred MPa but not obstructing the nutrient flow, we expect mechanical restriction of cell growth without its metabolic suppression. Therefore, the addition of the number of PE layers would increase force opposing the cell growth and its division, which will result in the prolonged generation time.
- ii. Free phospholipids in the cytosol are known to signalize the cell size and can determine division time. By the confinement of a cell inside such a PE capsule, we expect to interfere with the cell size regulation by the increase of free cytosolic phospholipids. Through this effect, we suppose that the confined cell gets a signal to constantly engage anabolic metabolism. It is expected that the anabolic part is less regulated and it will result in a non-specific increase in transcription. Therefore, we hypothesize that the leakage expression of genetically produced constructs will be increased.
- iii. According to published research reports, we found ambiguities where, on the one hand, PEs, especially positively charged, are toxic, and on the other hand, the same PEs are used for the surface modification of different prokaryotic and eukaryotic cells. Accordingly, we expect that toxicities can be attributed to the PE type and cell choice. Since these two aspects are not determined, we expect that, on the one hand, the type of cell will determine which PE with its physio-chemical properties will be the most suitable to preserve viability when confined. On the other hand, we expect that the major contribution is the total charge of PE.

- iv. Most surfaces that can be populated by bacteria are negatively charged and electrostatically repelling them. From a physical point of view, there is a characteristic distance from the surface that these electrostatic forces are not taking action anymore. By breaching this distance, short-range forces come into action. Therefore, if we alter cells with the opposite charge to the surface, we will immediately decrease the distance, enabling a more intimate and longer interaction with the surface. The most important time for transforming reversible attachment of cells to irreversible is within 20 minutes. Therefore, the increased time and stronger interaction with the surface can induce more efficient biofilm formation of biofilm forming bacteria, as well as it can facilitate attachment and biofilm formation of biofilm non-forming bacteria.
- v. Since we wanted to observe how bacterial cells are acting when we combine all steps, i.e. to keep cells alive, to control their growth, to visualize and to modify the surface with paramagnetic NPs into one complex solution, we chose the case of preparing cells for remote guiding in a living organism to make bacterial therapy safer. Different bacteria were demonstrated to be used as tumor healing agents (Forbes, 2010). Currently, cancer treatment is based on using pathogenic strains, which are growing in immunosuppressed conditions, occurring inside many tumors. Although many bacterial strains are attractive candidates for inducing the immune system, it is hard to control cell dissemination and train cells to target tumor. By surface manipulation, we expect to remotely control their position within the tumor tissue being not merely dependent on the bacterial ability to colonize the tumor region.

Chapter 3

Scientific Publications

The publications, composing the core of the dissertation are listed in such a way that they consequently introduce the solution of the problems and follow the dissertation content. The candidate's contributions for each manuscript are described in article's preface. In the present work, articles appear in the following order:

- Rybkin, I., Gorin, D., Sukhorukov, G., & Lapanje, A. (2019). Thickness of polyelectrolyte layers of separately confined bacteria alters key physiological parameters on a single cell level. *Frontiers in Bioengineering and Biotechnology*, 7, 378. <https://doi.org/10.3389/fbioe.2019.00378>
- Rybkin, I., Gorin, D. A., Sukhorukov, G. B., & Lapanje, A. (2017). Method for Synthesis of Protein in Culture of Bacterial Cells: Patent Application TMA170199738. Federal Institute of Industrial Property.
- Rybkin, I., Mirtic, J., Majaron, H., Hrescak, J., Ceh, M., Strancar, J., ... Lapanje, A. (2021). Strain-dependent selective antimicrobial action of cationic polyelectrolytes on Gram-negative bacteria. *BioRxiv*. <https://doi.org/10.1101/2021.08.31.458386>. The manuscript is in the BioRxiv repository and is under final revision for submission to: ACS Nano
- Deev, D., Rybkin, I., Rijavec, T., & Lapanje, A. (2021). When Beneficial Biofilm on Materials Is Needed: Electrostatic Attachment of Living Bacterial Cells Induces Biofilm Formation. *Front. Mater.* 8: 624631. <https://doi.org/10.3389/fmats.2021.624631>
- Gusev, A., Zakharova, O., Muratov, D. S., Vorobeva, N. S., Sarker, M., Rybkin, I., ... Kuznetsov, D. V. (2019). Medium-dependent antibacterial properties and bacterial filtration ability of reduced graphene oxide. *Nanomaterials*, 9(10), 1454. <https://doi.org/10.3390/nano9101454>
- Gusev, A., Zakharova, O., Vasyukova, I., Muratov, D. S., Rybkin, I., Bratashov, D., ... Kuznetsov, D. (2019). Effect of GO on bacterial cells: Role of the medium type and electrostatic interactions. *Materials Science and Engineering: C*, 99, 275–281. <https://doi.org/10.1016/j.msec.2019.01.093>
- Rybkin, I., Pinyaev, S., Sindeeva, O., German, S., Koblar, M., Pyataev, N., ... Lapanje, A. (2021). *In vivo* capture of bacterial cells by remote guiding. *BioRxiv*. <https://doi.org/10.1101/2021.08.06.455395>. The manuscript is in the BioRxiv repository and is under final revision for submission to: Small

3.1 Thickness of Polyelectrolyte Layers of Separately Confined Bacteria Alters Key Physiological Parameters on a Single Cell Level

Rybkin, I., Gorin, D., Sukhorukov, G., & Lapanje, A. (2019). Thickness of polyelectrolyte layers of separately confined bacteria alters key physiological parameters on a single cell level. *Frontiers in Bioengineering and Biotechnology*, 7, 378. <https://doi.org/10.3389/fbioe.2019.00378>

In fact, all bacterial cells experience the effect of confinement in natural conditions. Over time, the cells accumulated on the surfaces get entrapped in various PE matrices, resulting in the formation of stable multicellular systems, as biofilms or flocs. This property of immobilization found important application in biotechnology since confined cells are protected from mechanical influence as well as the product separation is facilitated. Taking into consideration that bacteria require efficient mass transfer and appropriate spatial arrangement to maximize the efficiency of nutrient distribution we could artificially immobilize bacteria. However, conventional approaches such as matrix entrapment are not suitable to provide such conditions. As an alternative, we have designed a system where each microbe should be individually immobilized in strong permeable tailor-made coatings. To reach this purpose, we have developed an artificial confinement of bacterial cells by deposition of several PE layers on the cells, which resulted in the formation of durable coatings at the single cell level. We observed the dynamics of physiological changes of separately confined cells, which also affected the colony development.

Since cell aggregation was observed during the PE application, the authors modified the conventional approach by reducing the number of centrifugation steps and lowering centrifugal force. The protocol was further compared with previously developed protocols by analyzing the polydispersity index of coated cells in both systems, which showed a 2.4 times lower aggregation. Prior to assessing the physiological changes of confined bacteria, the authors determined electrophoretic mobilities of *Escherichia coli* TOP 10 at different stages of cell growth in a gradient of salt concentrations in order to define the most suitable time for PE deposition. To improve the coating procedure, we used the Oshima's soft particle model, which demonstrated that the cells in the exponential growth stage had a 1.1 times higher negatively charged surface than the cells from lag or stationary phases and were thus more suitable for coating.

The deposition of each PE layer was monitored by determining the electrophoretic mobilities using a dynamic light scattering device. To study the effect of PE confinement, coated cells in up to 4 bilayers were observed under a Time Lapse Laser Confocal Microscopy (TLCM) experiment in a specially designed chamber, which provided the most suitable growth conditions. The authors found that PEs were not toxic to the cells of *E. coli*. However, using a growth curve model, it was found that due to the strength of the capsule, the physiological properties of the cells, such as lag phase and colony formation, were altered. Accordingly, analysis of the growth dynamics at a single cell level showed that the lag phase of the coated bacteria was prolonged up to 1.7 times, depending on the number of deposited layers. Over a certain time, the cells left the shells by dividing in a distal position in weaker capsules, while in stronger capsules, the cracking process of the PE wall was randomly distributed. It was shown that due to the interaction of new-born cells with partially coated bacteria, microcolonies were more spherical than with non-coated cells. For analysis of protein expression in cells, both methods, microscopy and spectrophotometric determination were used. Mechanical confinement was found to alter

molecular physiology, as the authors observed an increase in the GFP fluorescent signal up to 5 times and an increase in cell size up to 2 times when the division process was stalled.

Summarizing all the findings, the authors developed a method for the surface modification that allows to individually coat bacterial cells, control the cell growth, surface charge and metabolic activity and allows studying the cell physiology under confinement.

The candidate performed the experimental part, partially performed the statistical analysis, partially prepared the drawings, and also participated in the writing of the manuscript.



Thickness of Polyelectrolyte Layers of Separately Confined Bacteria Alters Key Physiological Parameters on a Single Cell Level

Iaroslav Rybkin^{1,2,3,4}, Dmitry Gorin^{2,5}, Gleb Sukhorukov^{2,6} and Aleš Lapanje^{1*}

¹ Department of Environmental Sciences, Jožef Stefan Institute, Ljubljana, Slovenia, ² Remote Controlled Theranostic Systems Lab, Institute of Nanostructures and Biosystems, Saratov State University, Saratov, Russia, ³ Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, ⁴ Department of Reactive Transport, Helmholtz Zentrum Dresden Rossendorf, Institute of Resource Ecology, Leipzig, Germany, ⁵ Center for Photonics and Quantum Materials, Skolkovo Institute of Science and Technology, Moscow, Russia, ⁶ School of Engineering and Materials Science, Queen Mary University of London, London, United Kingdom

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East China University of Science and
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*Correspondence:

Aleš Lapanje
ales.lapanje@ijs.si

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Confinement of bacterial cells in a matrix or in capsules is an integral part of many biotechnological applications. Here, the well-known layer-by-layer method of deposition of a polyelectrolyte film a few nanometers in thickness to confine separated bacterial cells in permeable and physically durable shells has been examined. Due to the physical properties of such a confinement, we found that this method enables investigation of effects of physical barriers against mass gain and cell division. Using the method of time-lapse confocal microscopy, we observed a prolonged lag phase, dependent on the number of polyelectrolyte layers. In the confinement, both the GFP fluorescent signal from the leaking T7 promoter and the cell size were increased by factors of more than five and two, respectively. This creates a paradigm shift that enables use of mechanical entrapment for control of bacterial cell physiology and opens possibilities of controlling the division rate as well as gene expression. These effects can be attributed to the perturbation of the sensing of the cell size, which results in disproportional synthesis of a cell envelope impinging the intracellular material and compels cells to grow rapidly. In addition, the charged surface of cells enables prolonged intercellular physical interaction and results in spherically shaped microcolonies.

Keywords: time-lapse confocal microscopy, polyelectrolytes, layer-by-layer encapsulation, electrostatic interactions, cell surface modification

INTRODUCTION

Confinement of bacterial cells in matrices or capsules is an integral part of many applications in biotechnology. The development of different confinement methods of microbial cells is in demand in many industrial and agricultural processes such as microbial based biofertilization in sustainable agriculture, bioaugmentation for water processing or wastewater treatment using carriers in packed, fluid bed reactors or sand filters. In addition, such confinement can be used in medicine to providing protective barriers for probiotic bacteria against harsh conditions in the human gut. Protection of probiotic bacteria is currently gaining much interest due to the most recent discoveries of the importance of healthy human microbiome on human health

(Everard and Cani, 2013). Currently, there are many different well-established methods of microencapsulation of microorganisms that are based on extrusion, emulsion, spray drying, electrospinning and other approaches that have been reviewed in the literature (Martín et al., 2015). One of the less conventional methods used for bacterial encapsulation, contrasts with other methods, which enables tailor-made surface modification of bacterial cell, is based on deposition of polyelectrolytes over the surface of the cell using a layer-by-layer (LBL) approach (Hillberg and Tabrizian, 2006). Recently, this approach of electrostatic modification of the bacterial surface has resulted in better adherence of probiotic bacteria to the surface of gut epithelia (Anselmo et al., 2016) and when cells are made in an LBL manner they can enhance the efficiency of vaccination due to the better presentation of the vaccine to immune cells (Speth et al., 2016). The surface modification of single bacterial cells by such a simple method can be a good alternative to the genetic modifications designed to equip cells with different antigen presenting enhancers. When bacterial cells are entrapped within a matrix it has an impact on their physiology, which is mostly attributable to the lower diffusion of nutrients, metabolites, and other charged molecules (Lieleg and Ribbeck, 2011). Using well-established matrix entrapment methods the induction of fermentation activities, the ability to externally induce expression of specific proteins, for example green fluorescent protein (GFP) and the germination of spores have been observed and it was shown that it causes the “skin effect” of encapsulated cells. It is speculated, but not further investigated, that the skin effect is attributable to the increased permeability of the cell lining as a response to the less permeable capsule wall. It has also been shown that confinement induces quorum sensing genes to reach characteristic cell densities sometimes even at the single cell level (Carnes et al., 2010).

LBL entrapment of bacterial cells is however different from entrapment in a matrix such as hydrogel (e.g., alginate, chitosan). LBL entrapment represents the entrapment of a single cell within a strong mesh of thickness in the nanometer range with pores that enable nutrients to pass freely but which keeps cells mechanically separated from the surrounding microenvironment. The polyelectrolytes (PE) deposited on the bacterial cell surface by the LBL method result in tailor-made layered capsules in which each of the layers is only 2–3 nm thick (Hillberg and Tabrizian, 2006; Franz et al., 2010; Fakhrullin and Lvov, 2012). The number of layers determines the thickness and strength of the final capsule (Kolasinska et al., 2007). The capsules formed in this way can tolerate a force of more than 300 MPa (43,500 psi) (Gao et al., 2001; Vinogradova, 2004; Fery and Weinkamer, 2007), and the layers are porous, enabling approximately 4–10 kDa large polar molecules to pass unobstructed (Georgieva

et al., 2004). Under physiological conditions they are permeable for molecules of up to 75 kDa (Tong et al., 2005). In contrast to the diffusion properties of entrapment matrices based on gels such as alginate, increasing the thickness of the capsule by incorporating more nano-sized layers of PEs (polyelectrolytes) does not significantly affect the diffusion of molecules (Georgieva et al., 2004; Kozlovskaya et al., 2011).

In view of these properties of the LBL layers, our main aim was to determine effects of an LBL shell consisting of different numbers of layers on growth and division, metabolism and colony establishment of separated *Escherichia coli* cells. It is known that cationic polyelectrolytes can be toxic for bacteria (Kügler et al., 2005), and accordingly we used here a strain that does not show toxic response after being exposed to the highly charged polyelectrolytes and enables us to observe the aforementioned physiological parameters resulting from the mechano-physical interactions of polyelectrolytes with bacterial cells. We also adapted an LBL procedure since in most cases when bacterial cells are used as an LBL template, formation of aggregates usually results (Hillberg and Tabrizian, 2006; Franz et al., 2010; Fakhrullin and Lvov, 2012), and precludes observation of single cells. Accordingly, our specific aims were to (i) prepare a method for time-lapse observation of the physiology of single cells covered with polyelectrolytes, (ii) determine the effects of physical constraint on growth, division and constitutive expression, and (iii) assess the effects of the LBL shell on the process of the microcolony formation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

In all experiments we used non-motile cells of *Escherichia coli* top 10 strain [F[−] mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG], transformed with pRSET-emGFP plasmid (Thermo Fisher Scientific Corp.) and standard electroporation procedures (Sambrook et al., 1989). The plasmid contains T7 promoter regions upstream of the emGFP reporter gene and ApR cassette. Since the cells are deficient in T7 polymerase, the GFP is transcribed only on a basis of the leakage of the promoter leakage. The transformants were cultivated at 37°C on nutrient agar (NA) plates (Sigma-Aldrich) supplemented with ampicillin (100 μg/ml, Sigma-Aldrich)—NAamp.

Prior to the experiments, we prepared overnight liquid cultures from a single colony in NBamp medium. One milliliter of this culture was transferred into 100 mL of the fresh medium and incubated until optical densities appropriate for conducting the particular experiments were obtained. All liquid cultures were incubated with shaking at 37°C and 150 rpm.

Determination of the Appropriate Growth Phase of Bacterial Cells for PE Deposition

For efficient polyelectrolyte deposition, the electrostatic properties of the surface of bacterial cells in different growth stages were determined. The charge densities (ZN) and the electrostatic softness parameter ($1/\lambda$) of bacterial cells were determined from a non-linear regression analysis of the ionic

Abbreviations: ApR, Ampicillin Resistance; auc, area under the curve; ELS, Electrophoretic Light Scattering; GFP, Green Fluorescent Protein; ISDEM, Ionic Strength-Dependent Electrophoretic Mobilities; LBL, Layer-by-Layer; NA, Nutrient Agar; NB, Nutrient Broth; OD, Optical Density; PE, polyelectrolytes; PEI, Poly(Ethyleneimine); ppGpp, Guanosine pentaphosphate; PSS, Poly(Sulfonate); RNAP, RNA polymerases; TLM, Time-lapse confocal microscopy; TRITC, Tetramethylrhodamine isothiocyanate; ZN, Charge densities.

strength-dependent electrophoretic mobilities (ISDEM) of bacterial cells using Ohshima's soft particle equation as a model (Ohshima, 1995).

To obtain the ISDEM, the cultures were washed 3 times in 0.00062 M NaCl solution. Then 100 μ l of washed culture was mixed with 900 μ l of the 0.00062 M NaCl solution. The electrophoretic mobilities of bacterial cells were measured using an ELS device (Zetasizer Nano, Malvern, USA). The ionic strength of the suspension of bacterial cells within the measurement cuvette was automatically altered by titration with an MPT-2 titrator. Measurements were made within the linear gradient of ionic strengths of NaCl from 0.00062 to 0.11 M in 12 steps of 0.0091 M per step by addition of a 0.155 M solution of NaCl. The data was obtained from 3 experimental replicates in which each of the measurement was acquired from the accumulated values of 70 separated ELS values. In all ELS measurements the polarities of electrodes were fixed, with an automatically adjustable voltage. The approximate ionic strengths were obtained from the titrator and the exact values for the cell suspensions within the cuvettes and were determined on a basis of conductivity values obtained in each ELS measurement. The ionic strengths in the ELS measurements were calculated on the basis of the standard curve values obtained from the measurement of the conductivities of different concentrations of NaCl solutions in water.

Preparation of PEs for Layer-by-Layer Encapsulation

We used the negatively charged sodium poly(styrene sulfonate) (PSS) with MW = 70,000 and poly(ethyleneimine) PEs (PEI), with MW = 750,000, both from Sigma-Aldrich, to encapsulate bacteria based on electrostatic principles (Sukhorukov et al., 1999). The solutions of PEs in Milli-Q (ultrapure water type 1) water (2.5 mg/ml, pH7 adjusted by NaOH or HCl) were prepared by solubilizing PEs, initially by stirring and then by the sonication (35 kHz, 100 W) for 15 min. In experiments using labeled PEI with tetramethylrhodamine isothiocyanate (TRITC) the PEI was labeled using NHS ester labeling of amino biomolecules (Lomant and Fairbanks, 1976). Briefly, the TRITC (11 mg in 11 mL DMSO solution) was added to a PEI solution (2.5 mg/ml in 20 mL of water) in a 50 mL tube and incubated for 4 h at room temperature with constant stirring. To remove the residual dye, the solution after labeling, was dialyzed for 3 days using a dialysis tube (Orange Scientific) with nominal molecular weight limits between 12 and 14 kDa.

Encapsulation Procedure

Before the procedure, cells were grown at 37°C by shaking at 150 rpm until OD₆₆₀ reached 0.2. Cells were concentrated by centrifugation of 50 mL of the culture at 5,000 g for 6 min. To wash out residues from the medium the pellet was washed three times by resuspension in 30 mL of 0.9% NaCl solution and centrifugation of the suspension at 3,000 g for 3 min.

To prevent formation of aggregates of the cells, we determined the parameters associated with efficient single cell PE deposition. First, we deposited PEI on cells by adding a 0.25% solution of PEI at pH 7 (adjusted with HCl) in Milli-Q water to the washed cells

(OD₆₆₀ 1.2) in a 1:1 v/v ratio. This suspension was incubated at room temperature for 5 min. Unattached PEI was removed from the suspension by centrifugation at 900 g for 2 min. The obtained pellet was washed twice by gentle addition of 1 ml of 0.9% NaCl over the pellet taking care to avoid dissolving it. After washing, the PEI covered cells were resuspended in 0.9% NaCl solution. We then added a PSS layer to the PEI covered suspension using a solution in Milli-Q water of PSS at pH 7 (adjusted with NaOH) in a 1:1 v/v ratio. The washing step was the same as was used with PEI, except that 3 min centrifugation at 1,500 g was used to obtain a sufficiently firm pellet to permit washing by pipetting. By repetition of these two steps, we were able to deposit up to 4 such bilayers on the surface of bacterial cells while keeping their aggregation low. The aggregation was monitored by microscopy.

Effect of Encapsulation on Growth of Cells and GFP Fluorescence Signal

To measure the growth of the population of bacterial cells we inoculated 5 μ l of the (OD₆₀₀ = 0.38) uncovered, the control population, or PE covered with the 1, 2 or 3 bilayers, into the microplate wells containing 200 μ l of fresh and sterile NBamp media. During the 16 h incubation period at 37°C with vigorous discontinuous shaking prior to each measurement, we measured the OD₆₀₀ every 20 min and obtained the fluorescence signal at 485/528 nm. The fluorescence signals and optical densities obtained in this way were normalized to the fluorescence signal from the control population and the OD₆₀₀ of the media, respectively. Each experiment was performed in triplicate and Student's *t*-test was used for statistical evaluation.

Time-Lapse Confocal Microscopy (TLCM)

To enable precise measurement by TLCM, it was necessary to distribute cells on a thin planar surface and provide them with appropriate growth conditions to support a few hours of observations. Accordingly, cells were distributed on an NA solid medium within a simple chamber prepared according to a published method (de Jong et al., 2011), with modifications. Briefly, each chamber was created on a surface of a clean cover slip (76 mm $\times$ 26 mm) attaching one surface of a rectangular frame made from the double-sided tape 5 cm long $\times$ 2 mm wide by cutting out a rectangular shaped 1 cm $\times$ 3 cm aperture. The sterile NA was solvated by heating in a microwave oven, then rapidly distributed inside the frame by pipetting. Before the NA gelled, the slide glass was put on top of the medium touching the frame. This formed a smooth and even NA surface confined between two glass surfaces and separated by the thickness of the tape. We left this provisional chamber in a refrigerator for 10 min at 4°C to form a solid NA matrix. The non-attached slide glass was then removed, the protective layer of the double-sided tape was stripped off and then 4 μ l of culture, with a final OD₆₆₀ of $\sim$ 0.4 was evenly distributed over the exposed NA surface. Finally, the chamber was sealed with by attaching the cover slip to the sticky surface of the frame enclosing the NA.

The chamber with the cells was equilibrated at 37°C for 15 min and then transferred to a pre-warmed confocal microscope (a Leica TCS SP8X confocal laser scanning microscope equipped with temperature control system cube and a box thermostated at

37°C). The TLCM was performed at 1000x magnification using an objective lens (HCX PL APO 100x/1.44 OIL) immersed in oil. We observed cell growth and division using the excitation at 525/36 and emissions 525/41 and 605/60 with 850–900 and 750–800 V gains for emGFP and TRITC fluorescence, respectively. The morphology of cells and the quantity of cells were observed using white light and a condenser as the objective lens.

Analysis of TLCM Micrographs

To determine the growth properties of the cells entrapped in the PE layers, we analyzed the pictures using Fiji software (Schindelin et al., 2012). Prior to the experiment, we selected fields occupied by similar amounts of cells under the microscope. From the fluorescent images that were obtained forming a hyperstack we discarded those that were of low contrast or unfocused. The prepared hyperstacks of the images were then converted to a stack of binary images using the “make binary” plugin. On such prepared images we measured the surface area of cells at consecutive time points using the “analyze particles” plugin to determine temporal changes of the cell biomass. The experiments were performed in triplicate and *t*-test was used for statistical comparison of amount of cell biomass per time of each group of cells with different numbers of deposited PE layers. Based on the data that was obtained, we plotted the growth curves and analyzed them with the R package, Growthcurver (Sprouffske and Wagner, 2016). We calculated growth curve properties such as the growth rate (*r*), the maximal population size (*K*), the time (*t*_{mid}) needed to reach *K*/2, the time at the point of the fastest division rate (*t*_{gen}) and the area under the curve (AUC) parameters. The λ _{lag} parameter was obtained from the intersection point calculated from linear equations of two regression lines from data points of exponential and lag phases, respectively. The last data point belonging to the set of points within each of these two phases was determined by calculation of the regression coefficient of lines fitted to the data points (*R*). The lag phase points were determined by starting linear regression calculations from the first point on the graph and using all points until reaching the data point that significantly decreased the *R* value. In the linear regression calculations of the exponential phase we used the point that was not included in the lag phase as the starting point and all the points up to the last point that did not significantly decrease *R*.

RESULTS

Effects of the Entrapment on the Cell Growth and Division

By analysis of the growth curves on the population level we observed a significantly prolonged lag phase (Figure 1A) for cells on which we deposited more than one PE layer. The longest delay of the lag phase ($\lambda = 5$ h) was observed when 4 bilayers had been deposited. Although the durations of the lag phase of the populations of cells covered with 2 or 3 bilayers were not significantly different from each other, they both showed significantly longer lag phases than the control population of untreated cells (control $\lambda = 1.5$ h, 2, and 3 bilayers $\lambda = 2.5$ h).

The delay of the exponential phase is caused by either (i) the toxicity of PEs that increases the proportion of killed cells within the inoculum of entrapped cells or (ii) the interference of entrapment with the growth and division of live cells. Accordingly, to resolve this ambiguity, we performed growth experiments on the single cell level using a TLCM approach.

Since covering of the cells with PEs tends to form aggregates (see **Supplementary Material Data Sheet 1**), we optimized the conventional method to avoid aggregation that can interfere with the diffusion of nutrients. Precise coverage using the updated method resulted in a switch of the surface charge of the bacterial cells from the initial -30 mV to the 20 mV. Using this approach, we were able to avoid aggregation (see **Supplementary Figure 1**) when we deposited as many as 10 layers of PEs (Figures 2A,B).

Using TLCM on such covered cells, we observed growth in more than 99% of cells within each of the fields of view in both the control and entrapped population of cells and we failed to observe any toxicity attributable to the LBL entrapment. However, we observed a prolongation of the lag phase which was proportional to the number of the PE layers (Figure 2C). The longest delay of the lag phase was observed for cells entrapped in 8 layers ($\lambda = 111.5 \pm 1.3$ min), whose lag phase is almost 2 times longer than that in the control population of cells ($\lambda = 65.2 \pm 9.9$ min) (Figure 2D). The acceleration of the growth, which is the transition from the lag to the exponential phases, is overall lower for cells covered with PEs ($p < 0.1$)

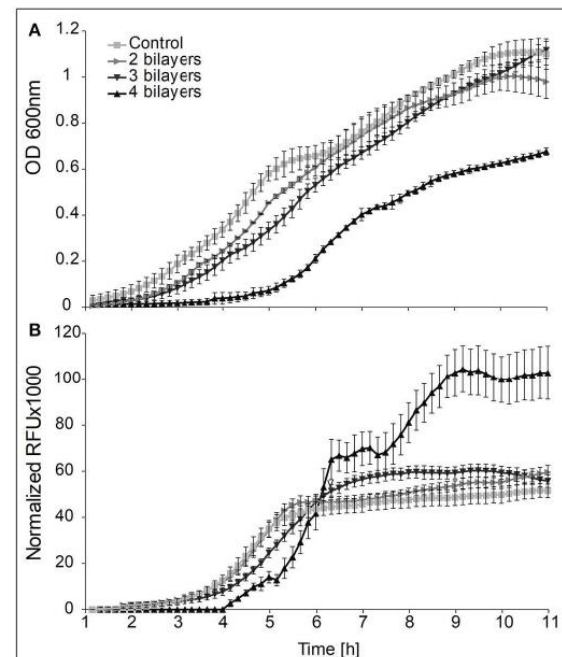
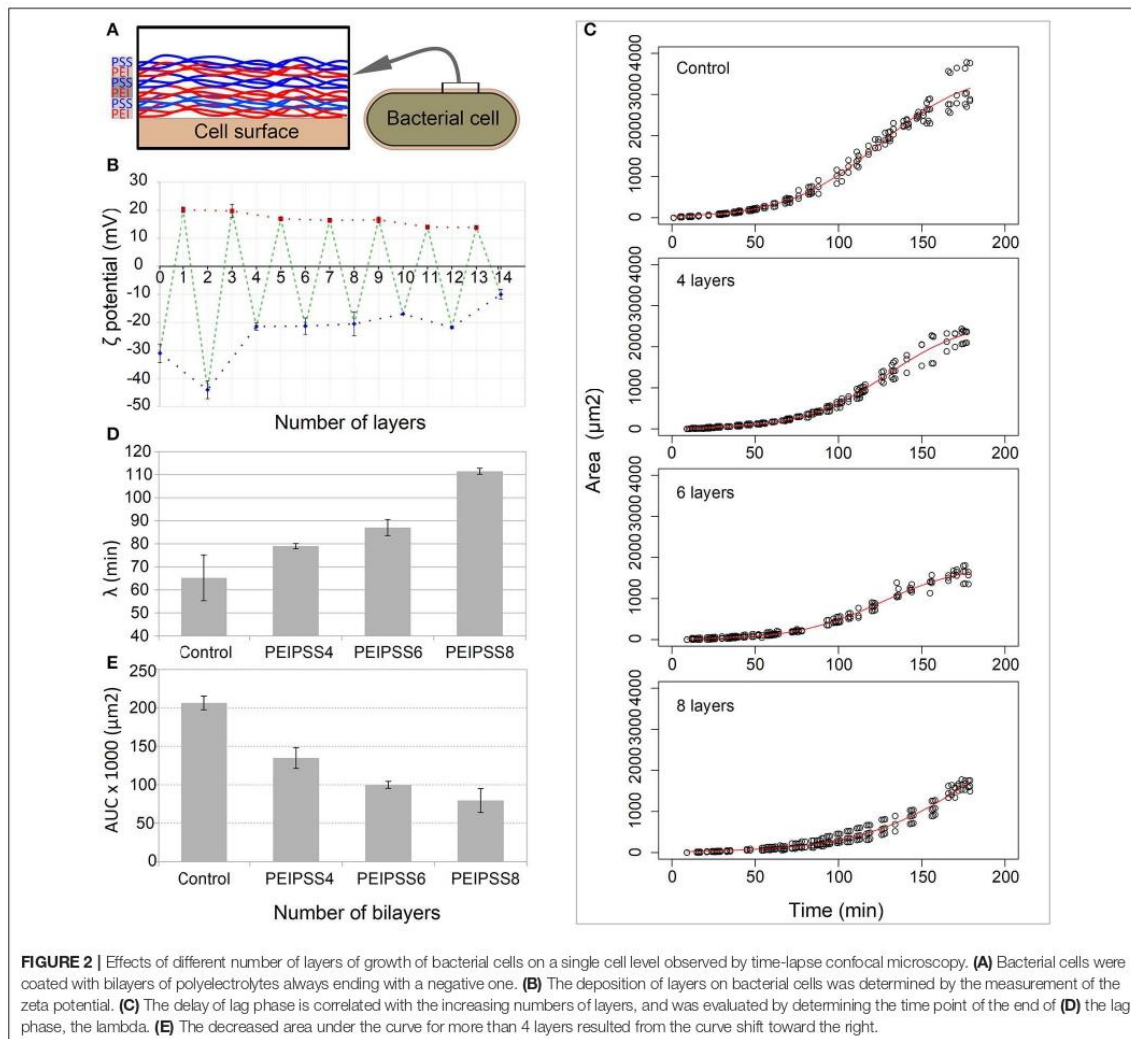


FIGURE 1 | (A) Effects of the entrapment of cells in layers of polyelectrolytes on growth and **(B)** GFP fluorescence produced from the promoter leakage without induction of the bulk population of bacterial cells.



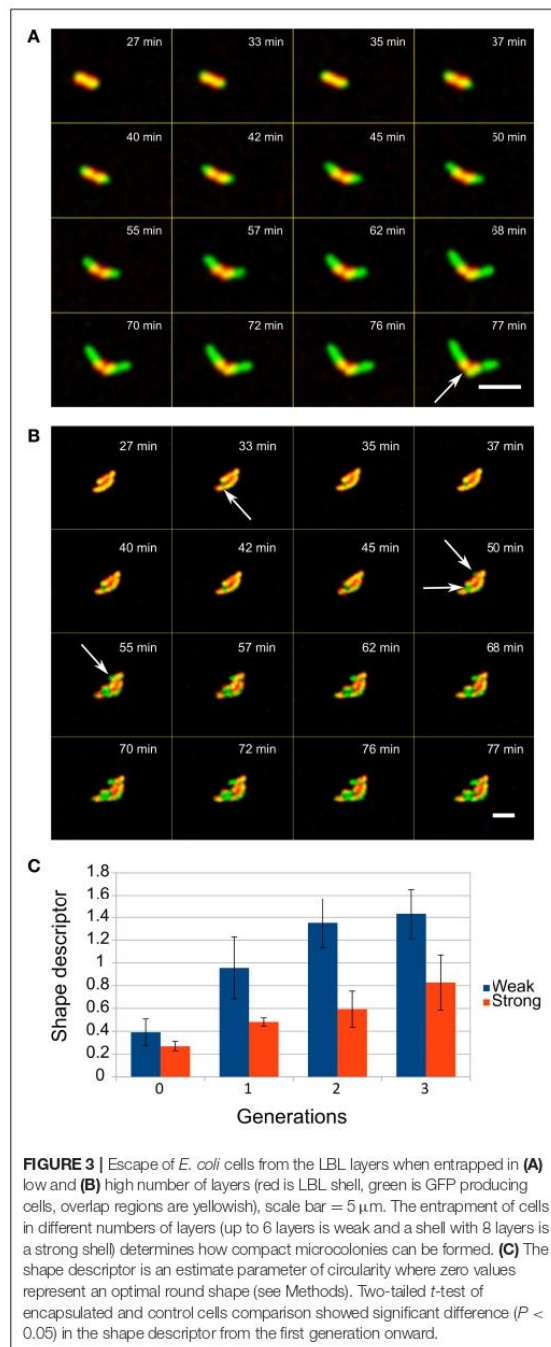
and decreases proportionally with the increasing number of added layers (see **Supplementary Table S1**). Other growth curve properties such as the t_{mid} and AUC parameters (**Figure 2E**) of the populations of cells covered with the PEs were significantly smaller than in the control population of cells. On the other hand, the maximum growth rate (r) and generation times were not significantly different in all experiments except those in which the cells were covered with 8 layers of PEs where we observed slower growth rate and consequently longer generation times (**Supplementary Table S1**, $p < 0.05$).

Escape of the Cells From the LBL Layers

We measured the number of layers determining the escape of the cells from the capsules by analyzing the growth of each of the

covered cells. With up to 6 layers, cells escape by their elongation which initiates cracking of the capsule at the distal positions above the cell tips leaving a belt of PE layers surrounding the cell (**Figure 3A**). However, when more layers were deposited on bacterial cells this resulted in the escape of the cells by decreasing the overall integrity of the capsules, not only on distal part, which was observed as occurrence of several green fluorescent patches of the cell material within the yellow-reddish walls of the capsules (**Figure 3B**). Then small bulges of the cell material were formed, from which cells began to grow outward from the capsules.

Analysis of the events after the initial escape of cells showed that a deposit of 8 layers of PEs on cells results in formation of significantly more spherical



microcolonies with smoother edges than in populations of cells where 6 or fewer layers had been deposited (*t*-test, $p < 0.05$, Figure 3C).

Effects of Entrapment on the Shape of Bacterial Cells and GFP Fluorescence

On the population level we obtained the maximum intensity of the fluorescent signal in the middle of the exponential phase from all of the cultures with either entrapped or free cells. However, the culture with cells entrapped in 3 and 4 bilayers showed 1.2 and 2.1 times higher fluorescence signals, respectively, than the signals obtained from suspensions of untreated cells (Figure 1B).

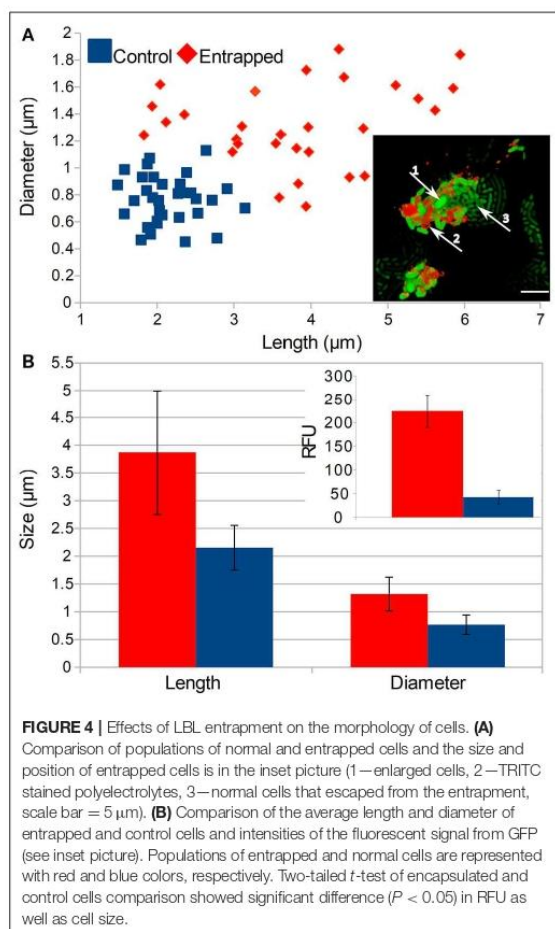
On the single cell level, we observed that numerous cells entrapped inside the more than 6 PE layers were aggregated and failed to divide (Figure 4A, insert). These cells formed a distinctive population of cells that were either (i) of the same length as the control but with larger diameters (ii) longer than the control, but of the same diameter or (iii) bigger in both dimensions than the normal *E. coli* cells (Figures 4A,B). On average, both the size and diameter of entrapped cells were significantly larger than those of normal cells ($p < 0.01$). Those larger non-dividing cells also showed 5 times higher intensity of the fluorescent signal than normal *E. coli* cells that were not entrapped ($p < 0.01$).

DISCUSSION

The cell acts as a micrometer sized particle with a persistent negative electrostatic charge. We found this property to be of high biotechnological value since different oppositely charged molecules can be deposited on the cell surface, enabling simple and robust surface modification of the cell. The modified cellular surface can be positively charged due to the polyelectrolyte deposition, and this can enable fast attachment of cells on the surfaces, resulting in an immobilized biomass. The control of division by polyelectrolyte capsules can determine the exact and synchronous start of the biotechnological process. Mechanical forces opposing the division process increase the expression that can result in higher yields. By mixing oppositely charged cells the aggregates can be formed and perhaps can enable more efficient growth of co-cultures due to the spatial distribution of the different cells. For the most efficient encapsulation it should be considered that the polyelectrolytes and the bacterial cells must be carefully chosen to prevent aggregations in any particular encapsulation experiment.

In our experiments it was observed that physical constraints cause delay in division and excessive gain of cell size as well as an increased transcription rate of the constitutively expressed operon. Since the confinement modifies the gene expression without chemical induction, it offers new opportunities to control bacterial cell physiology, which has not been possible previously. On the one hand this paradigm shift opens possibilities of controlling the division rate as well as gene expression with mechanical constraints, and on the other hand, since cells become electrostatically adhesive, surface modification can be used for spatial positioning of individual cells on surfaces or in aggregates involving either single species or complex consortia (e.g., surface attached biomass or artificially formed flocs).

The observed delay in the onset of the division of cells (see Figure 2) until disruption of the PE capsule and their escape



from the entrapment by the cell division (see **Figure 1**) is caused only by either decreased diffusion flux of nutrients or increased mechanical strength of the PE shell, since the third possibility, toxicity of the PE layers, was not observed. The deposited polyelectrolyte film can affect the physiology of the cell due to the (i) diffusion, resulting from thickness of the film and its pore sizes, and (ii) strength, which is dependent on the charge densities of the polyelectrolytes, the number of their layers and branching as well as ionic strength of the media. The diffusion property of LBL walls is less likely to cause delayed growth, since it has been shown in several studies of activities of LBL encapsulated enzymes that enzymatic substrates larger than 6 kDa (in some cases up to 75 kDa), such as short peptides and dextran molecules, can freely pass the PE wall (Antipov et al., 2002; Qiao et al., 2005). Since we used NA, more than 70% of which consists of peptides with mass below 1 kDa, the PE layers cannot affect the flux of the nutrients. Moreover, the limiting diffusion flux for growth of *E. coli* cells should be two orders of magnitude lower than the lowest reported diffusion rate for LBL layers ($D = 10^{-12}$

$\text{cm}^2\text{s}^{-1} = 10^{-4} \mu\text{m}^2\text{s}^{-1}$, $t \approx 80$ ms of small molecules of 332 Da) (Kozlovskaya et al., 2011) since only $\sim 10^9$ glucose molecules are transported in 2,400 s during the most active growth (Milo et al., 2009) and the PE layers are thin (1 bilayer is 4 nm) (Sukhorukov et al., 1998; Kolasinska et al., 2007). Therefore, during the most intensive growth, up to 8 PE layers (16 nm thick) can adequately provide the requisite amount of the necessary glucose molecules. In addition, in our experiments, since cells were in a lag phase, a stage of slow growth, they needed a lower flux of nutrients, and the NA medium #2 is composed mostly of short proteins and amino acids that are transported and metabolized more slowly than glucose. These assumptions are also consistent with our observations indicating that growth of cells covered with a single bilayer was not significantly different from that of the control.

Although each separate bilayer in the 8 bilayer structure does not obviously contribute to the decrease of the diffusion properties (Kozlovskaya et al., 2011), the strength of the capsule is significantly increased by 0.05 μN per bilayer (Vinogradova, 2004). In LBL capsules composed of PEs similar to those used in our study, a Young modulus of between 170 and 300 MPa was determined (Gao et al., 2001; Vinogradova, 2004; Fery and Weinkamer, 2007). Hence, it is intuitive that the forces which counteract the cell growth result in a prolonged lag phase, although the LBL cores in the aforementioned studies are not cells and the absolute values cannot be directly applied to our system. However, we do observe the growth delay and number of layers are therefore significantly correlated, probably due to the progressive increase of the strength of the capsule with the number of layers (see **Figure 2**). In addition, the number of layers also determines the type of the escape. Cells can escape from the distal parts of capsules when they are enclosed in a few layers but if more than 4 bilayers are added, they will tear the capsule in the middle. Although the tips of the *E. coli* cells are expected to be more negatively charged due to the increased levels of cardiolipins, which contribute to stronger interaction with the positively charged PEI, the curvature of the capsule is larger at the tips than in the remainder of the cell surface and this causes relaxation of layers due to the Ostwald ripening. Since *E. coli* cells grow from the central region, the surface of the cell must slide along the capsule wall to break the capsule at the tip. This sliding might be possible on account of the lower negative charge densities along the middle part of the cell, since the membrane in these regions contains higher amounts of less negatively charged phosphatidylethanolamine phospholipids (Matsumoto et al., 2006).

When a strong, thick layer is deposited on the surface of the cell, it appears that the adaptive properties of the shape of the *E. coli* cells help the cells to escape from the capsule whose wall is partially perforated by the tearing. The squeezing properties of the *E. coli* cells reported by Männik et al. (2009), are crucial for the escape of the cell through the 300 nm narrow pores within the capsule wall (see **Figure 3B** at 50 min). However, this feature alone would be insufficient for a successful escape, since the initial pores can occur randomly anywhere along the capsule wall and the flexibility and rigidity of the cell should be also considered (Amir et al., 2014). For example, we observed that the partial escape from the pores bends cells in one direction (see **Figure 3B**

at 55 min). The cell then preserves its shape by its elastic response and the remodulation or regrowth of the cell wall, which causes increased forces on the occupied pore on the opposite side of the cell. This pushes the cell through the pore, and also increases the widening of the pore as it enables insertion of the opposite tip of the cell into the pores, which are formed during the consecutive events in the continuous expansion of the cell volume and tearing apart of the capsule wall.

Since different sorts of interferences with the physical intercellular interaction result in the formation of diverse colony shapes (Ishii et al., 2004; Seminara et al., 2012), we showed that with polyelectrolytes we can also interfere with the formation of microcolonies. Here the capsule wall is not uniformly strong (see **Figures 3A,B**) and after its disintegration, patches of the capsule wall stay attached to the cell surface. This keeps the cells together due to the increased electrostatic adherence of one cell to another, resulting in formation of compact microcolonies (see **Figure 3C**).

When we added more than 6 PE layers, we made capsules that were strong and not ruptured by the growth of cells. In such strong capsules we observed increases in both the size of cells and GFP expression from the T7 promoter without its induction. Since the cells were not deprived of nutrient, but were constrained, we expected a paradox in which the mechanical constraint prevents cells from growing but at the same time induces their growth. This paradox can occur because bacterial cells sense membrane capacity which can support the normal activity of the cell with the amount of available free membrane lipids in the cytosol. The presence of membrane lipids inhibits ppGpp activity, which results in increased central metabolism involved in the synthesis of cellular components (Vadia et al., 2017). Hence, in our system it is expected that the amount of the free membrane lipids is in excess in the cytosol and this results in constantly engaged anabolic metabolism, forcing cells to synthesize cellular components continuously. This can indirectly increase GFP synthesis (see **Figure 4**) through the increased number of (i) plasmids and (ii) RNA polymerases, resulting in the decreased specificity of the RNA polymerases (RNAP) transcription.

The process of cell division and protein dilution rate as well as gene copy numbers are usually disproportionate in one cell, since chromosomes can be synthesized faster than cell division can occur (Klump et al., 2009). Consequently, in rapidly dividing cells, which contain the most disproportionate levels of cellular components, it was observed that the level of constitutive expression is higher due to the disproportionate number of chromosomes in one cell. In our GFP expression system, the T7 regulated operon leaks without any regulation, and its transcription can be dependent only on the cell status, the operon therefore acting as a constitutive promoter. Moreover, when using physical constraint by depositing PE layers, the growth and division of the cell are limited, and one can expect even more chromosomes producing more RNAP and at the same time due to the disproportionation more plasmids per cell can be expected as well. Prevention of separation into daughter cells decreases dilution of the intracellular proteins including RNAP, which can in turn decrease the specificity of transcription and increase leakage,

resulting in an increased GFP signal. The amount of RNAP is therefore dependent on the growth rate. Faster growth rate produces more RNAP, either free, transcribing or non-specifically bound (Klump and Hwa, 2008). Since the dilution of proteins by division is prevented and anabolic metabolism is increased as a result of the entrapment, the levels of unbound RNAP are even higher, inducing higher rates of non-specific transcription including the GFP gene in this simple genetic system.

In addition, we demonstrated here for the first time the effects of physical constraints on bacterial physiology on the *E. coli* model organism. Our results open a new vista of the physiological responses of bacteria in different environments such as biofilms, porous materials, infected tissues, and intracellularly inside amoebas or macrophages, where mechanical constraint can play a role.

CONCLUSION

Careful deposition of polyelectrolytes enables a single cell encapsulation in a capsule with the wall thickness of a few nanometers. The polyelectrolytes are not toxic to *E. coli*, but due to the high mechanical strength of the capsule wall the physiological properties of the cell such as lag phase, colony formation and leakage expression are affected. Accordingly, measurements at a single cell level showed that the number of polyelectrolyte layers correlates positively with the duration of the lag phase. Over time, cells can escape from the shells at the distal position in weaker capsules and in stronger capsules the cracking process of the polymer wall is randomly distributed. Due to the intracellular electrostatic interactions, the escaped bacteria formed more spherical microcolonies than non-encapsulated bacteria. The mechanical stalling of the division also effects the cellular molecular physiology and biochemistry, since we observed high increase in GFP fluorescent signal from the leaking operon and 2–3 times increased length and width of the encapsulated cells when their division process was stalled. We can conclude that the encapsulation of cells described here can be a versatile instrument for the modification of the cell surface without applying genetic tools and might be suitable when the genetically modified organisms may not be used.

DATA AVAILABILITY STATEMENT

All data generated and analyzed during this study are included in this article/**Supplementary Material**. Raw datasets are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

IR executed the experimental part and performed statistical analysis as well as contributing partially to the writing. DG and GS were involved in the conceptual design of the experiments and they contributed to the critical reading of the manuscript.

AL developed the idea and concept, designed and executed the experiments, and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbioe.2019.00378/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Thickness of polyelectrolyte layers of separately confined bacteria alters key physiological parameters on a single cell level

1 Supplementary results

1.1 Entrapment procedure

Our goal was to observe the effects of mechanical restriction of the PEs while avoiding diffusion restrictions as much as possible and consequently, it was necessary to keep cells separated. The current procedures of deposition of PE on the surface of bacterial cells are prone to the formation of aggregates after separating out the nonattached PEs by centrifugation (Fig. S1). We improved the method by selecting the cells in an appropriate growth stage and modification of the centrifugation step. The cells in the exponential phase showed the highest electrophoretic mobilities (Figure S1A) and significantly more negative surface charge (Figure S1B) than those in the lag phase as determined through Ohshima's soft particle model (Duval and Ohshima, 2006). Although we also tested the LBL procedures in all three phases, according to our expectations we were able to prepare the most dispersed cell suspension when the cells were in the exponential phase.

The most critical step was the repeated washing out of the excess PE by centrifugation. This resulted in excessive aggregation of cells and consequently, we developed a protocol in which just one centrifugation was used after PEI deposition on cells and then the supernatant was exchanged 3 times for fresh physiological solution. With this method, cells were efficiently covered with a deposited layer of PE (see changed potential after each deposited layer in Figure 2B). Alternatively, we observed the change of the color of cells from green to red-orange due to the presence of TRITC stained PEI on the cell surface (Figure 3A). Using this modified approach, we were able to prepare up to 14 layers, or 7 bilayers of PEI/PSS. After more than 7 bilayers were deposited, the bacterial suspension became less stable, which can be observed by steady decrease of zeta potential toward 0 mV with -30mV to -10mV and 20mV to 13mV values for first to the last bilayers, respectively (see Figure 2B). This developed approach resulted in the suspension of LBL covered cells similar to the control (Figures 1S, C and D), which enabled single cell confocal time lapse microscopy experiments.

2 Supplementary discussion

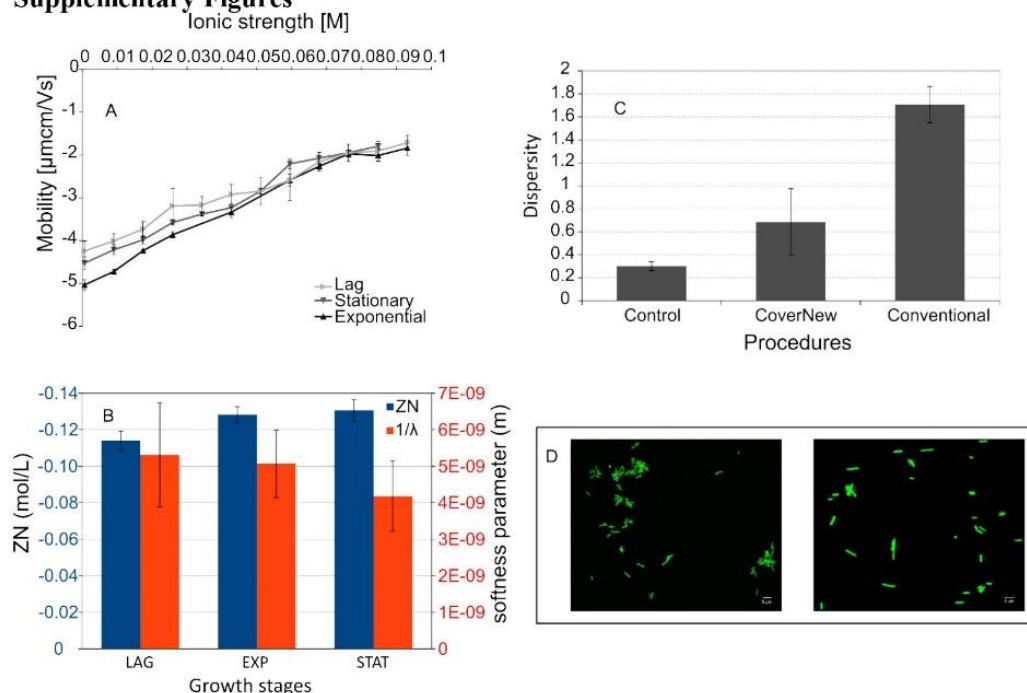
From micrographs of previous reports (Balkundi et al., 2009; Franz et al., 2010; Hillberg and Tabrizian, 2006) and our preliminary tests, it can be deduced that aggregation is a common problem during the deposition of PE on bacterial cells. The removal of the PE by centrifugation and washing out is the most critical step. We suppose the bacterial cells are of lower mass with less momentum than the bigger eukaryotic cells, since the formed clumps of cells can be disintegrated into smaller aggregates containing as few as 10 cells that is equivalent to the size of the average eukaryotic cell. Hence, we decreased the number of centrifugation steps as well as centrifugation force.

Supplementary Material

The charge density on a surface of bacteria seems to play a role, since we observed that the cells in the exponential phase were easier to encapsulate without aggregation. On one hand this can result from higher charge densities at the same softness parameter than in the lag phase (see Figure S1B) resulting in better PE interaction with the cell surface. On the other hand, it may be due to the lower amount of debris of dead cells and thinner surface layers of extracellular polysaccharides on cells than in the stationary phase. Cells were also needed to be vital whereas in the stationary phase culture cells are deprived of nutrient and the proportion of death cells is increased thus not appropriate for using in our experiments.

However, the larger numbers of layers results in a higher aggregation rate which was observed under the microscope in experiments using more than 8 layers. The results of zeta potential measurements showed the inclination toward zero, which is a sign of a less stable colloidal solution (see Figure 2B). The increased number of aggregates after 8 PE layers can be a result of the more efficient sedimentation of aggregates than separated cells under low sedimentation forces, due to the higher buoyancy and lower charge densities compared to individual cells. Higher charge densities of cells repeal individual cells from surfaces more efficiently than aggregates are repealed, decrease sedimentation and consequently increase the amount of aggregates at each PE deposition step.

3 Supplementary Figures



Supplementary Figure 1. The figure legends are required to have the same font as the main text, 12 point normal Times New Roman, single spaced. Please use a single paragraph for each legend and prepare the figures keeping in mind the PDF layout.

Supplementary table S1. Parameters of the growth curves obtained from time lapse microscopy. N0 - predicted number of cells at the beginning of the experiment, r - maximal growth rate, tmid - time

point at which the maximal growth is reached, tgen - generation time at the maximal growth speed, AUC - area under the growth curve, λ - time when culture switch from the lag to the exponential phase, $dS(\lambda)/dt$ - acceleration of growth at the λ .

Samples	N0	r	tmid	tgen	AUC[S*dt	λ	$dS(\lambda)/dt$
Control	34.39	0.0377	114.20	18.46	206,498.40	65.22	12.42
PEIPSS4	15.32*	0.0401†	130.60*,†	18.54	134,904.89*,†,‡	79.37†,	11.40*
PEIPSS6	11.54*	0.0404†	135.19*,†	17.35	99,922.18*,‡	87.05*,†,	10.83*
PEIPSS8	11.65*	0.0302*	181.11*	22.99*	79,612.24*	111.48*	8.77*

* significantly different than the control

† significantly different of cells covered with 8 layers of samples from 4 and 6 layers

‡ significantly different between 4 and 6 layers

p<0.05 and grey symbols p<0.1

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3.1.1 Method for synthesis of protein in culture of bacterial cells

Rybkin, I., Gorin, D. A., Sukhorukov, G. B., & Lapanje, A. (2017). Method for Synthesis of Protein in Culture of Bacterial Cells: Patent Application TMA170199738. *Federal Institute of Industrial Property*.

The invention is related to the field of biotechnology, in particular to the cultivation of bacteria on a nutrient medium in order to increase the protein content in cell cultures. Current strategies and solutions to increase protein yield are based on using genetically modified organisms. Since the size and stability of genetic elements is very limited, it narrows the choice of metabolic pathways in bacteria. Therefore, in order to expand the repertoire of the method for increasing protein synthesis, we adapted the LbL method for application on bacterial cells. After consequent deposition of positively and negatively charged PEs on bacterial surfaces, the coated cells were incubated in medium to initiate metabolism. Later, it was observed that the coated cells had an increased protein content after the incubation period compared to the uncoated ones. The increased protein content was determined using spectrophotometric and confocal studies on coated and uncoated cells.

The candidate executed the experimental part, prepared the application form and took care of communication with the expert.

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Mail address:

410012, g. Saratov, ul. Astrakhanskaya, 83, SGU,
TSPU, Romanovoj N.V.

(72) Inventor(s):

Sukhorukov Gleb Borisovich (RU),
Gorin Dmitrij Aleksandrovich (RU),
Rybkin Yaroslav Andreevich (RU),
Lapanie Alesh (SI)

(73) Proprietor(s):

Federalnoe gosudarstvennoe byudzhethoe
obrazovatelnoe uchrezhdenie vysshego
obrazovaniya "Saratovskij natsionalnyj
issledovatel'skij gosudarstvennyj universitet
imeni N.G. Chernyshevskogo" (RU)**(54) PROTEIN SYNTHESIS METHOD IN BACTERIAL CELL CULTURE**

(57) Abstract:

FIELD: biotechnology.

SUBSTANCE: invention refers to biotechnology, particularly to a method for protein synthesis in cultures of bacterial cells. Method involves modifying the cell surface by layer-by-layer adsorption of the oppositely charged polymers and subsequent thermostating of the cell culture. Cell culture is used in amounts of 10^6 – 10^{20} CFU/ml. Prior to application and after application of polymers cells are washed with solutions with ionic strength in range of 0–0.155 mol/l. Positive charged polymer used is polylysine, protamine sulfate, polyallylamine hydrochloride, polyethyleneimine, polydiallyldimethylammonium chloride or chitosan. Negatively charged polymer used is alginate, hyaluronic

acid, polyacrylic acid, sodium polystyrene sulphonate, polylactic acid, polyglutamic acid or cellulose sulphate. Concentrations of polymers are selected in range of 0.1–10 mg/ml. Cells are incubated in polymer solution for 4–15 minutes. If centrifugation is used, the reaction volume of the mixture is selected in range of 0.5 ml – 1 l and centrifuged in range of 700–10,000 g for 30 s – 5 minutes. In the case of filtration, the reaction volume of the mixture is selected in range of 1–50 l and filtering materials with pore size of at least 0.45 μ m are used.

EFFECT: invention allows to increase the amount of synthesized protein in different types of cultures of bacterial cells without reducing cell survival.

1 cl, 4 dwg, 4 ex

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The invention relates to the field of biotechnology, in particular to the cultivation of bacteria on a nutrient medium in order to increase the protein content in cell cultures.

It was found a method of industrial cultivation of *Escherichia coli* strains obtained on the basis of strain BL21 (de3), carrying the T7 RNA polymerase gene under the control of the lacuv5 promoter, with increased biomass synthesis and the yield of the target protein (see patent for invention RU 2473683, IPC C12N 1/21). The method involves growing an inoculum based on *E. coli* BL21 (DE3) strain, carrying the T7 RNA polymerase gene under the control of the lacUV5 promoter. Cells are cultivated until the middle of the logarithmic growth phase on a nutrient medium, using glucose as a carbon source at a concentration of 10 g/L at 38 °C, stirring 200 rpm for 3 hours, inoculating the fermenter with an inoculum with a seed dose of 10% at 38 °C and dissolved oxygen concentration 80% at a maximum stirring speed of 1200 rpm, a maximum aeration level of 1 volume of air/min : 1.2 volume of culture liquid, pH 7.00; the fermentation is finished after 12-13 hours of cultivation; the cells of the *E. coli* producer strain are later centrifuged. At the beginning of cultivation, a 50% (wt%) glucose solution is added, in the middle of the logarithmic growth phase, after, portionwise an 80% (wt%) glycerol solution is added, and from the end of the logarithmic phase of culture growth, fractional additions of a 25% (wt%) lactose solution. After cultivation, the biomass containing the recombinant target proteins is precipitated in the insoluble form. According to this method, it was achieved an increased yield of biomass and target protein per unit of volume of culture medium.

Closest to the claimed is a method for increasing protein production (see application for invention WO 8606410, IPC C12P 21/02). The method allows to increase the amount of the obtained protein by culturing *E. coli*, having a vector that contains the structural gene of the protein. The method includes growing *E. coli* on a medium containing a source of iron, manganese or its mixtures, and a naturally occurring nitrogen source to increase the amount of methionine-free protein corresponding to a translational ATG starting donor. The protein, free of a methionine residue, can be obtained in an increased amount and corresponds to the ATG start codon.

However, a significant disadvantage of these methods is the use of genetically modified organisms to increase the amount of synthesized protein on selective nutrient media. The technical problem of the present invention is to develop a method for increasing the protein synthesis without the introduction of genetic vectors, providing an expansion of the arsenal of methods for protein synthesis in biotechnology. The technical result of the claimed invention is to increase the amount of synthesized protein in various types of cultures of bacterial cells by coating the surface of bacterial cells with polyelectrolyte layers and subsequent growth of cells on a nutrient medium.

The technical result is achieved by growing cells on a nutrient medium, deposition of a positively charged polyelectrolyte, incubation in a positively charged polyelectrolyte solution, centrifugation or filtration, washing, deposition of a negatively charged polyelectrolyte, incubation in a negatively charged polyelectrolyte solution, centrifugation or filtration, washing; the deposition of layers is repeated until the required number and a given size of cell aggregates are obtained; after, the coated cells inside the polyelectrolyte layers are placed on a nutrient medium, grown and the resulting product is separated, while the culture is used in amounts of 10^6 - 10^{20} CFU/mL, the cells are washed before and after the application of polyelectrolytes with solutions with ionic strength in the range of 0-0.155 mol/L, polyelectrolyte concentrations are selected in the range of 0.1 mg/L - 10 mg/mL, cells are incubated in a polyelectrolyte solution for 4 - 15 minutes. Polylysine or protamine sulfate or polyallylamine hydrochloride or polyethylencimine or polydiallyldimethylammonium chloride or chitosan is used as a positively charged polyelectrolyte. Alginate or hyaluronic acid or polyacrylic acid or sodium polystyrene sulfonate or polylactic acid or polyglutamic acid or cellulose sulfate are used as a negatively charged polyelectrolyte. The optimum temperature for coating is selected in the range from 2 to 46 °C. In the case of using centrifugation, the reaction volume of the mixture is selected in the range of 0.5 mL - 1 L and centrifuged in the range of 700-10000 g for 30 seconds - 5 minutes, in the case of using the filtration method, it is selected in the range of 1 L - 50 L with using filter materials with the pore size not less

RU 2 688 383 C2

than 0.45 microns. Cell aggregates vary in size from 5 to 200 microns. The invention is illustrated in several figures.

FIG. 1 - the relative intensity of green fluorescent protein in cells coated with polyelectrolytes and control cells (*E. coli* TOP 10) observed under a confocal microscope: a) the fluorescence of control cells, which is at the level of 40-50 relative units, b) cells fluorescence with increased content, which reach a value of 255 relative units.

FIG. 2 and FIG. 3 - cells with increased protein content (the brightest with increased size) and control cells.

FIG. 4 - A spectrophotometric measurement of fluorescence of a population of polyelectrolyte-coated and control cells in a nutrient medium. The protein fluorescence values of the coated cells were 103027 ± 11659 in the stationary growth phase, the protein fluorescence values of the control cells were 51492 ± 2980 , meaning that the increase in protein content was about 2 times. The measurements were carried out to further confirm the increase in the amount of synthesized protein in the cells with the modified coating in comparison to the control cells.

Examples of practical implementation of the method.

Example 1.

Preparation of polyelectrolytes.

For this encapsulation example, oppositely charged polymers polyethyleneimine and sodium polystyrene sulfonate were used, which were diluted in deionized water at a final concentration of 2.5 mg/mL. The resulting solutions are placed in an ultrasonic bath for 15 minutes. Subsequently, the polymers are titrated to pH = 7.

Encapsulation.

Initially, a solution of polyethyleneimine is added to the washed cells, in an amount 10^{12} CFU/mL in a volumetric ratio of 1:1 (reaction volume of the mixture is 2 mL) and incubated for 5 minutes at room temperature (25 °C). Then the cells are centrifuged at 700 g for 2 min. The culture deposited on the surface is washed 2 times in a sodium chloride solution (0.155 mol/L) and the cells are resuspended in the volume of the solution by washing. After coating with the first polyelectrolyte, cells are incubated in a solution of sodium polystyrene sulfonate in a 1:1 volume ratio using the same conditions. Washing is carried out in the same way, except for the time and force of centrifugation, which were 3 min and 1500 g, respectively, for the formation of a sufficiently dense layer of cells. This procedure is repeated until 6 layers are reached.

Example 2.

Preparation of polyelectrolytes.

In this example, a pair of oppositely charged polyelectrolytes, polyallylamine hydrochloride and polyacrylic acid, were used, which were diluted in deionized water at a final concentration of 10 mg/mL. The resulting solutions are placed in an ultrasonic bath for 15 minutes. Subsequently, the polyelectrolytes are titrated to pH = 7.

Encapsulation.

Initially, the polyallylamine hydrochloride solution is added to the washed cells, in an amount of 10^{20} CFU/mL in a volume ratio of 1:1 (the reaction volume of the mixture is 500 mL) and incubated for 15 minutes at a temperature of 46 °C. Then the cells are centrifuged at 6000 g for 5 min. The culture deposited on the surface is washed 2 times in a sodium chloride solution (0.0775 mol/L) and the cells are resuspended in the volume of the solution by washing. After coating with the first polyelectrolyte, cells are incubated in a 1:1 volumetric polyacrylic acid solution using the same conditions. Washing is carried out in the same way, except for the time and force of centrifugation, which were 5 min and 10000 g, respectively, to form a sufficiently dense layer of cells. This procedure is repeated until 4 layers are reached.

Example 3.

Preparation of polyelectrolytes.

RU 2 688 383 C2

For this example, a pair of oppositely charged polyelectrolytes chitosan and polyglutamic acid were used, which were diluted in deionized water at a final concentration of 0.1 mg/mL. The resulting solutions are placed in an ultrasonic bath for 15 minutes. Subsequently, the polyelectrolytes are titrated to pH = 7.

Encapsulation.

Initially, the chitosan solution is added to the washed cells in an amount of 10^6 CFU/mL in a volume ratio of 1:1 (the reaction volume of the mixture is 1 mL) and incubated for 10 minutes at a temperature (2 °C). Then the cells are centrifuged at 2000 g for 4 minutes. The culture deposited on the surface is washed 2 times in deionized water and the cells are resuspended in the volume of the solution by washing. After coating with the first polyelectrolyte, cells are incubated in a 1:1 volumetric polyglutamic acid solution using the same conditions. Washing is carried out in the same way, except for the time and force of centrifugation, which were 6 min and 4000 g, respectively, to form a sufficiently dense layer of cells. This procedure is repeated until 8 layers are reached.

Example 4.

Preparation of polyelectrolytes.

In this example, a pair of oppositely charged polylysine and polylactic acid polyelectrolytes were used, which were diluted in deionized water at a final concentration of 3 mg/mL. The resulting solutions are placed in an ultrasonic bath for 15 minutes. Subsequently, the polyelectrolytes are titrated to pH = 7.

Encapsulation.

Initially, the polylysine solution is added to the washed cells, in an amount 10^{10} CFU/mL in a volume ratio of 1:1 (reaction volume of the mixture 1 L) and incubated for 10 minutes at a temperature (15 °C). The culture is washed by filtration in a sodium chloride solution (0.03975 mol/L) and the cells are resuspended in the volume of the solution by flushing. After coating with the first polyelectrolyte, cells are incubated in a 1:1 volumetric polylactic acid solution using the same conditions. Washing is carried out in the same way using a filter material with a pore size of 0.45 microns. This procedure is repeated until 10 layers are reached.

An increase in the amount of synthesized protein.

Preparation of culture.

As an example of increasing the amount of synthesized protein, *E. coli* TOP 10 culture was chosen as a model organism of the behavior of bacterial cells. It follows that this method can be applied to other bacterial cell strains as well (Neidhardt EC, Ingraham JL, Schaechter M. Physiology of the bacterial cells: a molecular approach. - Sunderland: Sinauer, 1990. - T. 20, Ankeny RA, Leonelli S. What's so special about model organisms? // Studies in History and Philosophy of Science Part A. - 2011. -T. 42. -No. 2.-C. 313-323 .; <http://study.com/academy/lesson/escherichia-coli-e-coli-as-a-model-organism-or-host-cell.html>; <https://www.emlab.com/s/sampling/env-report-03-2010.html>; Lee ES., Lee K.H. Escherichia coli-a model system that benefits from and contributes to the evolution of proteomics // Biotechnology and bioengineering. - 2003. - T. 84. - No. 7. - C. 801-814.). The culture was transformed with pRSET-emGFP plasmids by electroporation using standard protocols. It should be noted that the introduction of the pRSET-emGFP plasmid was used only to test the present hypothesis. The cells were cultured in nutrient broth (volume 100 mL, Nutrient Broth No. 2) with the addition of ampicillin at a concentration of 100 mg/L. This mixture was placed on a rotary growth shaker for about 3 hours at 37 °C until an optical density of 0.2 was reached. Subsequently, 50 mL of the resulting culture was concentrated by centrifugation at 5000 g for 6 min. Then, the bacterial culture was washed from the remains of the nutrient medium in 0.09% sodium chloride solution three times and centrifuged at 3000 g for 3 min.

The prepared cells after the application procedure (see Example 1) were placed in a volume of 4 µl on a glass slide containing a nutrient agar medium, and covered with a cover slip. A Leica TCS SP8X confocal microscope equipped with a heating box ($t = 37$ °C) was used for observation. The

RU 2 688 383 C2

sample was placed in a chamber, where it was thermostated to a predetermined temperature during the entire experiment using a 100x magnifying objective (HCX PL APO 100x/1.44 OIL) (Figs. 1-4). Preliminary experiments using other cultures of *P. stutzeri*, *S. aureus*, *B. subtilis* and various strains of *E. coli* also showed a positive effect of using this method to increase the amount of synthesized protein.

The claimed invention can be implemented not only in the variants that are presented in the present description for illustration, but in various combinations of parameters, which are limited only by the claims in the present invention.

In addition, it should be understood that the various features, aspects and functionality described in one or more of the individual examples are not limited in their applicability to various embodiments of a given biotechnological process, and may instead be applied, separately or in various combinations, to one or more ways of implementing the invention.

It should be noted that the possibility of applying polyelectrolytes to the surface of bacterial cells was shown in the following works; see WO 200845806, IPC A61K-009/48; A61K-035/00; A61K-035/12; B01J-013/22 et al., See Franz B. et al. Layer by Layer Nano Encapsulation of Microbes: Controlled Cell Surface Modification and Investigation of Substrate Uptake in Bacteria // Macromolecular bioscience. - 2010. - T. 10. - No. 2. - P. 164172., see Balkundi S.S. et al. Encapsulation of bacterial spores in nanoorganized polyelectrolyte shells // Langmuir. - 2009. - T. 25. - No. 24. - C. 14011-14016, however, these methods were not used to increase the amount of synthesized protein and effects similar to those proposed in the patent were not observed.

This method allows to expand the arsenal of methods for protein synthesis and can also be used in combination with genetic engineering methods.

(57) Claims

1. A method of protein synthesis in a culture of bacterial cells, includes growing cells on a nutrient medium, application of a positively charged polyelectrolyte layer on the surface of the grown cells by incubation in a solution of a positively charged polyelectrolyte, centrifugation or filtration, washing, then application of negatively charged polyelectrolyte layer on the cell surface by incubation in a negatively charged polyelectrolyte solution, centrifugation or filtration, washing; the application of layers is repeated until the required number of layers and a given size of cell aggregates are obtained; after which the cells, coated with polyelectrolyte layers, are placed on a nutrient medium, grown and the resulting product is separated, while the culture is used in amounts of 10^6 - 10^{20} CFU/mL, the cells are washed before and after the application of polyelectrolytes with solutions with ionic strength in the range of 0 - 0,155 mol/L, polylysine or protamine sulfate, or polyallylamine hydrochloride, or polyethyleneimine, or polydiallyldimethylammonium chloride, or chitosan is used as a positively charged polyelectrolyte, alginate or hyaluronic acid, or polyacrylic acid, or sodium polystyrene sulfonate, or polylactic acid, or polyglutamic acid, or cellulose sulfate is used as a negatively charged polyelectrolyte, polyelectrolyte concentrations are selected in the range 0.1 mg/mL - 10 mg/mL, the cells are incubated in a polyelectrolyte solution for 4-15 minutes, in the case of centrifugation, the reaction volume of the mixture is selected in the range of 0.5 mL - 1 L and centrifuged in the range of 700-10000 g for 30 s - 5 minutes, in the case of using the filtration method - selected in the range of 1 L - 50 L using filter materials with a pore size of at least 0.45 microns.

2. A according to claim 1, the optimum temperature for coating and incubation is selected in the range from 2 to 46 °C.

RU 2 688 383 C2

FIGURES

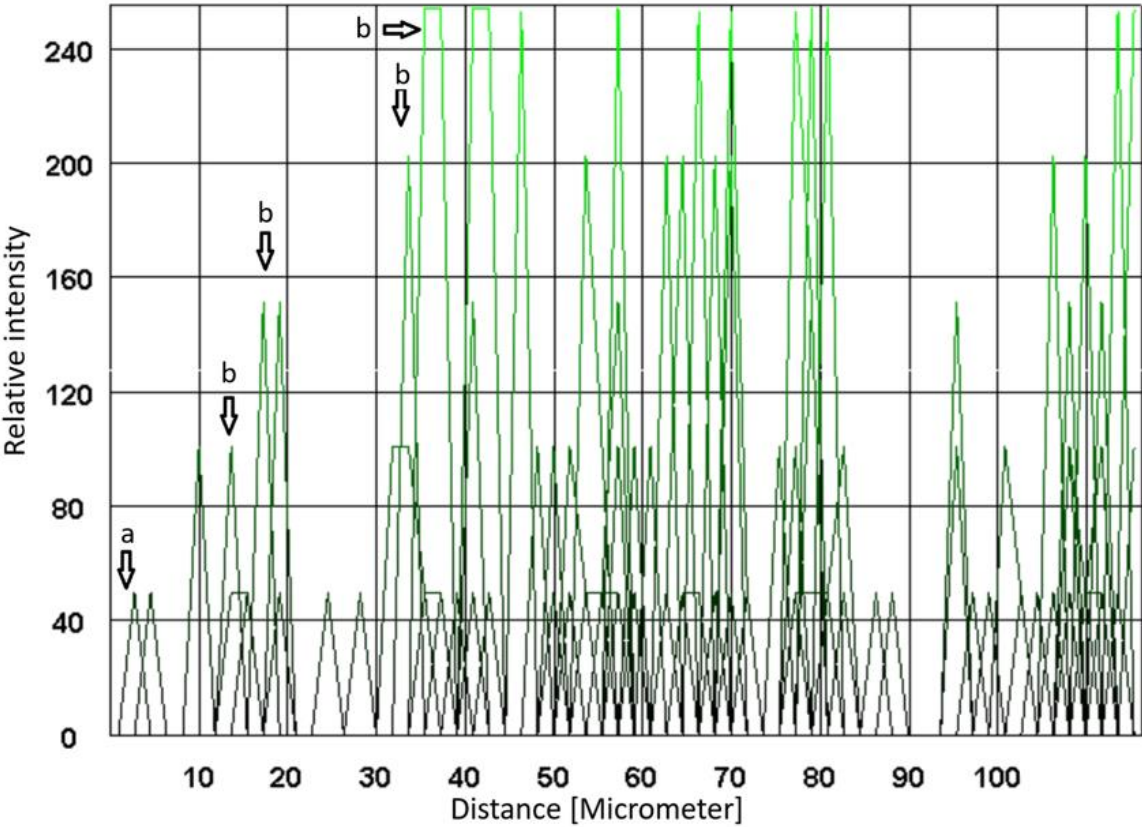
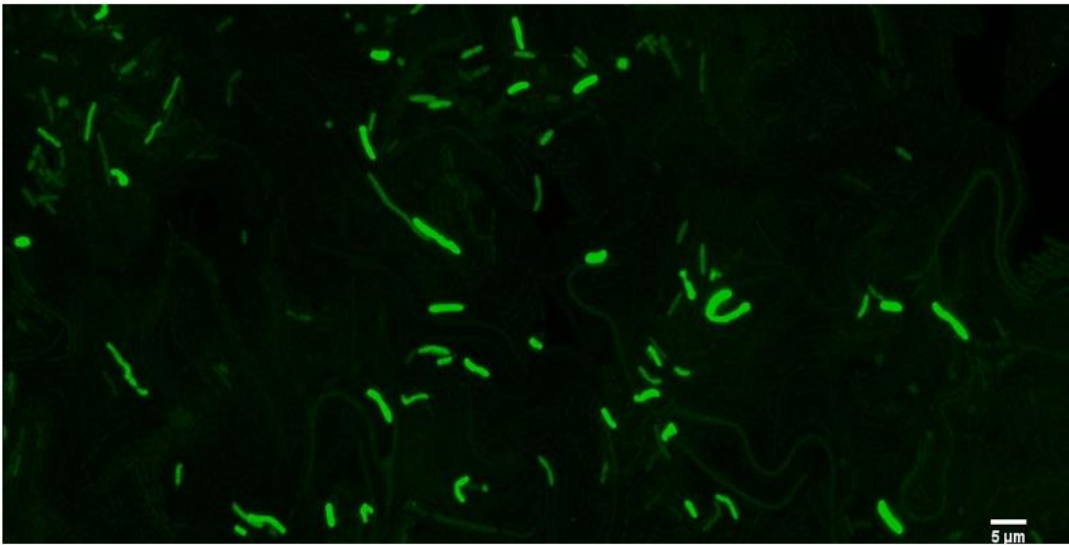


FIG. 1



RU 2 688 383 C2

FIG. 2

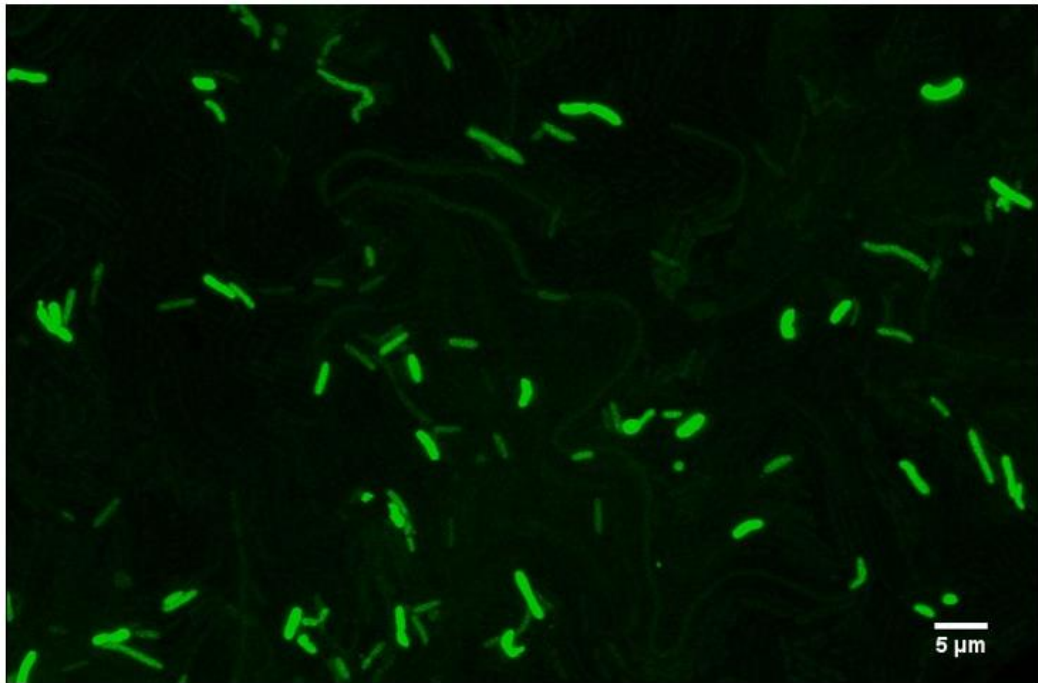


FIG. 3

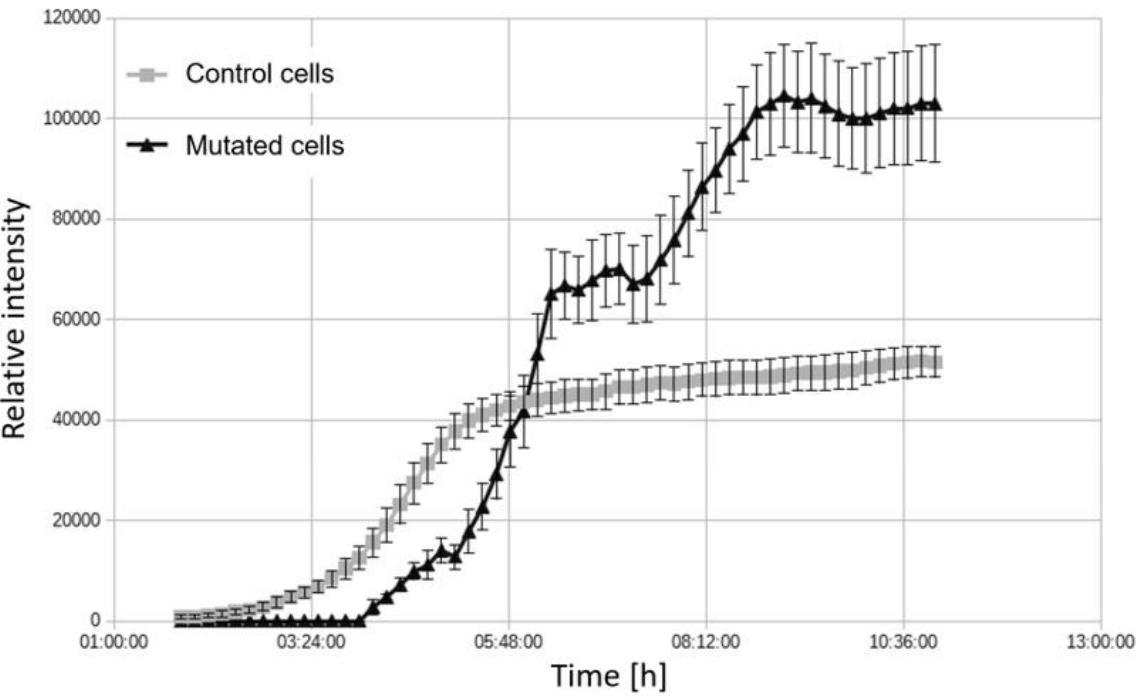


FIG. 4

3.2 Strain-dependent Selective Antimicrobial Action of Cationic Polyelectrolytes on Gram-negative Bacteria

Rybkin, I., Mirtic, J., Majaron, H., Hrescak, J., Ceh, M., Strancar, J., ... Lapanje, A. (2021). Strain-dependent selective antimicrobial action of cationic polyelectrolytes on Gram-negative bacteria. *BioRxiv*. <https://doi.org/10.1101/2021.08.31.458386>. The manuscript is in the BioRxiv repository and is under final revision for submission to: ACS Nano

To precisely control the properties of the cell surface, which can help introduce new methods of immobilizing cell biomass in biotechnological processes, bacteria can be ornamented with PEs during self-assembly. However, the lack of understanding of the effects caused by introducing PE coatings to bacteria limits the development and further implementation of the method. In special concern, the ambiguous effect in viability of cells when positively charged PEs cause toxicity. It has been found that the toxic effect is inconsistent between studies and depends on the particular strain. In order to shed light on toxic behavior of PEs and to study cell response, we selected two bacteria, representing the same Gram architecture *Escherichia Coli* and *Pseudomonas Stutzeri*, but with different susceptibility to positively charged PEs. To determine the contribution of structural differences to toxicity, two positively charged PEs, poly(ethylenimine) (PEI) and poly(allylamine) hydrochloride (PAH), with different molecular weights were chosen. Since in our preliminary experiment we also observed the ambiguity of the viability of the coated cells in different media, we performed tests in liquid and on solid media.

To assess susceptibility, we performed a Minimal Inhibitory Concentration (MIC) test with continuous exposure to PEs. Since an excess of PEs does not provide the same conditions as PEs deposited on the cell surface due to interaction with media, we also analyzed the MIC test, where PEs are present only on bacteria. In case, when PEs were in excess in solution, it resulted in toxicity for both strains, while *E. coli* was 2 times more resistant to low molecular weight PEs than *P. stutzeri*. PEs deposited just on the cells revealed a difference in susceptibility between the strains, as no toxicity to *E. coli* was observed in liquid or on solid media. However, the coatings were still toxic for *P. stutzeri*, depending on the incubation conditions. In particular, primary amines were more toxic in liquid media, while PEs containing quaternary amines were more toxic in solid media.

To test whether PEs can penetrate the cell wall of bacteria, the coated cells were analyzed using Stimulated Emission Depletion Microscopy (STED). All data were evaluated using image processing software to determine the ratio of PE signals, which were detected inside or outside the membrane. The authors observed that after the PEs were deposited onto the cell surface, PEs were mostly present inside *P. stutzeri* cells. However, for *E. coli*, they were mainly present on the surface. The intensities of the deposited PEs at the highest concentration were 7.8 ± 3.5 times lower on the membrane and 20.8 ± 12.8 inside the cells of *E. coli* than *P. stutzeri*. In addition, the authors did not observe any differences in the PE branching or molecular weights for these types of cells.

To lower the toxic effect in *P. stutzeri*, cells were coated with PEs at various concentrations and the viability of cells was assessed by evaluating Colony Forming Units (CFU). The authors found that at 0.039 $\mu\text{g/mL}$, the electrophoretic mobility remains positive (1.83 ± 0.22) with the highest viability of the coated cells ($73\% \pm 23\%$ of viable cells), which creates the possibility for manipulation with sensitive strains, while using 2.5 $\mu\text{g/mL}$ keeps only $46\% \pm 8\%$ of viable cells.

The authors conclude that modification of the bacterial cell surface in Gram-negative bacteria demonstrated different outcomes in viability, despite the similar membrane

architecture. Differences in structural elements of the membrane revealed drastic differences in viability, where one strain became a suitable template for the PE deposition (no significant reduction in the viability), while the other demonstrated high death rates ($46\% \pm 8\%$ of viable cells), requiring mild coating conditions. By careful manipulation, they found suitable concentrations, where positively charged PEs can be introduced onto the surfaces of the cells to create a coating.

The candidate prepared the protocols for deposition of PEs, performed toxicities studies, partially performed statistics, partially prepared drawings and partially contributed to writing. The manuscript was prepared in collaboration with co-authors.

Strain-dependent selective antimicrobial action of cationic polyelectrolytes on Gram-negative bacteria

Iaroslav Rybkin^{1,2,3,4}, *Janja Mirtič*⁵, *Hana Majaron*⁶, *Jitka Hreščak*⁷, *Miran Čeh*⁷, *Janez Štrancar*⁶, *Julijana Kristl*⁵, *Tomaž Rijavec*², *Aleš Lapanje*²

¹Department of Reactive Transport, Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf, Leipzig 04318, Germany

²Department of Environmental Sciences, Jožef Stefan Institute, Ljubljana 1000, Slovenia

³Remote Controlled Theranostic Systems Lab, Institute of Nanostructures and Biosystems, Saratov State University, Saratov 410012, Russian Federation

⁴Jožef Stefan International Postgraduate School, Ljubljana 1000, Slovenia

⁵Faculty of Pharmacy, University of Ljubljana, Ljubljana 1000, Slovenia

⁶Laboratory of Biophysics, Condensed Matter Physics Department, Jožef Stefan Institute, Ljubljana 1000, Slovenia

⁷Center for Electron Microscopy and Microanalysis, Jožef Stefan Institute, Ljubljana 1000, Slovenia

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KEYWORDS: Cationic polyelectrolytes, Layer-by-Layer assembly, antimicrobial coatings, surface modification, cell encapsulation, Gram-negative, polyelectrolyte coating, stimulated emission depletion (STED) microscopy, polyethyleneimine, poly(allylamine hydrochloride)

Abstract

Although electrostatic modification of bacterial surfaces using polyelectrolytes (PEs) is a convenient and versatile tool for biotechnological processes, the ambiguities in toxicity of PEs between various bacteria and the insufficient understanding of the mechanism of action of cationic PEs and their nano-thick shells formed around the bacteria create a bottleneck of the approach. Here, we show how the viability of two bacterial strains, *Escherichia coli* and *Pseudomonas stutzeri*, both from the Gram-negative group differs, when the cells are exposed to cationic PEs under different conditions. Although the cell wall architecture of the strains should be structurally similar, we found that the viability of *E. coli* was not affected by the electrostatic deposition of polyethyleneimine (PEI) or poly(allylamine) hydrochloride (PAH), whereas for *P. stutzeri* the deposition resulted in high death rates. The cells of *E. coli* proved to be suitable templates for Layer-by-Layer (LbL) modification, while in *P. stutzeri* a modified protocol with mild conditions had to be used to ensure the viability of the cells. Super resolution stimulated emission depletion (STED) microscopy allowed us to clearly visualize that after PE deposition onto the surface of the cells, the PEs could penetrate inside the cells of *P. stutzeri*, while forming a capsule around *E. coli* as expected. Therefore, this knowledge will help us select the most appropriate combinations of strains and PEs, for biotechnological processes or biomedical application, preventing unwanted toxicity.

Introduction

There is a substantial need to find alternative antimicrobial approaches to fight against microorganisms with antimicrobial resistance (AMR), on the one hand, due to a high speed of their dissemination, and on the other hand, due to a dramatic reduction in the number of antibiotics that can eradicate such infections. The highest impact on the health is made by biofilms as bacteria present within these films are unattainable from the action of antibiotics and antimicrobials compounds.^{1,2} Moreover, cells exchange genes containing AMR resistance between members of biofilms, making the biofilm even more resistant for treatment.³

One of the approaches to make a surface bactericidal is to coat the surface with positively charged PEs. However, PEs can be used either of two ways to prevent colonization of unwanted microbes (i) by aiding probiotic or beneficial bacteria to attach the cells on surfaces by electrostatic attachment⁴⁻⁶ or (ii) to induce a toxic effect that prevents attachment of unwanted microbes.^{7,8} These two approaches are diametrically opposing since one requires the preservation of bacterial viability, although another is based on the PE toxicity to inhibit cell growth. Therefore, we expect tremendous ambiguity in the conditions and reasons for selection of one sort of PEs over others, either for coating or killing cells.

In the case when PEs are used as mild coatings deposited in the LbL manner,⁹ it allows the formation of ultra-thin (10-20 nm in thickness), but extremely strong nano-thick capsules that are permeable to nutrients.^{10,11} LbL nanocapsules consisting of PEs, firstly functionalize the cell's surface, by introducing positive charges and secondly facilitate cell's attachment to surfaces. Since the cells stay under physical constraint in the permeable capsules, it also allows primary studies of cellular physiology.⁶ Although bacterial cells have been suitable templates for PEs

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deposition, the latter has been noted to negatively affect the viability of various strains in several cases.^{7–11}

In the case when PEs are used as coatings, inhibiting cell growth,^{7,12–14} they are known to be particularly toxic to bacteria.^{15–17} However, the toxicity of these coatings was ambiguous as the susceptibility of various bacteria to cationic PEs differed from one strain to another, as well as between Gram groups.^{15,16,18–22} Thus, we hypothesize that toxicity can be dependent either on the structural differences of PE molecules or on the diversity of the structure of the cell envelope. In particular, cationic PEs can have different molecular weights (MWs) or distances between functional groups, their hydrophilic-hydrophobic balance and the nature of counterions, which all can contribute to the observed toxic effects.^{12,15,22–24}

On the other hand, we can expect that the architecture of the cell envelope of Gram-negative or Gram-positive bacteria, as well as the presence of appendages on the cell surface, can affect the binding with PEs.^{25,26} In particular, both groups of cells can have mechanisms of resistance to cationic PEs by incorporating molecules, which make the membrane charge more neutral.^{16,25,27,28} Cells of Gram-positive bacteria lack an outer membrane however, they have a thick cross-linked layer of a peptidoglycan.^{29,30} In contrast, cells of Gram-negative bacteria possess two cell membranes, an intermediate peptidoglycan cell wall and the outermost lipopolysaccharide layer (LPS).^{31,32} The latter creates a barrier that prevents the penetration of cationic molecules.^{20,32} Although the architecture of Gram-positive bacteria is quite uniform, the composition of the outer membrane and the LPS of Gram-negative bacteria is very diverse. Therefore, we can expect that due to this diversity, the efficiency of PE deposition onto the surface of Gram-negative cells can vary to different extents, even causing a reduced viability of cells. Although it has not been examined in past studies, Gram-negative bacteria might be a

suitable study system, because the differences in the cell envelope architectures can be examined in relation to the toxicity of certain PEs.

In addition, we suppose that the duration of the cell's exposure to PE is a factor of toxicity, which conventional toxicity tests rarely consider. The minimal inhibitory concentration (MIC) test exposes the cells to the PE present in the growth medium throughout the growth incubation period (a case we refer to as long-term or "continuous" exposure). This differs from the LBL modified system, where PEs are only deposited onto the surface of a cell forming a nano-thick capsule, while it is absent from the growth medium during incubation, because the encapsulated cells are washed by a buffer (a case we refer to as "short-time" exposure).

Therefore, we hypothesize that the toxicity of PEs depends on both physical and biological factors, i.e.: (i) the physicochemical characteristics of PE (branching, amount of the amino groups, MW, amount of PE, conditions of exposure), and (ii) the particular structure of the cell envelope. To determine the effect of coatings on the viability of bacterial cells, we selected two Gram-negative stains with different architecture of the outer layers of the well-studied *Escherichia coli* TOP10 and *Pseudomonas stutzeri* DSM 10701 based on their difference in susceptibility to positively charged PEs. Accordingly, to reveal the ambiguities in PE susceptibility we aimed: (i) to assess the interaction of the PEs and the surface of the bacterial cells, (ii) to determine the effect of the toxicity of positively charged PEs on two Gram-negative bacteria (*P. stutzeri* and *E. coli*) and (iii) to estimate changes in survivability depending on exposure conditions.

Results

Cationic PEs enter the *P. stutzeri* bacterial cells

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In our experiments we selected two Gram-negative bacteria of *E. coli* and *P. stutzeri* to test the difference in susceptibility to positively charged PEs. Since MWs were expected to contribute to toxicity, each PE was used either in high or low MW forms. In order to find the most suitable concentration for susceptible bacteria, the experiments were performed in a gradient of PE concentrations. We also tested different conditions of cell exposure (continuous and short), since in one case, PEs can be applied as antimicrobial films, and in another they are used to make cell coatings (Table 1).

Table 1. Inhibition of bacterial growth estimated as the Minimal Inhibitory Concentration (MIC) for two cationic polyelectrolytes polyethylenimine (PEI) and poly(allylamine hydrochloride) (PAH) with different molecular weights. The bacterial cells of *P. stutzeri* and *E. coli* were exposed to the polyelectrolytes dissolved in the medium (continuous exposure) or they were deposited on the cell surface, washed and then resuspended in the medium (short time exposure) to determine inhibition of growth, which was quantified after 72 h.

	<i>P. stutzeri</i>		<i>E. coli</i>	
Polyelectrolyte	Continuous [µg/mL]	Short [µg/mL]	Continuous [µg/mL]	Short [µg/mL]
PEI 600-1000 kDa	70.3-17.6	78.1-39.1	70.3-17.6	not observed
PAH 120-200 kDa	35.2-17.6	39.1-19.5	35.2-17.6	not observed
PEI 25 kDa	35.2-17.6	78.1-39.1	70.3-17.6	not observed
PAH 17.5 kDa	17.6-8.9	39.1-19.5	35.2-17.6	not observed

In the case of continuous exposure, we observed similar, statistically insignificant, toxic effects on both strains either using high or low MW PEI or high MW PAH (see Table 1). However, *E. coli* was found to be more resistant to the low MW PAH, where MIC concentrations were twice higher than in *P. stutzeri* (see Table 1). In short time exposure tests, the toxicities were strain-dependent. Accordingly, in *E. coli*, there were no toxic effects on the bacteria from both PEs either high or low MWs. We also did not observe any significant differences in the duration of the lag phase (data not shown) between cells exposed to the highest concentrations of the PEs and the control cells ($P > 0.05$). In *P. stutzeri* cells, we only observed higher resistance with a 2-fold increase in MIC concentrations for the low MW PAH than in continuous exposures. There were no significant differences ($P > 0.05$) in the duration of the lag phase (data not shown) between the PEs treated cells and the control cells.

Prior to analysis of the effect of cationic PE coatings on cell integrity, we deposited a single layer of high MW PEI on resistant cells of *E. coli* and a sensitive strain of *P. stutzeri* to observe if PEs can cause disturbance of the cell envelope (Figure 1). Although we did not see obvious disintegration of the *P. stutzeri* cell envelope, the coated cells of *P. stutzeri* had indistinct membrane boundaries compared to the coated cells. We did not observe any difference between coated and uncoated cells of *E. coli*, as they both preserved cell integrity with a clear membrane boundary.

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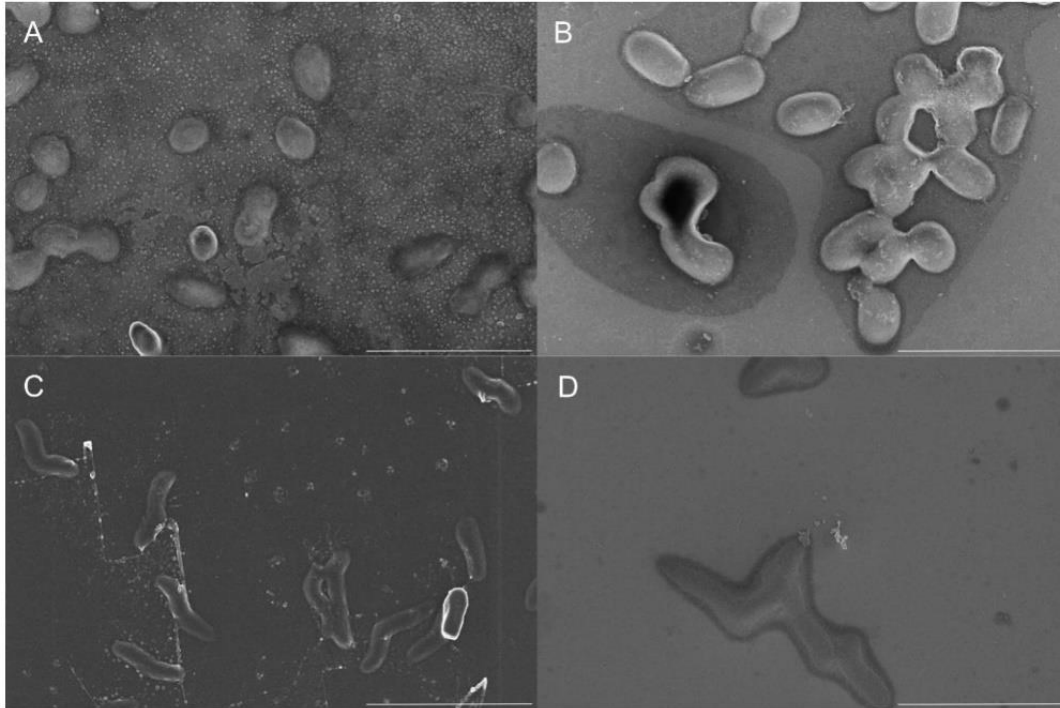


Figure 1. Deposition of cationic polyelectrolyte on the surface of bacterial cells. The SEM image represents uncoated cells of *E. coli* (A) and *P. stutzeri* (C) and coated cells of *E. coli* (B) and *P. stutzeri* (D) in a layer, composed of PEI 600-1000 kDa. Scale bar is 5 μ m.

Since we observed a difference in susceptibility to PEs between the two strains in short time exposure, we hypothesized that PE might somehow interfere with the bacterial envelope, causing toxicity. Therefore, we assessed in detail the distributions of PEs within a single cell using super resolution STED microscopy (Figure 2). We observed that PEs penetrated the cells of *P. stutzeri* in large quantities, forming bright shining areas despite the type or MW of applied PEs. Also, the shape of *P. stutzeri* after exposure to PEs remained altered due to the formation of up to 2 bulges on bacterium at the planar plane of the membrane. Although we did not observe statistical differences ($P > 0.1$) between the normal cell diameter and the diameter of the region where the bulge was formed, the diameter with bulges was on average 1.17 ± 0.14 larger than the diameter

of the unaltered region of cells. With *E. coli*, the deposited PE layer was clearly visible on the cell surface, forming a bright intensive coating for each type of PE. Despite the signals detected inside the cells of both strains, they were on average 1.9 times lower in *E. coli* than inside *P. stutzeri* ($P < 0.05$).

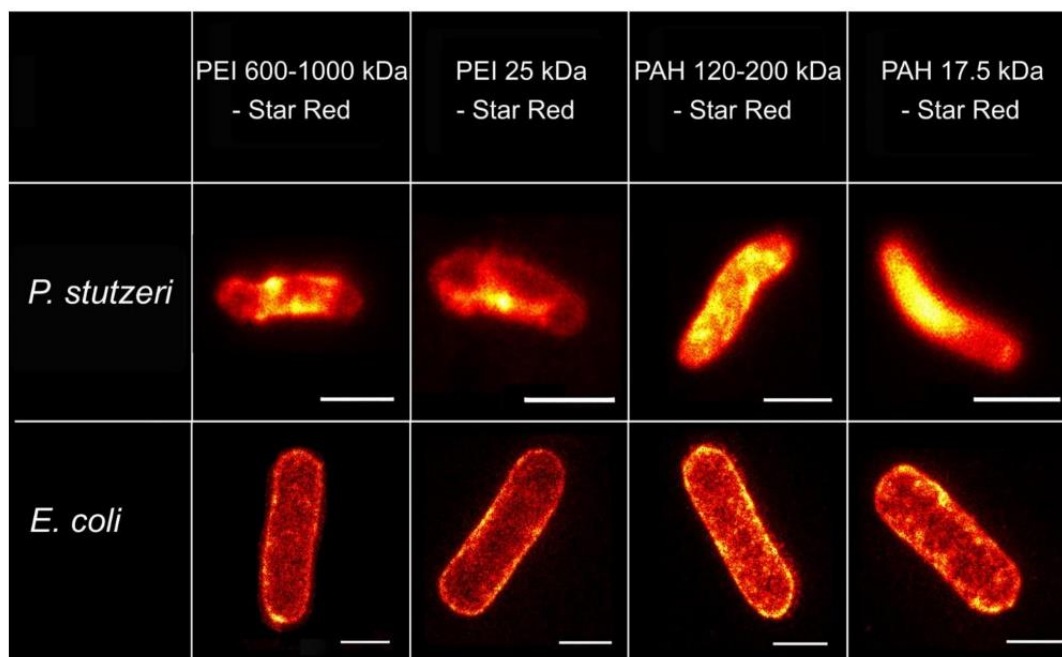


Figure 2. STED images of *P. stutzeri* and *E. coli* coated with polyethylenimine (PEI) and poly(allylamine hydrochloride) (PAH) polyelectrolytes with two molecular weights labelled with STAR RED STED dye. The deposited cationic PEs penetrate cells of *P. stutzeri*, while forming a capsule around *E. coli*. Scale bar for all images is 1 μm .

Analyzing external and internal signals, we determined the ratio, defining the penetration of PE inside the bacterium. The ratio was expressed as the amount of PEs on the outer layers to the amount detected inside the cells (Figure 3). In general, all PEs were distributed on the surface of

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E. coli cells (ratio > 1) and no significant changes of the ratios were observed when concentrations of PEs were increased. With *P. stutzeri* the PEs penetrated inside the bacterium (ratio < 1), and the ratio was reduced on average 1.9 times when the highest concentrations of PEs were applied ($P < 0.02$).

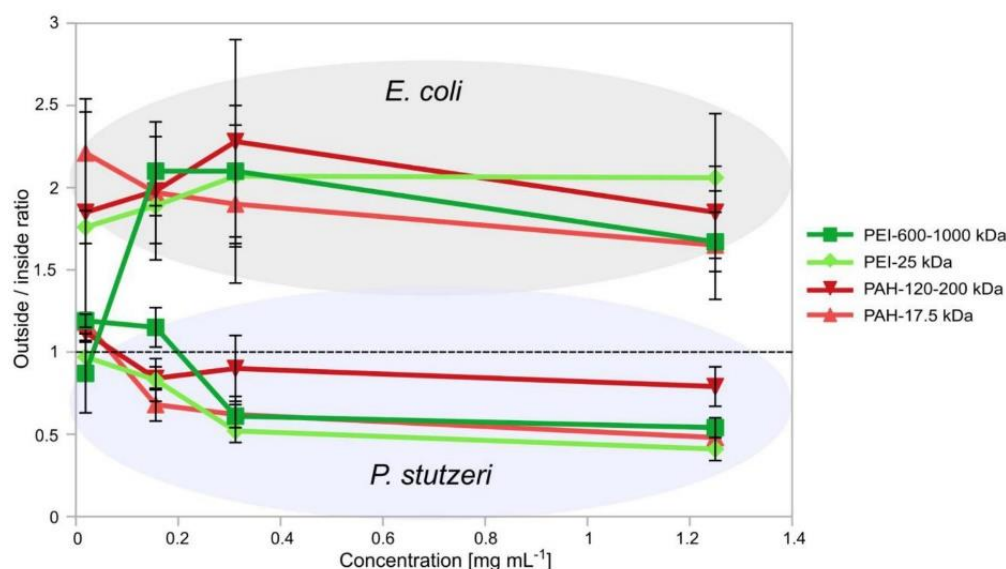


Figure 3. Quantitative determination of the ratio of polyelectrolytes on the outer membrane vs. the intercellular surrounding of the bacterial cells for two types of Gram-negative cells (*E. coli* – labeled in grey and *P. stutzeri* labeled in blue). The dashed line represents the boundary, above which polyelectrolytes are located on the outer layers, and below they are protruded through the cells, respectively. Two polyelectrolytes polyethylenimine (PEI) and poly(allylamine hydrochloride) (PAH) were used with two molecular weights at different concentrations. The analysis was performed on the obtained STED images.

We did not find any significant differences between the molecular weights of PEs deposited on the surface of *E. coli*, however, the intensities of both PEI were on average 2.5 times lower than

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PAH ($P < 0.01$), (Figure 4). With *P. stutzeri* the highest intensity of PEs was observed, when a low MW PAH was applied inside (358 ± 67) and outside (168 ± 14.2) the membrane. The intensities of the deposited PEs at the highest concentration were 7.8 ± 3.5 times lower on the membrane and 20.8 ± 12.8 inside the cells of *E. coli* than *P. stutzeri* ($P < 0.05$). In addition, the correlation coefficient between the concentration and intensities of PEs for *P. stutzeri* (0.80 ± 0.12) was, on average, higher for all PEs than for *E. coli* (0.25 ± 0.26).

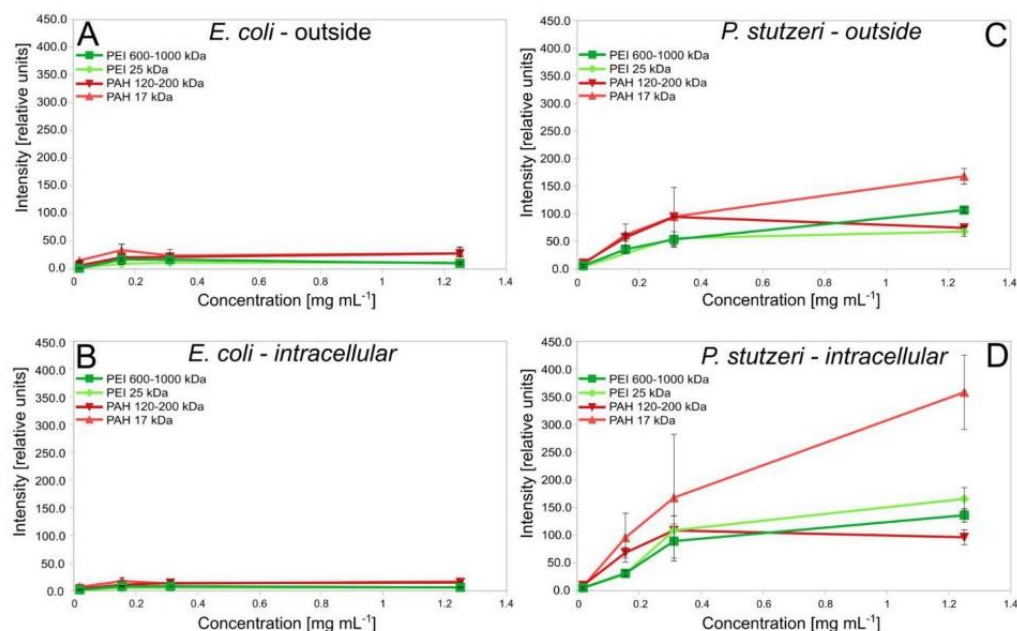


Figure 4. Quantitative determination of the intensities of polyethylenimine (PEI) and poly(allylamine hydrochloride) (PAH) polyelectrolytes with two molecular weights obtained from the surface or internal surrounding of bacterial cells.

Reduction in charge densities of PEs improves viability of *Pseudomonas stutzeri*

Since the PEs were found to penetrate inside the cells of *P. stutzeri*, we tested whether there is a window, which allows to sufficiently coat the cells not being bactericidal, but keeping a

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positive charge suitable for further manipulations. By making serial dilutions, we estimated how the surface charge of the formed coatings depends on concentration (Figure 5A). Although the concentrations of the PEs were diluted by a factor of 2, we did not observe a two-fold decrease in the surface charge. The highest decrease of positive mobilities (1.59 times on average) from the initial concentration of PEs was only observed at 0.039 mg mL⁻¹ (64 times diluted solution). The number of viable cells of *P. stutzeri* was strictly dependent on the concentration of PEs and, therefore, on the change in the electrophoretic mobility of the coated bacteria (Figure 5B). The highest reduction in Colony Forming Units (CFU) (46% ± 8% of viable cells) was at the highest concentrations of PEs (2.5 mg mL⁻¹). In contrast, there was no significant reduction in growth when mobilities were negative (0.019 and 0.009 mg mL⁻¹ for all PEs). Thus, in the range between the highest and lowest concentrations, we found a compensation point (above the isoelectric point) at 0.039 mg mL⁻¹, where we observed the highest possible viability (73% ± 23% of viable cells) at the highest possible positive electrophoretic mobility (1.83±0.22).

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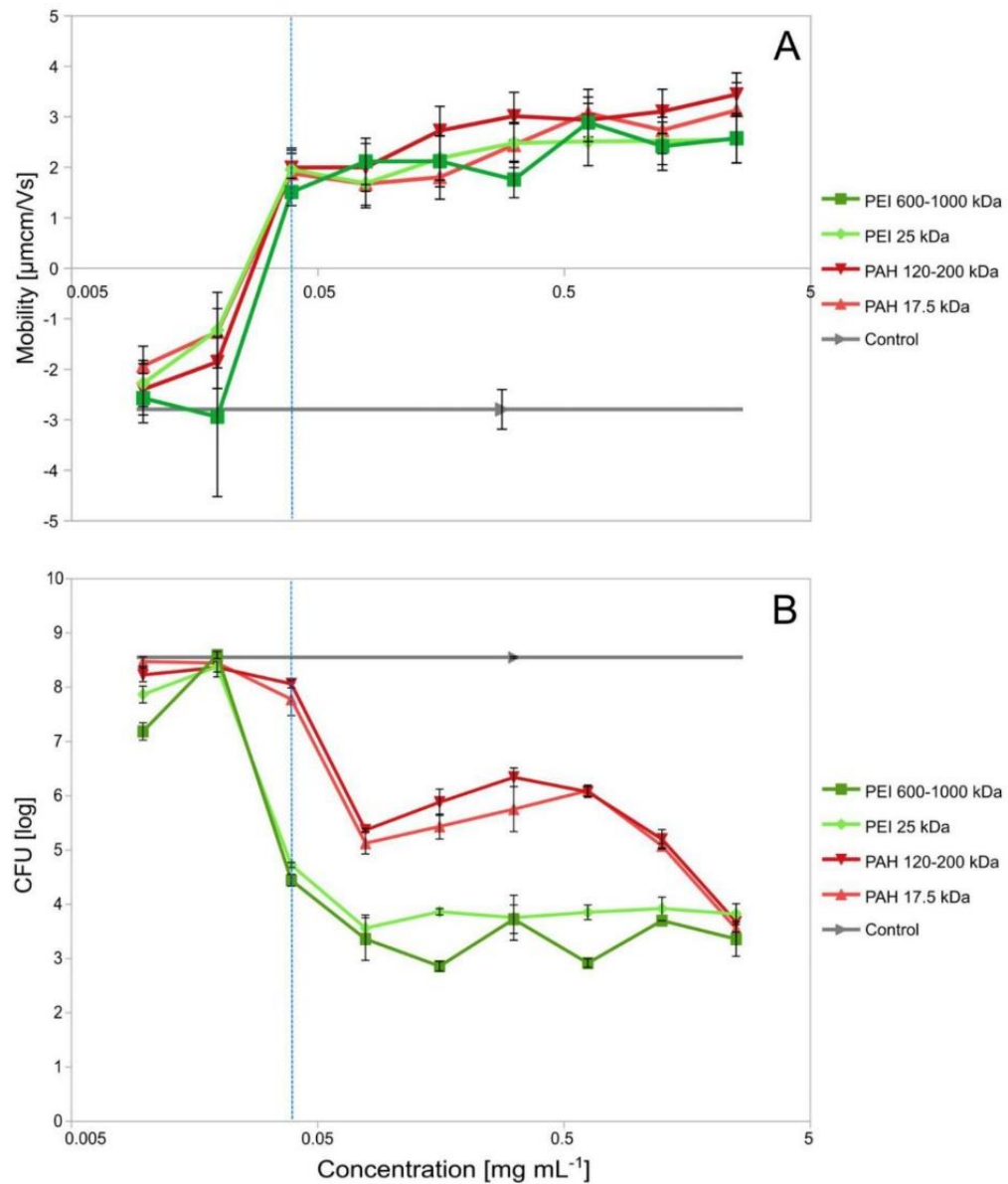


Figure 5. The effect of coating bacterial cells of *P. stutzeri* in a single layer of polyethylenimine (PEI) or poly(allylamine hydrochloride) (PAH) polyelectrolytes with two MWs on A) the change in electrophoretic mobility and B) viability, which was determined using plate counting of

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colony forming units (CFU). The dashed line indicates the compensation point, where the cells still have a positive charge and have a better viability.

The viability of the coated *P. stutzeri* cells was dependent on the particular type of PE. In general, PAH-coated cells showed significantly higher ($P < 0.05$) viabilities than PEI-coated cells between 0.625 – 0.019 mg mL⁻¹. Comparisons between the same type of PEs (PEI or PAH) but of two different MWs showed no significant difference. With *E. coli*, we did not observe any significant reduction of the cell growth between control and exposed to PEs cells in all tested concentrations. Based on both experimental approaches, determining toxicities in liquid or on solid media, we observed ambiguities in susceptibility to the PEs between the tests. Accordingly, the cells were more resistant to both types of PEI in liquid surrounding, since the range of MIC concentrations was two times higher than with PAH (see Table 1). However, with using solid media, the cells were more resistant to both types of PAH than PEI (see Figure 5B).

Discussion

Our results clearly show that PEs entered the cells of *P. stutzeri* however, they could not penetrate inside the cells of *E. coli* (Figures 2 and 3). Since we used the same PEs for two different strains, we can expect that the major contribution to permeability was related to the difference in the structure of the cell envelope. Accordingly, we summarized the specific differences in membrane composition of each bacterium as systems of protein export, LPS biosynthesis, cationic antimicrobial peptide resistance (CAMP), which can affect PE penetration (Table 2).^{33–36} The cells of *E. coli* have been found to have a much wider repertoire of mechanisms, which can help to overcome the exposure to cationic PE molecules and mask the membrane, compensation the effect of PEs. In particular, a decrease in cell-PE binding can be achieved through a decrease in the membrane charge and affinity to the PE either involving

specific proteins³⁷ or activating genes, which modify lipid A by ethanolamine and aminoarabinos.^{27,28} Our results also support that the membrane is somehow masked since the overall intensities of PEs were much lower in *E. coli* than in *P. stutzeri*, indicating a lower binding ability (Figure 4) and possibly, lower membrane damage (Figure 1). However, the contribution of each gene, making the cells more resistant, has to be further investigated. Although some amount of PEs was also found inside *E. coli*, it could be related either to the noise from the top or the bottom of bacterium and thus, axial limit of detection³⁸, or the cells maintain this amount of PEs inside not losing the viability. Since PEs entered the cells of *P. stutzeri*, we observed the formation of bulges (Figure 2), which can be a result of damage to the cell envelope by penetration of PEs and crosslinking of the peptidoglycan layer.³⁹ In addition to morphological properties, metabolic pathways could also contribute to resistance. Both bacteria are facultative anaerobes. However, in the absence of oxygen, *E. coli* can ferment, whereas *P. stutzeri* requires nitrate. From this perspective, *E. coli* can be more resistant to hindering respiration, which can be caused by PEs.

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Table 2. Differences in the composition and metabolism of two Gram negative bacteria. Core genes present in both strains (not shown), only unique genes for a particular bacterium are presented.

Structural feature	<i>Pseudomonas stutzeri</i>	<i>Escherichia coli</i>
Gramm staining	Gram-negative	Gram-negative
Metabolism	Facultative anaerobe in presence of nitrate, strict respiration, no fermentation	Facultative anaerobe Respiration & fermentation
Protein export	zero genes	3 genes (<i>SecM</i> , <i>Ffc</i> , <i>TatE</i>)
Lipopolysaccharide biosynthesis	Single gene (<i>PagL</i>)	12 genes [<i>Lpx(P,M,T)</i> , <i>PagP</i> , <i>AmT</i> , <i>Ept(A,B)</i> , <i>GmhD</i>]
Cationic antimicrobial peptide resistance	Single gene (<i>DltA</i>)	23 genes [<i>PagP</i> , <i>Pho(P,Q)</i> , <i>ept(A,B)</i> , <i>am(A,B,D,T,E,F)</i> , <i>Pmr(A,B)</i> , <i>Sap(A,B,C,D,F)</i> , <i>ycfS</i> , <i>marA</i> , <i>cpxA</i> , <i>TolC</i> , <i>NlpE</i>]
Oligosaccharides composition	5 combinations* (GlcNac, Kdo, L-D-Hep, D-GalN-L-Ala, D-Glc May include 5 genes : L-Rha, D-Fuc-NAc, D-ManNAc3NAcA and D-ManNAc3Nama)	9 combinations L-Rha, Kdo (I, II), Hep (I, II, IV), PEtN, Glc, Gal

* - data obtained from *P. aeruginosa*

The cells of *P. stutzeri*, coated with PEs at $39 \mu\text{g mL}^{-1}$, reached the compensation point at which the coated cells had positive values of mobilities (Figure 5 A) and the highest viability (Figure 5B). The number of PE molecules in this case for high MW PEI would be at least $7.3 \cdot 10^4$ per single cell (at $\text{OD}_{600}=0.4$). However, according to our estimation, the compensation point for all PEs can be shifted even further to $32.5 \mu\text{g mL}^{-1}$ (or $6.1 \cdot 10^4$ molecules per cell) in order to increase the safety of introduction of PEs to bacteria, where viability and mobility would be $\lg=7.93$ cfu and $0.98 \mu\text{mcm/Vs}$, respectively. By analyzing the number of CFU, it was found that PEI PEs were more toxic than PAH (Figure 5B) when the cells were incubated on solid media. PEI molecules have a larger amount of the amino groups (1N:2C atoms against 1N:3C in PAH) and higher branching, which could lead to increased binding within a thin layer of water and thus causing more damage to cells. In particular, we assume that the surface tension of a thin liquid layer around the cells on an agar plate, first, spatially arranges PE molecules to be oriented in the same plane as bacteria, and second, reduces distance between PEs and the cells, thus, a greater amount of amino groups can interact with the cell envelope. On the contrary, when cells were incubated with the PEs in a liquid medium, PAH with its primary amino groups (Table 1), exceed the quaternary counterparts in toxicity, possibly due to a more beneficial spatial orientation, which could cause stronger electrostatic forces and hydrogen bonds.^{12,23} In addition, it can be expected that excessive amount of PE could also interact with the components of the medium, reducing availability of nutrients or increasing osmolality. Accordingly, the mixture of bacteria-PE was diluted to reduce the interaction of PEs with nutrients and at to maintain the same bacteria-PE ratio in the continuous exposure test. As a result, we did not observe suppression of the growth of *E. coli*, while *P. stutzeri* still exhibited decreased viability,

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indicating the contribution of the constant exposure. There was also no change in the viability of *E. coli*, even by depositing multiple consecutive layers of PEs on the cell surface.⁴⁰

The results of continuous exposure and the measured intensities on STED showed that major significant difference in PEs, which contributes to the toxicity, is the type of the amino group. The molecular weights or the amount of amino groups were not shown to contribute to the toxicity, since both PAH were not different in toxicity although they were more toxic than PEI in liquid media. In order to reduce the susceptibility of the different bacterial cells and, therefore, toxicity we propose several approaches: (i) acetylation of the amino groups in PEs, for reduction of the positive charge,⁵ (ii) the use of divalent cations, which can additionally cross-link the membrane and protect from permeabilization,⁴¹ (iii) genetic construction of strains, producing longer LPS tails with lowered affinity to PEs and lowered negative charge, or (iv) optimize the concentrations of PEs for keeping a small positive charge.

To summarize, LBL is an important and versatile technique for medical and biotechnological applications. Here, we showed new solutions for cases when the combination of PE and bacterium results in toxicity. Electrostatic entrapment also sheds light on differences in the membrane structure of Gram-negative cells, expanding the possibility for biomedical applications. Careful selection of PEs as well as lowering the overall concentration, can facilitate the preparation of PE coatings. Such coatings can induce expression of specific proteins (e.g. GFP), or genes responsible for quorum.⁴² Oppositely to matrix entrapment, LBL coating of an individual cell into strong porous nanometer range thick mesh enables nutrients to pass freely, but keeps cells mechanically separated from the surrounding microenvironment.⁴⁰

Conclusions

Our findings show new solutions for cases when the combination of PE and bacterium results in toxicity. Cell surface modification of Gram-negative bacteria has shown different outcomes in cell viability. Despite the membrane architecture being similar, structural features drastically affected the cell response. Single layer coating of *E. coli* with cationic PEs did not show any significant effects of toxicity, making them suitable templates for LbL deposition. However, *P. stutzeri* were extremely sensitive to changes in surface charges with high cell death, implying the use of careful and mild conditions for keeping the cell activity. All tested PEs could protrude through the membrane of this bacterium being detected intracellularly. We did not find any differences in toxicity with various MWs for the LbL case, however, the primary amines were more toxic in the liquid surrounding and had a higher ability to attach on the membrane based on the data from STED microscopy. In addition, we showed how variabilities in exposure affect the cell viability and how the toxicity can be reduced by manipulating the surface charge of bacteria.

Materials and methods

Bacteria strains and growth conditions

In our preliminary experiments (data not shown) on the coating of the cells of *Pseudomonas stutzeri* DSM10701 and *Escherichia coli* TOP10, we observed that after encapsulation, two strains exhibited opposite results in terms of viability. Therefore, we selected these strains, both from the Gram-negative group, for our experiments to determine the conditions, which make PEs toxic to one strain but not to the other. The optical density (OD_{600 nm}) of each cell suspension was determined at 600 nm in a shorter light path of 200 µL well in a 96 well plate of a microplate reader (Synergy H4, Biotec). A total of 1 mL of each overnight liquid cultures (OD_{600 nm} = 1) was inoculated into a 100 mL of nutrient broth1 (NB1), (Carl Roth) and incubated by shaking at 28 °C and 37 °C, respectively, at 150 rpm until the cells reached the stationary phase (OD_{600 nm} = 1 -

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1.5). Finally, the cells were collected in 50 mL tubes, centrifuged at 5000g for 5 min and washed 3 times in 30 mL sterile 0.9% NaCl. In all experiments the number of cells in suspension was normalized to $OD_{600\text{ nm}} = 0.4$ in a 96 well plate with 200 μL volume.

Preparation of PE solutions for coating bacteria and determination of toxicities

Positively charged PEs with different MWs were used in toxicity experiments: poly(ethyleneimine) (PEI, MW=600 - 1000 kDa Cat.No. 03880), PEI (MW=25 kDa, Cat.No. 408727), poly(allylamine hydrochloride) (PAH, MW=17,5 kDa, Cat.No. 283215), (all obtained from Merck) and PAH (MW=120-200 kDa, Cat.No. 43092, Alfa Aesar). The PEs were suspended in MQ water (2.5 mg mL^{-1} , pH = 7) and then sonicated (35kHz, 100W, 15min). Dissolved PE solutions were titrated to pH 7 using 1 M NaOH and then sterilized by filtration through a 0.2 μm sterile filter.

Fluorescent labeling of PEs for STED imaging

Abberior STAR RED NHS carbonate was used for labeling all four PEs (PEI 600 KDa, PEI 25 KDa, PAH 120 KDa and PAH 17.5 Kda). Esters of N-hydroxysuccinimide (NHS-) react with primary amines under mild alkaline conditions (pH 7.2 to 9) and form stable amide bonds. Briefly, PEs were dissolved in MQ water at a concentration of 3.5 mg mL^{-1} and pH was adjusted to 8.5. To 3 mL of PE solution 250 mg of STAR RED dissolved in 100 μL DMSO (without water) was added under a magnetic stirring and cooled with ice. The reaction mixture was left on stirring for 18 h at 4 °C. To remove residual dye after labeling, the solution was dialyzed against purified water for 7 days using a dialysis tube with MWCO 100 kDa or MWCO 8 - 10 000 Da (SpectraPor), changing water every 12 h. After the dialyzed solution showed no traces of blue coloring, the solution was transferred into the glass vial at -40 °C and lyophilized in order to

obtain dry labeled PE. Such prepared PEs were dissolved in water, pH adjusted at 7 and solutions were then diluted to obtain 0.1% (w/w).

Deposition of PE on the cell surface

The electrostatic deposition of PEs on the cell surface was carried out as previously described.⁴⁰ Briefly, prepared cells were exposed to one of the PE solutions with the concentration in the range of 2.5 – 0.0097 mg mL⁻¹, pH 7 in MQ water, adding it to the washed cells in 1:1 v/v ratio. The highest concentration was chosen as the most suitable for coating *E. coli* in our previous study,⁴⁰ while the lowest one was chosen because no change in electrophoretic mobilities was observed when PEs were applied to the bacteria. PEs were gradually diluted from the highest to lowest concentrations by a factor of 2 using sterile MQ solution. This suspension was incubated at room temperature (25°C) for 5 minutes. Unattached PEs were washed out from the bacterial suspensions by centrifugation at 900 g for 2 min and removing supernatant. When the PEs with lower concentrations (lower than 0.156 mg mL⁻¹) were used for coating, it was more difficult to centrifuge the bacteria. Therefore, we adjusted the centrifugation speed by increasing it to 1200 g and increasing the time up to 3 min. The obtained pellet was 2 times washed by carefully adding 1 mL of 0.9% NaCl over the pellet without dissolving it. After washing, the PEs coated cells were resuspended in 0.9% NaCl solution. Both strains at the lowest concentration had a greater tendency to aggregate, so in some cases it was difficult to find a separate single cell.

Measurement of electrophoretic mobility of PE coated cells

To determine electrophoretic mobilities the coated cells in different concentrations were washed 2 times and resuspended in 0.9% saline solution (NaCl). Prior to measurements, 50 µL of the suspension was added in 3 mL of MQ water, mixed by gentle vortexing and then measured

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by an ELS method (Particle analyzer, Delsa Nano, Beckman Coulter). Data were obtained from 3 replicates, where each of the measurements was produced from 15 accumulated values of the separated ELS values. In all ELS measurements the polarities of the electrodes were set fixed, with automatically adjustable voltage.

Determination of toxicities

Toxicity was measured in two approaches, in which the cells were initially exposed to PEs by mixing the PE solution and the cell suspension in a 1:1 ratio and then (i) the PEs were either left in the cell suspension or (ii) the PEs were washed out, which we named as continuous and short exposures, respectively. Since we also preliminary observed the ambiguities in toxicities between solid and liquid media, we performed an additional test, determining colony forming units on the solid media.

Minimal inhibitory concentration (MIC).

For testing continuous exposure, we prepared a series of two fold PE dilutions from 2.5 mg mL⁻¹ to 0.0097 mg mL⁻¹ by mixing 90 µL of sterile distilled water with 90 µL of PEs in a 96 well plate. Afterwards, 90 µL of twice concentrated NB was added. Finally, 20 µL of the cell suspension was added to 180 µL of a mixture of PE and medium (90 µL of PEs and 90 µL of media) and left to incubate. MIC was determined by measuring OD_{600 nm} every 30 min for 72 h by shaking and incubating at 28 °C and 37 °C for *P. stutzeri* and *E. coli* respectively with 30 s of shaking before each measurement.

Dilutions of the PEs for a short time exposure were prepared as described above. Then, diluted PE in the amount of 90 µL was mixed with an equal amount of bacteria and left for incubation for 5 min. Afterwards, the cell-PE suspension was diluted 160 times by consequent dilution of 5 µL in 195 µL of saline, shaking for 5 min, and the dilution was repeated. This dilution was

chosen in order to exclude the access of the PEs from the media. A total of 20 μL of this suspension was added to 180 μL of medium. MIC was determined under the same conditions as described above. The final concentration of PE started at a PE concentration of 1.125 mg mL^{-1} and ended at 0.009 mg mL^{-1} , prepared by dilution factor 2.

Colony Forming Units (CFU). CFU growth was assessed by plate counting at the appropriate culture dilution. The coating was performed by mixing 90 μL of PEs at different dilutions with an equal amount of bacteria and incubating for 5 min. After, all samples were diluted by mixing 10 μL of the sample and 90 μL of sterile 0.9 % NaCl for a suitable colony count. 10 μL of each dilution was transferred onto a nutrient agar plate and allowed to grow for 1 day at the appropriate growth temperature.

Scanning electron microscopy

The coated in PEI 600-1000 kDa (2.5 mg mL^{-1}) and uncoated cells (control) of both *E. coli* and *P. stutzeri* were transferred on a silicon wafer in an amount of 5 μL and left to air dry at least 2 h at room temperature (25°C). All the samples were mounted on SEM stubs and observed in a high vacuum SEM (Jeol JSM-7600F) with a field emission gun, at low voltages, to observe the structure of the cell.

Statistical analysis

For statistical comparison two tailed t-tests (LibreOffice Calc.) were used. All experiments were performed in triplicates. Graphs were prepared and visualized using LibreOffice Calc. (The Document Foundation), and Inkscape (Free Software Foundation). Analysis of the growth curves was performed using PRECOG.⁴³

Stimulated emission depletion microscopy (STED microscopy) studies

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For STED microscopy samples were prepared from a coated cell suspension, using 6 mL of cell suspension and 6 mL of Prolong Gold Antifade mountant (ThermoFisher Scientific) that were mixed on an object glass and then covered with a special STED cover glass. The samples were left for 2 h under hot air to obtain non-moving cells. The STED microscope set-up was custom build by the Abberior Instruments (lasers: 561 nm and 640 nm, STED laser: 775 nm; detectors: APD1: 560-630 nm; APD2: 630-720 nm). Image Acquisition and Analysis were performed using Inspector Software, v 0.1.⁴⁴ High-resolution images of bacteria coated with STAR RED-labelled PE were obtained using a STED microscope with a 60x water immersion objective (NA 1.2). Image acquisition parameters were kept the same for all samples (except those with a high/low intensity) in order to ensure reliable comparison between different samples: the fluorophore was excited using an excitation laser at 640 nm (with 3 μ W), its emission was depleted using a doughnut-shaped 2D STED laser at 775 nm (with 245 μ W) and was detected in the range of 650-720 nm (filters by Semrock) using an APD. The dwell-time in each pixel was 30 μ s, pinhole was 0.75 AU, pixel-size was set to 15 nm. The size and shape of the bacteria determined the field of view. In samples with extremely high/low amounts of PE, the excitation was accordingly decreased/increased, which was corrected in the later quantitative analysis to a laser power of the sample by multiplying or dividing to achieve the same values from a standard laser power.

A quantification of the amount and distribution of PE was programmed in Mathematica 12.0 (Wolfram Research): raw 2D STED images of bacteria were first slightly blurred using a Gaussian filter, and for each pixel the distance to the nearest background pixel was calculated using the inbuilt function “DistanceTransform” in order to obtain a list of pairs containing information on the raw intensity of each pixel and its distance to the background. The inner

intensity of bacteria was calculated as the average intensity of all pixels with $\frac{2}{3} d_{\max} < d_i < d_{\max}$ ($d_{\max}$ being the largest distance of any pixel from the background e.g. half of thickness of the bacteria). Edge intensity was calculated as the average intensity over a range of the edge of the bacteria. For more details, see supplementary information. Since all intensities were normalized to the laser power and derived in relative units, this allowed us to compare obtained intensities from inside and outside the bacterium as well as between two species.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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ASSOCIATED CONTENT

Supporting Information. The procedure of quantitative assessment of polyelectrolytes deposited on the cells as well as supplementary results of the coated bacteria are present in a PDF file. The following file is available free of charge.

AUTHOR INFORMATION

* Address correspondence to ales.lapanje@ijs.si

Author Contributions

The manuscript was prepared through contributions of all authors. IR prepared toxicity studies and developed protocols, partially performed statistical analysis and graphs, and partially prepared the manuscript. JM performed preparation of samples for STED studies and partially performed writing. HM performed STED studies, analyzed all the data and partially performed writing, JH performed SEM analysis, MČ critically revised manuscript and elaborated ideas for SEM studies, JŠ critically revised manuscript and elaborated ideas for STED studies, JK was involved in the conceptual design and critical reading, TR prepared samples for SEM microscopy and helped to write and to structure the manuscript, AL developed the idea and concept, designed the experiments, and prepared the manuscript.

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ABBREVIATIONS

PEs, polyelectrolytes; AMR, antimicrobial resistance; MIC, minimal inhibitory concentration; PEI, polyethyleneimine; PAH, poly(allylamine) hydrochlorid; LBL, layer-by-layer; MW, molecular weight; LPS, lipopolysaccharide; CFU, Colony Forming Units;

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3.3 When Beneficial Biofilm on Materials Is Needed: Electrostatic Attachment of Living Bacterial Cells Induces Biofilm Formation

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Usually biofilm formation is attributed to the presence of pathogenic microbes. Therefore, current strategies are dedicated for structuring surfaces in such a way that they prevent attachment of unwanted microbes. However, all these solutions are short-lived due to the fast conditioning of bactericidal surfaces from substances of milieu that reduce the bactericidal effects by blocking the active centers of the bactericidal surfaces. In contrast, biofilm formation is an important part of biotechnological processes, which allows cells to form a stable metabolic niche as well as remain firmly attached to different carriers. The lack of biofilm formation strictly limits the number of potential candidates, even though such cells might be very attractive for use because of their metabolic potential and biotechnological relevance. In the present manuscript, we have developed and tested an approach that allows to artificially attach viable bacterial cells to the surface including biofilm non-forming ones, using only electrostatic interaction. By depositing the beneficial coated cells, we have constructed a “living” surface, which prevents the attachment of pathogenic microbes.

In our preliminary toxicity studies of *Pseudomonas stutzeri* using a live/dead kit, we found that introduction of positively charged PEs caused a toxic effect on this strain. Since we found that viability can be lowered, we selected 8 strains with different cell architectures to test the toxic effect of positively charged PEI by using spectrophotometric determination of cell growth. It was found that many strains stay susceptible to PE. Since the toxicity was attributed to the positive charge, PEI PE was modified by introducing acetyl groups, reducing the electrophoretic mobility of coated cells on average 1.9 times. The electrophoretic mobility was determined using dynamic light scattering. Viability was assessed by spectrophotometric studies of cell growth. Analyzing the obtained growth curves, no toxic effect in cells with modified PEI was observed.

To test whether artificial attachment promotes surface population, the bacterial or glass surface were coated with modified and native PEs. Using a fluorescent microscopy, we have determined the longevity of cell attachment to modified surfaces during growth. Coating the glass with native PEI or cells with modified PEI resulted in significantly prolonged attachment for all bacterial cells including both biofilm forming and biofilm non-forming species on average 2.7 times.

Since we could safely introduce modified PEs, which did not cause toxicity, for the first time we demonstrated the case of preparing consequently assembled alive artificial biofilm that consisted of potentially probiotic and pathogenic bacteria. As a result, it revealed that the attachment of pathogenic cells to the artificial biofilm was reduced by 3.6 times and cell growth was halted, indicating controllable surface population. The estimation of pathogen and probiotic cell growth was made using fluorescence microscopy and analyzed using imaging software.

In summary, the author demonstrated a new case of biofilm management by artificial attachment of living bacteria. The surface modification of bacteria by PE deposition can expand the repertoire of strains, including biofilm non-forming for application onto different surfaces.

The candidate designed the experimental part with modification of PEs, toxicity studies and artificial attachment of cells. He partially performed experimental work, partially performed statistical analysis and partially prepared the figures and contributed to the writing of the manuscript. The manuscript was prepared in cooperation with co-authors.



When Beneficial Biofilm on Materials Is Needed: Electrostatic Attachment of Living Bacterial Cells Induces Biofilm Formation

Dmitrii Deev^{1,2}, Iaroslav Rybkin^{1,2,3,4}, Tomaž Rijavec¹ and Aleš Lapanje^{1*}

¹Department of Environmental Sciences, Jožef Stefan Institute, Ljubljana, Slovenia, ²Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, ³Remote Controlled Theranostic Systems Lab, Institute of Nanostructures and Biosystems, Saratov State University, Saratov, Russia, ⁴Department of Reactive Transport, Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf, Leipzig, Germany

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*Correspondence:

Aleš Lapanje
ales.lapanje@ijs.si

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Bacterial attachment is crucial in many biotechnological applications, but many important bacterial strains cannot form biofilms. Biofilms can damage materials, and current strategies to manage biofilms are focused on inhibition and removal of biofilm. Biofilm formation is inevitable when materials are exposed to microbes and instead of biofilm prevention, we propose management of microbial composition by formation of biofilms with beneficial microbes. Since bacteria need to overcome a high repulsive force to attach to the surface and later to grow and multiply on it, electrostatic modification of the surfaces of cells or the material by polyelectrolytes (PE) was used in our approach, enabling efficient attachment of viable bacterial cells. Since highly positively charged PEs are known to be bactericidal, they were acetylated to reduce their toxicity, while preserving their net positive charge and ensuring cell viability. In our study bacterial strains were selected according to their intrinsic capability of biofilm formation, their shape variety and cell wall structure. These strains were tested to compare how the artificially prepared vs. natural biofilms can be used to populate the surface with beneficial bacteria. Using an artificial biofilm constructed of the potentially probiotic isolate *Bacillus* sp. strain 25.2. M, reduced the attachment and induced complete inhibition of *E. coli* growth over the biofilm. This study also revealed that the modification of the surfaces of cells or material by polyelectrolytes allows the deposition of bacterial cells, biofilm formation and attachment of biofilm non-forming cells onto surfaces. In this way, artificial biofilms with extended stability can be constructed, leading to selective pressure on further colonization of environmental bacteria.

Keywords: artificial biofilms, polyelectrolytes (PEs), cell encapsulation, cell surface modification, biofilm management

Abbreviations: Ac-PEI, acetylated PEI; ELS, electrophoretic light scattering; emGFP, emerald green fluorescent protein; EPS, extracellular polymeric substance; gfp, green fluorescent protein; LPS, lipopolysaccharide; MQ, milliQ water; NB1, nutrient broth; OD, optical density; PE, polyelectrolyte; PEI, polyethyleneimine; RT, room temperature; SI, supplementary information.

INTRODUCTION

The detrimental effects of bacterial biofilms include surface biofouling and bio-corrosion making biofilms a threat to human health, environmental protection and industrial manufacturing costing more than \$500 billion per year (Alberte et al., 1992; Vigneron et al., 2018). The development of new strategies for the mitigation of biofilm formation is consequently the main priority in applied research. In most of the conventional approaches, attempts are made either to prevent the attachment of microbes, inhibit their growth, or even kill the cells after their contact with the surface. Studies have shown that structuring the surface (Lorenzetti et al., 2015; Linklater et al., 2020), applying metal oxide films (Upritchard et al., 2007), or inhibiting the functioning of adhesins (Bouckaert et al., 2005) all decrease the efficiency of bacterial attachment to the surface.

Although the current strategies preventing biofilm formation can be very effective for several days, solutions that would work in the long term have, to the best of our knowledge, not been developed to date. One of the major justifications for short-lived antibiofilm coatings is that a clean surface that is exposed in the environment or a closed microbial system, becomes immediately covered by a conditioning film (Dexter et al., 1975; Schneider 1997). This converts the original, less-hospitable or even antimicrobial surface to a surface that attracts cells and promotes their attachment (Palmer et al., 2007). Although biofilm formation on surfaces is an inevitable process, it can nevertheless be beneficial, which is demonstrated in cases such as water processing plants (Chaali et al., 2018), associations between plants and microbes in agricultural systems (Ghiasian 2020) and biofilm-based solid state fermentation processes (Cheng et al., 2010). Particularly in the latter, the benefits of biofilms are manifested by reduced microbial contamination of the fermentation process and the related need to renew cell biomass between fermentation cycles.

On the basis of the accumulated knowledge of the dynamics of biofilm development, we propose a shift of the biofilm removal paradigm. Rather than considering biofilms as undesirable structures that should be eradicated, we propose the onset of biofilm formation is used as an opportunity to construct a biofilm composed of protective bacteria that are not detrimental to the material. By selecting the first colonizers of the surface, we affect the development and composition of the biofilm structure, possibly making it become beneficial. A biofilm composed of desirable bacteria can protect the surface from corrosion, prevent the attachment of pathogenic bacteria and also be an integral part of the biotechnological process driven by the attached microbial biomass. In support of our hypothesis, studies of stainless steel surfaces (Oliveira et al., 2019), teeth (Marsh et al., 2011) and the urinary tract (O'May et al., 2008) have all shown that the presence and activity of one type of microbe is a prerequisite for the attachment and growth of a different sort of microbe, influencing the biofilm formation processes. Each microbe that becomes a part of the biofilm is contributing to its structure *via* the following mechanisms: 1) surface receptors enable physical interactions between the attached cells and compatible microbes attempting to populate the surface (Bucci et al., 2011; Liu et al., 2017), further

ensuring new physical interactions with other types of bacteria, such as anaerobic bacteria (Brooks et al., 1997), 2) selective conditions, such as pH or oxygen levels (Zuo 2007; Jakubovics 2015) provide the optimal conditions for the survival of strains that are sensitive to these factors (Bradshaw et al., 1998), and 3) a selective environment caused by extracellular metabolites can inhibit or promote the growth of other bacteria (Stadler et al., 2010).

In support of our hypothesis, Rijavec et al., 2019 have shown that the use of artificial biofilms consisting of a few selected strains can successfully inhibit the attachment and the growth of bacteria associated with microbially induced corrosion during exposure of about a month in environmental conditions. However, in the complex artificial biofilm of the previously described solution (Rijavec et al., 2019), each part of the artificial structure, the hydrophobic elastomer, the polyelectrolyte (PE) matrix, or the selected antibacterial-producing bacteria, can contribute to the observed shift in the composition of the microbial community. It is thus important to determine the contribution of each part of the artificial biofilm structure in order to further develop or redesign our initial solution for biofilm control. In order to carry out the construction of artificial biofilms using different bacteria, it is necessary to successfully attach the bacteria to the surface without reducing their viability.

Although there are many different strains of bacteria that can be used to manage and to control biofilm formation, many of them do not form biofilms and are thus not capable of attaching to many different materials. Furthermore, it has been demonstrated that many biotechnologically important bacterial strains may lose their intrinsic biofilm forming ability over time (TerAvest and Ajo-Franklin, 2016). In order to overcome this problem, it is necessary to implement new methods for cellular attachment that will promote the initial colonizers of the surface to stay attached and to form a biofilm. Bacteria must initially overcome very high repulsion forces from a surface (Hori and Matsumoto, 2010) and the conditions at the point of attachment must be favorable for growth. The surface must therefore be modified to promote initial attachment and to ensure the establishment of a relevant ecological niche, creating favorable conditions. After overcoming repulsion by decreasing the distance between the cell and the surface, it is expected that artificial attachment of bacterial cells will increase the likelihood of biofilm formation.

Our experience indicates that an approach that does not involve either genetic or chemical modification of the cell, is electrostatic modification of the surface, either of the material or the bacterial cell, using polyelectrolytes. However, highly charged PEs, especially those that are positively charged, have been used as antimicrobial coatings and prevented growth of bacteria due to their toxicity (Carmona-Ribeiro and De Melo Carrasco, 2013). Consequently, if PEs were chosen to be used for the attachment of live bacteria, either a selection of the appropriate combination of the PE and the bacterial strain must be made or the PE must be modified to mitigate its toxicity. The main goal of this study was thus to determine if the electrostatically modified cells of a selected strain, deposited onto the surface of a material could

TABLE 1 | The growth conditions and biological characteristics of the selected bacterial strains. All selected candidates were chosen from both Gram + or Gram– groups in order to distinguish the contribution of the cell wall structure, shape and division time to the process of biofilm formation. Since polyethyleneimine (PEI) is known to be toxic for some bacteria, strains were divided depending on their resistance to PEI.

No	Strain	Morphology	Generation time, min	Growth temperature(°C)	Biofilm formation ^{*,**}	Consecutive division plan	PEI sensitivity	Characteristics	References
1	<i>Staphylococcus epidermidis</i> DSM 20044	Cocci	20	37	+++*	Perpendicular	Sensitive	Pathogen, isolated from the human nose	Dice et al. (2009), Saraiva et al. (2020)
2	<i>Staphylococcus epidermidis</i> BH1	Cocci	100	37	-**	Perpendicular	Sensitive	Wild-type strain isolated from human skin	Kurečić et al. (2018), Saraiva et al. (2020)
3	<i>Bacillus subtilis</i> DSM 5547	Rod-shaped	120	28	++**	Parallel	Sensitive	n/a	Burdett et al. (1986)
4	<i>Escherichia coli</i> DH5a	Rod-shaped	30	37	-*	Parallel	Sensitive	Sensitive to PEI, unlike <i>E. coli</i> TOP10	Van Houdt et al. (2004), Banning et al. (2002)
5	<i>Pseudomonas putida</i> DSM 50198	Rod-shaped	20	28	++*	Parallel	Extremely sensitive	n/a	Li et al. (2007); Azoddein et al. (2017)
6	<i>Pseudomonas fluorescens</i> CHA0	Rod-shaped	33	28	+	Parallel	Resistant	Produce acidic extracellular polysaccharides	Bianciotto et al. (2001); Humair et al. (2009)
7	<i>Escherichia coli</i> TOP10	Rod-shaped	30	37	-*	Parallel	Highly resistant	n/a	Cowan et al. (2001), Stefan et al. (2003)
8	<i>Pseudomonas stutzeri</i> DSM 10701	Rod-shaped	58	28	-**	Parallel	Extremely sensitive	n/a	Bryan et al. (1985)
9 [†]	<i>Escherichia coli</i> TOP10	Rod-shaped	30	37	-*	Parallel	Highly resistant	Contains fluorescent pRSET-EmGFP vector	Cowan et al. (2001), Stefan et al. (2003)
10 [†]	<i>Bacillus</i> 25.2.M	Rod-shaped	70**	37	++**	Parallel	n/a	Has an antimicrobial activity** against <i>E. coli</i>	Zupančić et al. (2018)

*+ and - indicate the ability and disability, respectively, to form biofilms based on data available in this study (**) or elsewhere (*). The sign "†" denotes bacteria used only to study the interaction of the artificial biofilm with potentially pathogenic bacteria.

prevent the attachment and the growth of another selected undesirable bacterial strain. To achieve this goal, we adopted three specific aims: 1) to reduce the toxicity of PEs and to develop a method for the electrostatic modification and deposition of different viable bacterial cells onto a material surface, 2) to determine whether the electrostatic attachment of bacteria increased the possibility of forming biofilms, and 3) to demonstrate the proof of concept that the electrostatically deposited cells composed of a potentially probiotic strain can prevent the formation of the biofilm by potentially pathogenic bacteria.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Different bacterial strains were used in experimental tests on the basis of their intrinsic physiological properties. As presented in Table 1, the strains were used to: 1) test the toxicity of the modified PEs, 2) observe the effect of PE deposition on the formation and stability of biofilms and 3) test the proof of concept of the protective nature of artificial biofilms against the potentially pathogenic bacterial strains. Bacterial strains

were selected on a basis of different biological properties, including: 1) capability of formation of biofilms in the environment or in medical settings, 2) different shapes (e.g. bacillary *Escherichia*, *Pseudomonas* and *Bacillus*, and coccal *Staphylococcus*), 3) cell wall structure (Gram + and Gram –), and 4) differences in division time that contribute to the surface population. Since from our preliminary results the polyelectrolytes are known to be toxic for some bacteria, strains were differentiated according to their resistance to the polyelectrolytes, e.g. *E. coli* vs. *P. stutzeri* (see also Table 1).

Prior to experiments, the bacterial culture of each strain was pretreated by washing away the leftover components of the growth medium and obtaining comparable cell densities in the final suspension. The bacterial cell cultures were incubated overnight in a rotary shaker incubator (Multitron, Infors) at 150 rpm and the optimal growth temperature (Table 1) in 50 ml of Nutrient Broth 1 (NB1) medium (Carl Roth). The cells were washed 3 times with sterile 0.9% NaCl solution using centrifugation at 5000 g for 5 min. Before each experiment, the optical density of the cell suspension was normalized to optical density (OD)₆₀₀ = 0.1 (at the reduced optical path of the 200 µL volume of a well of a 96 well microtiter plate; VWR).

Reduction of Polyethyleneimine Toxicity by Acetylation

Since in our preliminary experiments PEI demonstrated toxicity when exposed to bacterial cells (see Supplementary Information – SI, **Supplementary Figure S1**), here it was tested to see if reducing the number of amino groups in the PEI molecule would decrease its toxicity. However, PEI should retain a sufficiently positive charge to be capable of successful covering of bacterial cells. PEI was modified according to methods described by Forrest et al. (2004) to produce acetylated PEI (Ac-PEI). Briefly, acetic anhydride (Sigma-Aldrich) and acetic acid (Merck KGaA) were used to acetylate the branched 600-kDa PEI (Sigma-Aldrich). The acetylation was carried out by mixing PEI with acetic anhydride 56% (w/v) in the presence of acetic acid, followed by incubation at 60°C for 4 h. The reaction solution was dialyzed using SnakeSkin™ Dialysis Tubing (10 K MWCO, 22 mm, Thermo Fisher Scientific) for 2 days in distilled water. The pH was monitored regularly and water was changed every 2 h until neutral pH was reached. The dialysis volume was allowed to exceed the reaction volume by a factor of about 100 to facilitate purification of Ac-PEI from the reaction mixture. Finally, the concentration of Ac-PEI was adjusted to 2.5 mg ml⁻¹ using milliQ water (MQ), the pH was adjusted to 7.0 using 1 M NaOH or HCl, and concentrations were chosen to prevent dilution of the polyelectrolyte solutions. The Ac-PEI solution was sterilized using 0.2 µm pore size filters (Sartorius). The degree of acetylation of the purified polymer was determined directly by potentiometric measurements during the preliminary work and indirectly by measuring the electrophoretic mobility values of bacterial cells coated with polyelectrolytes with different levels of acetylation (see SI, **Supplementary Table S1**). The most important aspect after the acetylation procedure used in this experiment is the correlation of electrophoretic mobility values, acetylation procedure and viability of cells, since our aim was to attach viable cells electrostatically.

Determination of the Suitability of the Ac-PEI for Surface Modification of Bacterial Cells

Since a surface modified by PEs should enable electrostatic attachment of cells but should not be toxic to viable bacterial cells, toxicity tests of the native PEI and Ac-PEI were performed. It was also determined the efficiency of the reversal of the surface charge of the bacterial cells when either PEI or Ac-PEI were deposited onto the surface of cells, and the cells were coated by each type of the PE. Three strains that showed different levels of resistance against the native, non-acetylated PEI were selected (see **Table 1**). The strain *E. coli* TOP10 was resistant to PEI, while the other two strains, *P. stutzeri* DSM 100701 and *P. putida* DSM 50198, were sensitive to PEI. The toxicities of PEI and Ac-PEI were evaluated by measuring the growth rate and by Live/Dead staining.

The growth rate was determined by measuring the optical density (OD₆₀₀) during the incubation. Before incubation, the sterile solution of PEI was mixed (concentration 2.5 mg ml⁻¹)

with the sterile two-times more concentrated medium NB1 (2X NB1) at a ratio of 1:1 (v/v) in wells of a sterile microtiter plate (VWR). A total of 20 µL of the bacterial cell suspension (OD₆₀₀ = 0.1) was added to each of the prepared dilutions. To determine bacterial growth for each well containing the Ac-PEI or PEI mixture OD₆₀₀ was measured every 30 min during the 24 h incubation. During this period the cell suspension was incubated in the plate reader/incubator (Synergy H4, Biotek) at a growth temperature appropriate for each strain (see **Table 1**). A 30 s shaking period was applied before each measurement to resuspend the sedimented cells.

In the same three strains, the toxicity of the PEs was determined by the observations under microscope after Live/Dead staining (Thermo Fisher Scientific) according to a commercially available procedure. In order to exclude possible effects from the interaction of PE with dye components rather than bacterial cells, it was also performed staining of the inanimate microcapsules of calcium carbonate coated with PEI or Ac-PEI according to the same protocol. Image acquisition was carried out using the inverted fluorescent microscope Zeiss Axio Observer Z1 (Zeiss, Germany), gfp (485 nm/528 nm) and dsRED filter sets (599 nm/619 nm) and the digital CCD camera Orca (Hamamatsu, Japan).

Since acetylation reduces the number of charged amino groups in the PEI, the charge differences between native PEI and Ac-PEI were analyzed, by measuring the electrophoretic mobility using electrophoretic light scattering (ELS) measurements (Delsa Nano HC, Beckman Coulter) of the cells coated with a single layer of PE as described by Rybkin et al., 2019.

The Electrostatic Deposition of Bacterial Cells and Biofilm Formation

Monitoring of biofilm formation promoted by the electrostatic deposition of bacterial cells onto the surface of a coverslip glass (Menzel) in a growth chamber described by de Jong et al., 2011 and Rybkin et al., 2019, was accomplished with modifications. According to the coverslip characteristics the roughness of surface is estimated to be 19 Å. Coverslip glasses from the same producer were used in order to exclude the effects on bacterial attachment of surface properties such as roughness or hydrophobicity. Electrostatic deposition of cells was achieved by changing the surface charge of either the glass surface or the bacterial cells themselves.

In the first approach the glass surface was coated with either PEI or Ac-PEI. A total of 30 µL of each PE (concentration 2.5 mg ml⁻¹) in MQ was applied to one side of the glass by smearing the liquid across the glass surface using the edge of a cover slip. After the PE was evenly distributed in this way, the sample was allowed to dry at room temperature (rt) (20°C). Subsequently, 20 µL of the bacterial cell suspension (OD₆₀₀ = 0.1) was pipetted onto the glass surface and left to incubate for 15 min at rt. This droplet approach produced the highest success of cell attachment in the various bacteria that were tested. The droplet approach was selected after different approaches including dip coating, smearing and application of droplets were examined, in combination with varying temperatures

(+4°C or +20°C), NaCl concentrations, which were 0.154 M or 0.015 M, and different incubation times of the cells, which were 5, 15, 30 or 45 min.

In the second approach, 20 µL of the suspension of the PE-coated cells was applied onto the unmodified glass surface within the prepared chamber and was allowed to incubate for 15 min at rt. Before executing the experiments, the unattached cells were in both cases washed off with 200 µL of sterile 0.9% NaCl, the chamber was filled with standard NB1 medium and was finally sealed by the cover glass. In order to culture only the attached cells on the glass surface, the side of the chamber upon which the bacteria were deposited, was turned upside down, so that the surface with the attached cells was facing downwards. The images were taken at the same physical location on the surface after 0, 3, 24, and 48 h. Recorded images were further processed in FIJI/ImageJ software (Schindelin et al., 2012). After the initial deposition it was observed that the cells coated with the PE or deposited on the PE-coated glass tend to form aggregates when attached to the surface. Thus, the analyzed surface of one aggregate was considered as a single bacterial unit (see SI, **Supplementary Figure S3**). As a result, the surface area occupied by the bacterial cells was mainly used to estimate the dynamics of the biofilms.

The Proof of Concept—Controlling Biofilm Development With the Artificial Biofilm

Using the glass chambers described above, an artificial biofilm composed of *Bacillus* sp. strain 25.2. M was prepared. The strain used herein previously had been isolated from the oral cavity and was showing strong inhibition of growth of potentially pathogenic Gram-negative bacteria *E. coli* and *Aggregatibacter actinomycetemcomitans*, respectively (Zupančič et al., 2018). Viable cells of strain 25.2. M were first stained with fluorescent dye Syto 64 (Thermo Fisher Scientific) by mixing the bacterial cell suspension ($OD_{600} = 1.0$) with 10 µM of Syto 64 fluorescent dye in ratio 1:1 (v/v) and incubating the mixture in the dark for 30 min. The cells were then washed 3 times by sterile 0.9% NaCl. Each time the cells were collected by centrifugation at 5,000 g for 5 min and finally, they were resuspended in sterile 0.9% NaCl. The artificial biofilm of *Bacillus* sp. 25.2. M was prepared by depositing a dense layer of cells of *Bacillus* sp. 25.2. M strain, Syto 64 stained and Ac-PEI coated on the glass surface. A total of 100 µL of the bacterial cell suspension ($OD_{600} = 1.0$), was incubated for 15 min at rt to allow the coated cells to attach to the glass surface. Using this protocol, it was managed to cover the surface with an average distance of 13.3 ± 5.5 µm between the bacterial cells.

To observe how the artificial biofilm of *Bacillus* sp. 25.2. M inhibits the attachment of Ac-PEI coated cells of a potentially pathogenic bacterium *E. coli*, it was used 20 µL of the bacterial cell suspension ($OD_{600} = 0.05$) of strain *E. coli* TOP10—emGFP (Emerald Green Fluorescent Protein, transformed by the expression vector pRSET-EmGFP; Thermo Fisher Scientific), which was spread over the surface of the artificial biofilm. In our experiment 100 times less *E. coli* than *Bacillus* sp. cells were used to simulate the initial colonization of the artificially formed

biofilm. The chamber was filled with NB1 medium and tightly sealed as described previously. The chamber was incubated for 24 h without being turned upside down. A negative control for biofilm formation at the absence of *Bacillus* sp. 25.2. M based artificial biofilm and a positive control were prepared by constructing the artificial biofilm using cells of two strains of *E. coli* TOP10, one with and one without the plasmid. Bacterial cells without the plasmid were stained by Syto 64 (red) to contrast the cells of *E. coli* TOP10 - emGFP (green) applied onto the surface forming an artificial biofilm such as with the *Bacillus* sp. 25.2. M. The cell growth and spatial distribution of *E. coli* TOP10 - emGFP on the surface were monitored at 0, 6 and 24 h by fluorescent microscopy imaging using gfp (485 nm/528 nm) for *E. coli* TOP10 - emGFP, and dsRED filter sets (599 nm/619 nm), for the *Bacillus* sp. 25.2. M and *E. coli* TOP10 (plasmid free) strains stained by Syto 64.

Image Analysis

In order to learn the growth dynamics of the biofilms, the shape, the size, the cell number and the gfp expression level as well as the extent of the surface covered with bacterial cells were determined from the obtained images, using the FIJI software (Schindelin et al., 2012). Only images with a contrast to noise ratio of 5.8 ± 2.1 were used for the analysis. Each stack of images was adjusted using the “subtract background” function with a rolling ball radius of 5 pixels and then was converted into a binary image using the “make binary” function. The boundaries of bacterial cells and their aggregates were outlined using the “find edges” algorithm (Burger and Burge, 2009) and this was followed by filling the inner area (Legland et al., 2016), using the “fill holes” function. The growth of biofilms was estimated by measuring the number and surface area of cells over time. Calculation of the surface area and number of bacterial cells was performed using the “analyze particles” plugin with the lowest size limit of $0.1 \mu m^2$. The “mean gray” value parameter was used to evaluate the gfp (green fluorescent protein) expression level.

Data Analysis

Unless otherwise specified, data are always reported as mean $\pm$ standard deviation calculated from three independent experimental measurements. Data sets were analyzed using the two tailed *t*-test. Statistical significance is represented as (*, †) for *p* value lower than 0.05. Graph plotting was prepared and visualized using LibreOffice Calc (The Document Foundation), Inkscape (Free Software Foundation) and Fiji. Growth curves were analyzed in PMAAnalyzer (Cuevas and Edwards, 2017).

RESULTS

Toxicity of PEI Is Decreased by Acetylation

In our preliminary experiments we observed that the toxicity of PEI varied between different bacterial strains (SI, **Supplementary Figure S1**). Using Live/Dead staining, PEI toxicity resulted in the death of $88 \pm 6\%$ of the cells of *Pseudomonas stutzeri*, $87 \pm 7\%$ of *Pseudomonas putida* and $55 \pm 4\%$ of *E. coli* TOP10 (*n* = 4 for each bacterial species) (**Figure 1**). In control experiments, in which

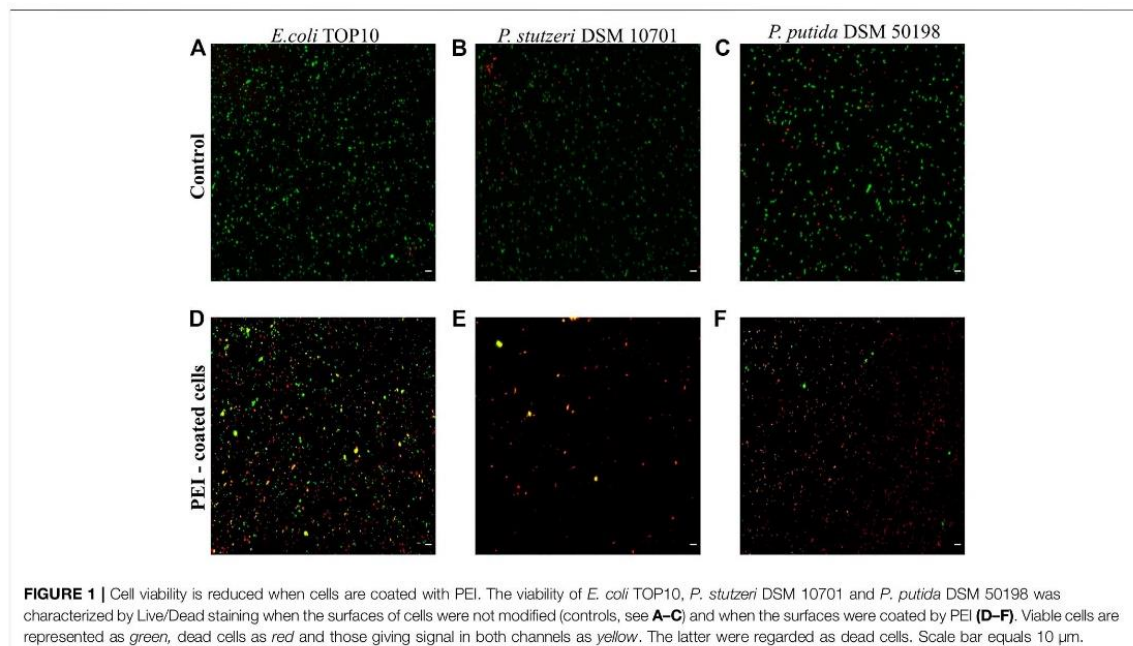
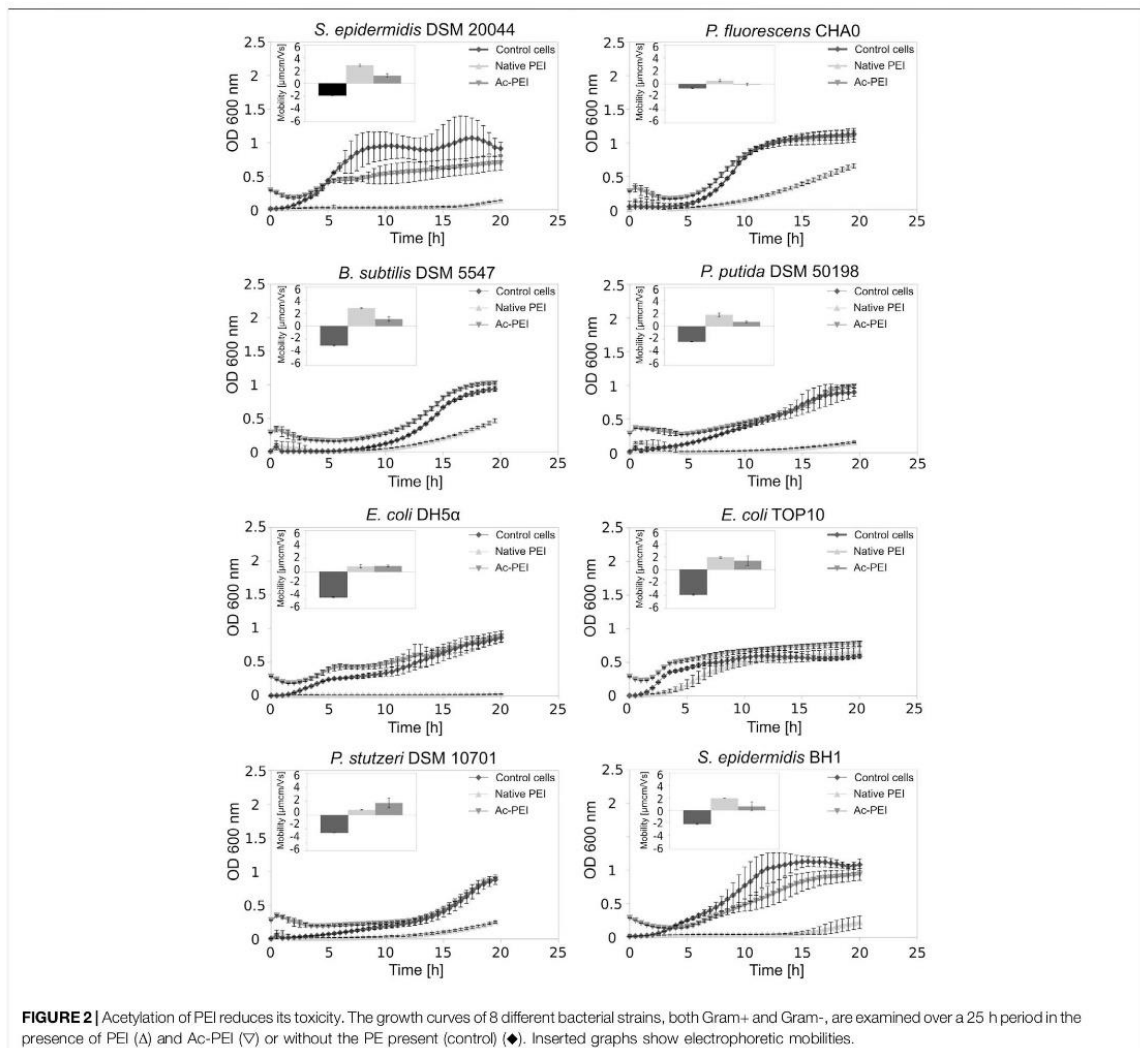


TABLE 2 | The parameters of the growth curves obtained from MIC tests. Asymptote factor-biomass yield obtained during stationary phase. Growth level - amplitude-weighted metric quantifying the amount of growth of selected bacterial strains (Cuevas and Edwards, 2018). Growth class – qualitative classification of growth based on growth level (+++ $g \geq 0.75$; ++ $0.5 \leq g < 0.75$; + $0.35 \leq g < 0.50$; - $0.25 \leq g < 0.35$; -- $g < 0.25$).

Strain	Sample	Asymptote [OD]	Growth level [OD]	Growth class
<i>S. epidermidis</i> DSM 20044	Control	1.0 ± 0.2	1.5 ± 0.4	+++
	PEI	0.05 ± 0.01^{ab}	0.04 ± 0.02^{ab}	--
	Acetylated PEI	0.7 ± 0.1	0.7 ± 0.2^a	++
<i>P. fluorescens</i> CHA0	Control	1.1 ± 0.1	1.5 ± 0.1	+++
	PEI	0.23 ± 0.004^{ab}	0.5 ± 0.2^{ab}	+
	Acetylated PEI	1.1 ± 0.1	$1.2 \pm 0.1^*$	+++
<i>B. subtilis</i> DSM 5547	Control	0.9 ± 0.03	1.1 ± 0.03	+++
	PEI	0.13 ± 0.005^{ab}	0.23 ± 0.02^{ab}	--
	Acetylated PEI	1.0 ± 0.02^a	1.0 ± 0.02^a	+++
<i>P. putida</i> DSM 50198	Control	0.9 ± 0.1	1.2 ± 0.1	+++
	PEI	0.1 ± 0.01^{ab}	0.1 ± 0.02^{ab}	--
	Acetylated PEI	1.1 ± 0.03^a	0.95 ± 0.03^a	+++
<i>E. coli</i> DH5 α	Control	0.8 ± 0.1	1.1 ± 0.1	+++
	PEI	0.02 ± 0.001^{ab}	0.01 ± 0.003^{ab}	--
	Acetylated PEI	0.9 ± 0.1	1 ± 0.15	+++
<i>E. coli</i> TOP10	Control	0.6 ± 0.05	1.0 ± 0.1	+++
	PEI	0.6 ± 0.05^b	1.0 ± 0.1	+++
	Acetylated PEI	0.7 ± 0.03^a	0.9 ± 0.1	+++
<i>P. stutzeri</i> DSM 10701	Control	0.8 ± 0.1	0.88 ± 0.11	+++
	PEI	0.08 ± 0.01^{ab}	0.11 ± 0.01^{ab}	--
	Acetylated PEI	1.0 ± 0.02	0.8 ± 0.03	+++
<i>S. epidermidis</i> BH1	Control	1.1 ± 0.1	1.6 ± 0.1	+++
	PEI	0.06 ± 0.01^{ab}	0.05 ± 0.01^{ab}	--
	Acetylated PEI	1.0 ± 0.1^a	1.1 ± 0.1^a	+++

^aSignificantly different than the control ($p < 0.05$).

^bSignificantly different than the acetylated PEI ($p < 0.05$).

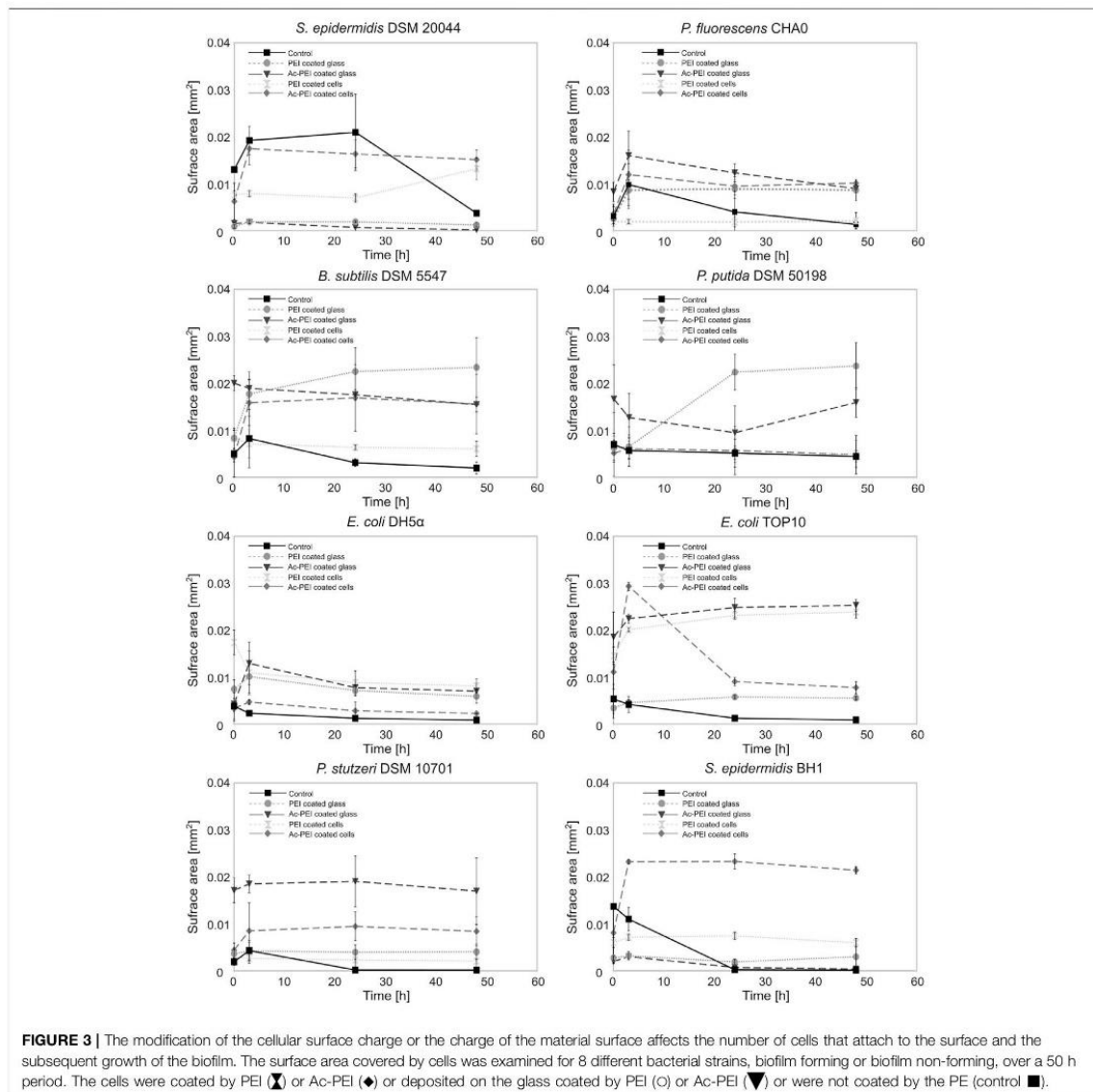


PEI was absent, the number of dead cells was approximately 3–10 times lower, $28 \pm 7\%$, $24 \pm 2\%$ and $5 \pm 1\%$, respectively. Both PE-coated and non-coated inanimate microcapsules propidium iodide did not interact with PEs (see SI, **Supplementary Figure S2**).

In our study, PEI inhibited the growth of all the tested strains within 24 h, which was manifested by a prolonged lag phase of the cell cultures. The highest sensitivity to PEI exposure was observed for *E. coli* DH5α, *S. epidermidis* DSM 20044 and *S. epidermidis* BH1 (see **Table 2**) and the growth levels were reduced by factors of 178.33, 36.93, and 29.81, respectively. On the contrary, *E. coli* TOP10 was found to be the most resistant to PEI toxicity and with this strain no reduction of the growth level was observed. The

PEI-coated cells of Gram-negative bacteria had values 2.06 times less on average than the electrophoretic mobilities of the Gram-positive strains.

Using acetylation, the positive charge of the PEI molecules (see SI, **Supplementary Table S1**) was decreased. In contrast to PEI, Ac-PEI failed to inhibit the growth of all tested strains during the 25 h period (**Figure 2**; **Table 2**). In the presence of Ac-PEI all strains consistently showed higher values of the initial OD₆₀₀ (**Figure 2**, see the first 5 h) than in the control experiments. Acetylation of PEI decreased the electrophoretic mobility of the coated cells of the majority of strains, by a factor of 1.86 on average, except for *E. coli* DH5α and *P. stutzeri*, where the values were unchanged or even increased, by a factor of 2.29.



Electrostatically Attached Bacterial Cells can Form Biofilms

Ac-PEI allowed us to manipulate the cell surface charge without its being toxic to the cells and it gave us possibility to determine how the electrostatic deposition of cells to the glass surface enabled both the biofilm forming and biofilm non-forming bacteria to occupy the freshly exposed surfaces (Figure 3).

After initial attachment, the surface area occupied by the majority of the control strains was reduced immediately after the initial stage (0 h) and was on average 28.76 times lower after

48 h. The non-modified cells of biofilm forming strains showed attachment patterns different from those of the biofilm non-forming strains. The biofilm non-forming strains did not show any significant increase in the surface area of the attached cells, while all three biofilm forming strains *P. fluorescens* CHA0, *B. subtilis* DSM 5547, and *S. epidermidis* DSM 20044 increased the surface area by a factor of 2.11 within the first 3 h. Among the biofilm forming bacteria, the cells of *S. epidermidis* DSM 20044 covered the largest surface area at the 24 h time point, and was increased by a factor of 1.6 ($p < 0.1$) from the initial stage (0 h) and was on average 5.7 times larger than the other two biofilm

TABLE 3 | Relative changes of the surface area of the attached biomass at 48 h. Obtained values illustrate the increase or decrease of the surface area occupied by the bacteria, relatively to the initial stage (0 h). Values in bold represent the most appropriate approach for the attachment and biofilm formation of the bacteria onto the surface.

Strain	PEI coated glass	Ac-PEI coated glass	PEI coated cells	Ac-PEI coated cells
<i>S. epidermidis</i> DSM 20044	1.0 ± 0.3	0.2 ^a ± 0.2	1.7^a ± 0.3	2.9^a ± 1.0
<i>P. fluorescens</i> CHA0	2.8^a ± 1.3	1.1 ± 0.6	1.2 ± 1.0	1.5 ± 0.4
<i>B. subtilis</i> DSM 5547	2.8^a ± 0.2	0.8 ^a ± 0.02	0.7 ^a ± 0.1	3.5^a ± 0.6
<i>P. putida</i> DSM 50198	4.3^a ± 2.1	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.3
<i>E. coli</i> DH5α	0.7 ^a ± 0.04	1.9 ± 1.2	0.3 ^a ± 0.3	0.7 ^a ± 0.1
<i>E. coli</i> TOP10	2.0^a ± 0.8	1.5^a ± 0.3	1.7^a ± 0.2	0.5 ± 0.4
<i>P. stutzeri</i> DSM 10701	1.1^a ± 0.1	0.8 ± 0.3	0.8 ± 0.5	2.1^a ± 0.9
<i>S. epidermidis</i> BH1	0.8 ^a ± 0.4	0.2 ^a ± 0.1	1.0 ± 0.03	2.4^a ± 0.7

^aSignificantly different than at the initial stage 0 h ($p < 0.05$).

forming strains. The attachment *S. epidermidis* DSM 20044 survived past 3 h, while the cells of other two biofilm forming strains gradually detached from the surface after 3 h.

Among the electrostatic modifications tested, coating the glass with Ac-PEI produced the highest number of bacterial strains with increased initial attachment of cells. The four bacterial strains were *E. coli* TOP10, *P. fluorescens* CHA0, *P. stutzeri* DSM 10701, *B. subtilis* DSM 5547. None of the electrostatic modifications showed any improvements at 0 h for both the *S. epidermidis* strains. The modification of the surface of cells or glass by PEI or Ac-PEI deposition significantly prevented detachment of bacterial cells and sustained the artificial biofilm on the glass surface for up to 48 h. Although the deposition of PEI on the glass surface as well as coating the bacterial cells with Ac-PEI were found not to be the most efficient for the initial attachment of cells, it sustained biofilm formation for up to 48 h for all strains except *E. coli* DH5α (Table 3).

The glass surfaces coated with PEI supported artificial biofilm formation for 5 strains (Table 3; column PEI coated glass). Among these strains, the cells of *P. putida* DSM 50198 showed the highest increase of the surface area (on average 1.94 times more than other strains) occupied by cells over the 48 h period. In contrast, coating of the bacterial cells with Ac-PEI was the most suitable approach for 4 strains (see Table 3; column Ac-PEI coated cells), among which, *B. subtilis* DSM 5547 showed the highest increase, on average 2.15 times that of other strains of the occupied surface area at 48 h. The other two approaches, coating the glass surface with Ac-PEI and coating the bacterial cells with PEI, were found to be less effective in keeping the bacterial cells attached, since only *E. coli* TOP10, and *S. epidermidis* DSM 20044 (only for Ac-PEI coated glass) showed a significant increase of the occupied surface area at the 48 h time point.

In contrast to the controls, in which almost all the cells of the biofilm forming bacteria were detached after 48 h, surface modifications by PEs resulted in a 4.66 times larger occupied surface area, and the occupied surface areas were 26.02 times larger for the biofilm non-forming strains. Finally, the temporal dynamics of cellular attachment, the increase or decrease in the numbers of the attached cells were in both groups, biofilm forming and non-forming, similar due to the PE treated surfaces.

Artificially Constructed Biofilm Inhibits growth of *E. Coli* on the Surface

To test the effects of the artificial biofilm in an *in vitro* system containing two different bacterial species, it was successfully constructed the biofilm with *Bacillus* sp. 25.2. M, a potential oral probiotic strain, and *E. coli*. In the presence of the artificial biofilm the cell numbers of *E. coli* TOP10 - emGFP were constant over time and no significant growth was observed on the surface of the artificial biofilm during the 24 h exposure period (Figures 4A–C). Already at the initial stage, the Ac-PEI covered cells of *E. coli* did not attach well when applied to the surface of the *Bacillus*-based artificial biofilm and even though the attachment of *E. coli* was also low on the raw glass surface (negative control), there were 3.64 times fewer cells on the surface of the *Bacillus*-based biofilm compared to the negative control. The number of cells of *E. coli* TOP10 - emGFP, did not change during the 24 h test period, while the gfp signal (green) was significantly reduced, on average by a factor of 1.19.

In the positive control (Figures 4D–F), where the cells of *E. coli* TOP10 were used instead of the strain *Bacillus* sp. 25.2. M to construct the artificial biofilm, it was observed a larger number of the attached cells in the initial stage, on average 18.46 times more than with the *Bacillus*-based biofilm and 5.1 times larger compared to the negative control. In the initial stage the number of attached cells in the positive control was higher compared to the negative control. But since the growth of *E. coli* TOP10 - emGFP was significantly lower by 4.65 times on average, in the positive control during the 24 h period (Figures 4G–I), there was no significant difference between the positive and the negative control in the number of the attached cells after 24 h. The gfp signal of *E. coli* - emGFP in the positive control was not changed over time, however it was already significantly lower at the initial stage (1.34) than in the negative control.

DISCUSSION

Our study clearly shows that the concept of using electrostatically deposited cells of *Bacillus* sp. 25.2. M, a potential probiotic strain, to construct an artificial biofilm controls further development of the biofilm by inhibition of *E. coli* growth (Figure 4). We need to

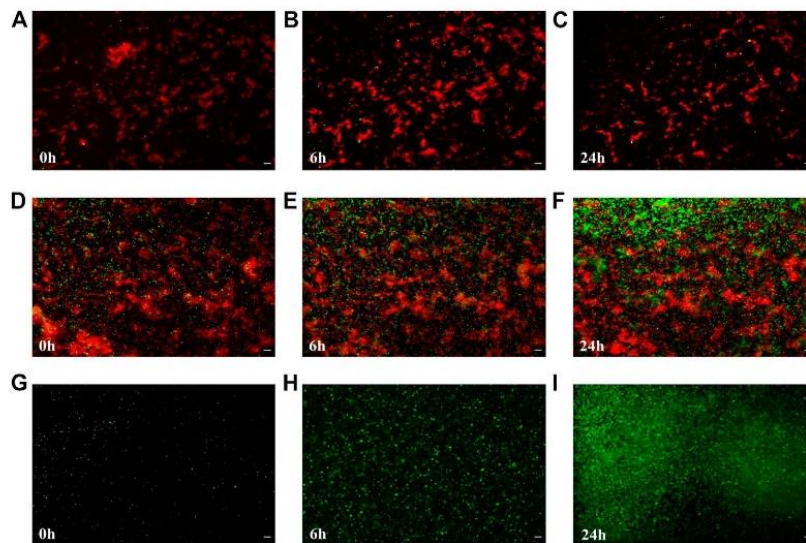


FIGURE 4 | The colonization and growth of potentially pathogenic *E. coli* is inhibited by the artificial biofilm made from the probiotic candidate strain *Bacillus* sp. 25.2.M. The number of attached cells of *E. coli* TOP10 emGFP, expressing the green fluorescent protein (gfp) (green), was determined after 0, 6 and 24 h of cultivation when the artificial biofilm was made from the antimicrobial-producing *Bacillus* sp. 25.2. M (cells are stained by Syto 64; visible in red) being deposited onto the glass surface (**A–C**) or from *E. coli* TOP10 (red) not producing gfp (positive control) (**D–F**), or no pre-applied biofilm on the surface (negative control) (**G–I**). Scale bar equals 10 μ m.

stress that the division rate of *E. coli* is 3 times higher at the experimental conditions used, meaning it could easily outgrow the film of *Bacillus* cells on the glass surface. Based on our previous study (Zupančič et al., 2018), we hypothesized that the bacterial cells that come into close contact with *Bacillus* sp. 25.2. M are inhibited by its extracellular antimicrobial products. Many different *Bacillus* strains are known to produce a wide range of antimicrobial substances, such as biosurfactants (El-Sheshtawy et al., 2015), antibiotics (Stein, 2005), bacteriocin (Bizani and Brandelli, 2002), lipopeptides (Ongena and Jacques, 2008), and antimicrobial peptides (Jayaraman et al., 1999). Additionally, *Bacillus* could inhibit the growth of bacteria that attach to the surface by competitive exclusion, which is the result of highly specific niches that are created by *Bacillus* (La Ragione et al., 2001; Morikawa, 2006). Apart from the antimicrobial substances, the formation of a specific niche is a result of combined effects of the production of exopolymeric substances (EPS), changes of local pH, oxygen depletion and exposure of incompatible receptors for attachment of other microbes (Xavier and Foster, 2007; Pham et al., 2009). Due to the production of spores *Bacillus* strains are also the most appropriate choice if the material surface is exposed to harsh environmental conditions. These strains can survive pHs up to 11 and temperatures as high as 80°C as well as high voltages during the electrospinning and other harsh conditions such as cold plasma treatment and pasteurization.

We reasoned that the highest efficiency of the biofilm for inhibiting the establishment and growth of harmful bacteria can

be achieved when the surface of treated material is completely covered with beneficial cells, leaving the surface with no free binding sites for new colonizers. In our experiments it was observed that regardless of the method used for cell deposition only up to 21.37% of the surface was covered by the cells (Figure 3, *B. subtilis* deposited on the Ac-PEI coated glass), meaning that the predominately uncovered surface would allow harmful bacteria to establish microcolonies. For initial attachment to the raw surface, bacterial cells must be in close proximity to the surface, needing about 20–200 nm distance to experience the effect of Van der Waals forces and about 20 nm to initiate even stronger electrostatic interactions (Kumar and Anand, 1998; Palmer et al., 2007). It can be expected that due to the electrostatic repulsion between PE-modified bacterial cells during deposition to the surface, the cells can cover the surface only partially. To improve the binding events, the surface could be dried after deposition so that the Van der Waals forces become more prominent and the electrostatic interactions are reduced, but cell viability can be significantly lowered this way (Nocker et al., 2012). Thus, it is more appropriate to promote cell division after deposition of viable cells to achieve overgrowth of the surface, since our deposition approach promoted the formation of biofilms in both biofilm forming and non-forming bacteria. In our results the density of cells of biofilm non-forming *E. coli* TOP10 increased by a factor of 3.95 after such incubation (Figure 3, Ac-PEI coated cells of *E. coli* TOP10). We find that the first 3 h after deposition are the most critical in the formation of dense films, since after 3 h the state of biofilm

density is probably the consequence of a dynamic equilibrium between detachment of bacteria and spreading of the biofilm over the surface. However, the timing of the formation of dense biofilms must be determined for each individual strain, especially if one aims to use bacteria with much longer generation times than those used in our study.

Since formation of dense biofilms is dependent on the process of cell division after the initial electrostatic attachment, it can be expected that the division plane of the bacteria could have an important role. If the division plane is perpendicular to the material surface, cells will divide and grow parallel to the material, staying very close to the surface, thus increasing the chance of keeping cells attached. In contrast, if bacterial cells divide in a plane parallel to the material surface, they tend to form columns or grape-like structures resulting in weakly attached biofilm and low occupation of the material surface. In our experiments two types of cells were used, with mixed parallel and perpendicular growth tendencies, coccoidal shaped cells of *Staphylococcus* strains, as well as those with only perpendicular division planes, rod shaped cells of bacteria like *Bacillus* (Saraiva et al., 2020; Bramkamp and van Baarle, 2009). For the former group where half of all the divisions happen in parallel to the surface and half perpendicularly, it can theoretically be expected that these bacteria spread over the surface with a two-time slower rate than those with perpendicular division plane. Unexpectedly, we observed that *S. epidermidis* DSM 20044 increased the occupied surface the most, up to 1.48 times within the first 3 h (Figure 3). This can be explained by the fact that bacillary cells can also grow over each other due to the formation of wrinkles in the biofilm, depending on the surface properties and cell density (DePas et al., 2013; Aguilar et al., 2018). Additionally, regardless of the spherically shaped cells dividing in different planes when the daughter cells are formed, they can occupy exposed empty space on the surface by settling at the most energetically favorable place, the oppositely charged surface of the material.

In our experiments it was also observed that for formation of dense biofilms it is more efficient to use a surface covered with PE than using the same PE to cover cells. According to Rybkin et al., 2019 it is expected that covering of cells with PE can increase the delay of the division and consequently slow down growth. In addition, the surface of the glass is still negatively charged when cells are released from the PE cover in contrast being present on the PE covered glass. Therefore, less bacterial cells produced after the replication can become attached to the non-covered glass surface (Figure 3). However, although the Ac-PEI coated cells were less efficient in the first few hours to form film than any of the PEI coated glass, the approach where cells were coated by Ac-PEI was the second-best approach just behind that using the PEI coated glass. This approach was the most efficient for the biofilm forming strains, which can be due to the electrostatic attachment of cells on the surface and placing of the whole process of biological attachment very close to the surface. In return, it increases the probability of attachment of the newly formed cells after the division although the PEs are not present either on the cell or on the glass surface. This is most obvious for all three strong biofilm forming strains since they can form biofilm without any assistance and it seems that the distance to the

surface is one of the key factors for the formation of such biofilms (Palmer et al., 2007). Since here it was used the combined approach of physical attachment and biological biofilm formation, where attached cells must be alive and capable of cell division, the toxicity of PE must be considered. In this relation, different combinations of PE, either surface deposited or cell coated, must be tested for each particular strain (Figure 2; Table 2).

Currently, we could not determine why PEs were more toxic for one strain than for others, however, several factors have been reported that contribute to the PE toxicity. These include hydrophobicity, molecular size of PE (Qiao et al., 2012) or electrostatic interactions with the membrane molecules (Rawlinson et al., 2010), since the positively charged amino groups can form pores in the membrane (Brogden, 2005), and cause cell lysis (Fakhrullin et al., 2014). We also can not eliminate the possibility that PEs can alter the activity of proteins or interfere with metabolic processes, causing metabolic changes and cell death, since in our previous studies we observed the induction of production of constitutive protein by the PE entrapped *E. coli* cells (Rybkin et al., 2019). By simply comparing Gram-positive with Gram-negative bacteria, it can be expected that the architecture of the bacterial cell wall plays a key role in different outcomes when the bacteria are coated by PEs. With an outer membrane, less negatively charged lipids (Qiao et al., 2012), and the presence of lipopolysaccharide (LPS) making it harder for the PE to permeabilize the inner membrane, Gram-negative bacteria should be more resistant to surface modification by PEs than Gram-positive bacteria (Song et al., 2011; Thoma et al., 2014). Our study revealed the opposite (Figure 2 and Table 2, see *Pseudomonas* strains) because high PEI toxicity was also observed in Gram-negative strains. The resistant strains either have a lower amount of PE deposited on the cell surface or the PE molecules are spatially oriented differently, avoiding toxicity. Since toxicity was observed for the PE-modified cells with low or high electrophoretic mobilities and since the mobilities of the Ac-PEI surface modified cells (i.e. Ac-PEI is non-toxic) were similarly low for all strains, we concluded that the amount of the deposited PE is not the reason for the toxicity, but rather the interaction of the PE with the cell surface is responsible. Therefore, a detailed study of cell architecture either within one Gram type or between Gram types is thus necessary to better understand this interaction.

To lower the toxic effects of PEI, we used acetylation to derivatize the free primary amines and lower their level (Forrest et al., 2004) to an extent which would improve cell viability without seriously compromising the positive charge of the molecule. According to our prediction, the decrease in the charge density of the PEI caused by the primary amines decreased the binding efficiency of PEI and consequently its toxicity (Figure 2; Table 2). However, due to the higher survivability of cells, the Ac-PEI enables enough strong attachment inducing further biofilm formation. Besides modification of the polyelectrolytes, attachment of the bacteria on the surface can be improved by manipulating the surface parameters, including roughness, surface charge and hydrophobicity. Higher roughness should be more beneficial for the bacterial cells since they will

have a larger area to which to attach, while combination of surface charge and hydrophobicity can improve binding of the polyelectrolytes with an inanimate surface.

In conclusion, we have demonstrated for the first time a new solution which does not prevent the inevitable establishment of the biofilm, but carefully manages its development by attachment of live bacteria. The electrostatic method is very useful since it enables artificial attachment of viable bacterial cells, including those unable to form a biofilm, by coating the cell surface with a positively charged non-toxic polyelectrolyte. The modification of PEs widens the repertoire of strains that can be used, and consequently the proposed approach can be applied to different surfaces in a variety of industries such as biotechnology, medical and other sectors, where prevention and control of biologically induced corrosion is important. The use of attached bacteria also prevents their detachment in the quick sand filter purification systems used for drinking and wastewater treatment and increases the success of bioaugmentation (Horemans et al., 2017).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DD executed the experimental part, partially performed statistical analysis and contributed partially to the writing. IR contributed to

the design of the experiments, partially performed statistical analysis, partially contributed to the writing and executed the experimental part. TR was involved in the design of the experiments, contributed to the writing and critical reading of the manuscript. AL developed the idea and concept, designed and executed the experiments, and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmats.2021.624631/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.4 Medium-Dependent Antibacterial Properties and Bacterial Filtration Ability of Reduced Graphene Oxide

Gusev, A., Zakharova, O., Muratov, D. S., Vorobeva, N. S., Sarker, M., Rybkin, I., ... Kuznetsov, D. V. (2019). Medium-dependent antibacterial properties and bacterial filtration ability of reduced graphene oxide. *Nanomaterials*, 9(10), 1454. <https://doi.org/10.3390/nano9101454>

Since we can structure bacterial biofilms and control their properties, we can use this to our advantage in possible application in microbial fuel cells. Since energy transfer is problematic, we can apply graphene materials that are known to be good conductors. For this purpose, we chose rGO NPs, which can be used to improve energy transfer from bacteria in this process. However, in many of the reported cases, we found big ambiguities in toxicity as, on the one hand, graphene was claimed to be nontoxic and, on the other hand, good bactericidal activity was observed. The toxicity of graphene materials might result from the interplay between various characteristics of the material with the biological aspects of a particular cell. Therefore, if we want to use such a material for surface functionalization, it would be needed to assess the potential toxic effects and the mechanisms causing it. To study toxicity, we chose the reduced form of GO and the model organism of *Escherichia coli* TOP 10.

To ensure the transition from GO to rGO, the particles of rGO were characterized using X-ray photoelectron spectroscopy, which showed a significant reduction of oxygen groups. To analyze changes in viability, the authors incubated bacteria with a suspension of rGO particles in nutrient media and a physiological solution, measuring the level of fluorescence. The introduction of rGO particles showed an almost twofold inhibition of GFP expression in a nutrient-rich media regardless of rGO concentration. The authors did not observe a decrease in fluorescence when cells were exposed to physiological solution in comparison with control. In order to test if rGO particles attach to bacteria, which might somehow affect viability, the suspension of cells and rGO particles was analyzed using confocal microscopy and a particle analyzer. Detailed analysis of this interaction showed that rGO particles are not necessarily toxic in direct contact since bacteria preserve fluorescent signals, indicating their activity.

The authors concluded that interaction of the rGO particles and bacteria depends on the type of media, since, on the one hand, it reduced the expression of GFP protein over time for bacteria growing in a nutrient-rich media (by half), but, on the other hand, it did not cause inhibition of cells since the decrease in the GFP signal was the same as control in nutrient-poor media. Thus, additional studies are needed to determine the reasons for toxicity and to introduce NPs for the application in microbial fuel cells.

The candidate performed toxicological studies, partially prepared confocal microscopic studies and partially contributed to the writing of the manuscript. The manuscript was prepared in collaboration with all the co-authors.



Article

Medium-Dependent Antibacterial Properties and Bacterial Filtration Ability of Reduced Graphene Oxide

Alexander Gusev ^{1,2}, Olga Zakharova ^{1,2}, Dmitry S. Muratov ², Nataliia S. Vorobeveva ³, Mamun Sarker ³, Iaroslav Rybkin ⁴, Daniil Bratashov ⁴ , Evgeny Kolesnikov ² , Aleš Lapanje ^{4,5}, Denis V. Kuznetsov ² and Alexander Sinitskii ^{3,6,*}

¹ Research Institute for Environmental Science and Biotechnology, Derzhavin Tambov State University, Tambov 392000, Russia; nanosecurity@mail.ru (A.G.); olgazakharova1@mail.ru (O.Z.)

² National University of Science and Technology “MISIS”, Moscow 119991, Russia; dmitrymrtv@gmail.com (D.S.M.); kolesnikov@isis.ru (E.K.); dk@isis.ru (D.V.K.)

³ Department of Chemistry, University of Nebraska—Lincoln, Lincoln, NE 68588, USA; nataliia.vorobeveva@huskers.unl.edu (N.S.V.); msarker39@huskers.unl.edu (M.S.)

⁴ Remote Controlled Theranostic Systems Lab, Saratov State University, Saratov 410012, Russia; rybkin.yaroslav@yandex.ru (I.R.); dn2010@gmail.com (D.B.); lapanje.ales@gmail.com (A.L.)

⁵ Jožef Stefan Institute, 1000 Ljubljana, Slovenia

⁶ Nebraska Center for Materials and Nanoscience, University of Nebraska—Lincoln, Lincoln, NE 68588, USA

* Correspondence: sinitskii@unl.edu; Tel.: +1-402-472-3543

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Abstract: Toxicity of reduced graphene oxide (rGO) has been a topic of multiple studies and was shown to depend on a variety of characteristics of rGO and biological objects of interest. In this paper, we demonstrate that when studying the same dispersions of rGO and fluorescent *Escherichia coli* (*E. coli*) bacteria, the outcome of nanotoxicity experiments also depends on the type of culture medium. We show that rGO inhibits the growth of bacteria in a nutrition medium but shows little effect on the behavior of *E. coli* in a physiological saline solution. The observed effects of rGO on *E. coli* in different media could be at least partially rationalized through the adsorption of bacteria and nutrients on the dispersed rGO sheets, which is likely mediated via hydrogen bonding. We also found that the interaction between rGO and *E. coli* is medium-dependent, and in physiological saline solutions they form stable flocculate structures that were not observed in nutrition media. Furthermore, the aggregation of rGO and *E. coli* in saline media was observed regardless of whether the bacteria were alive or dead. Filtration of the aggregate suspensions led to nearly complete removal of bacteria from filtered liquids, which highlights the potential of rGO for the filtration and separation of biological contaminants, regardless of whether they include live or dead microorganisms.

Keywords: graphene oxide; nanotoxicity; antibacterial properties; *Escherichia coli*

1. Introduction

Graphene and related materials are widely recognized for their potential for a multitude of biomedical applications, which include biosensing, bioimaging, anti-cancer therapy, cell growth, tissue engineering and antibacterial agents among others [1–3]. Among other materials from the graphene family, graphene oxide (GO) and its reduced form, reduced graphene oxide (rGO), are of particular interest for biomedical applications because of their high surface area, solubility in a variety of solvents—including water and aqueous solutions—and multiple opportunities for surface functionalization [4]. However, while GO, rGO and other graphene-based materials have been widely used in biomedical research [1–5], there

are different views on their nanotoxicity [5]. There was a considerable amount of in vitro and in vivo research on toxic effects associated with graphene-based materials [4,5]. Several studies showed that graphene and its derivatives are toxic for microorganisms [6–8] and some of the proposed mechanisms of toxicity involve membrane damage and oxidative stress [9,10]. Other authors argue that graphene-based materials do not exhibit inhibitory effects on bacteria [11–14].

The breadth of results obtained in various nanotoxicity experiments involving graphene and related materials suggests that generalized conclusions should be avoided as safety risks associated with graphene-based materials depend on a large number of factors [4,5]. These factors include the specific type of graphene-based material (GO, rGO, few-layer graphene, graphene quantum dots, etc.), the specific type of biological objects used in a particular study (cells, bacteria, various multicellular microorganisms) and the experimental conditions. The toxicity of graphene-based material of a certain kind, such as GO or rGO, can further depend on the flake size, surface functionalization and concentration, as was demonstrated in multiple studies [15–18].

In this work, we discuss another variable that should be added to the already multidimensional space of parameters that need to be considered in nanotoxicology experiments involving graphene-based materials. We show that in the experiments involving the same dispersions of rGO and same recombinant *Escherichia coli* (*E. coli*) bacteria expressing green fluorescent protein (GFP) the outcome of the nanotoxicity experiment strongly depends on the culture medium. The addition of rGO inhibited the growth of bacteria in a nutrition broth (NB) medium but showed little effect on the behavior of *E. coli* in a physiological solution (PS). These observations were valid for various concentrations of rGO, which ranged from 0.1 to 100 mg/L.

In order to gain insights into different behavior of bacteria in these experiments, we investigated the interaction of *E. coli* with rGO flakes in both NB and PS media. We observed instant flocculation when bacteria were introduced into high-concentration suspensions of rGO in both PS and distilled water. However, no such effect was observed when bacteria were added to suspensions of rGO in NB. Confocal microscopy and scanning electron microscopy (SEM) confirmed the accumulation of bacteria on rGO flakes. Interestingly, the *E. coli*-rGO flocculates were observed regardless of whether the bacteria were alive or dead. This observation, combined with the zeta potential measurements of the rGO suspensions before and after mixing with bacteria, which showed a significant drop from 40 to 14–15 mV, indicates the electrostatic nature of the interaction of graphene and bacteria, which could be mediated via hydrogen bonding between the cell walls of *E. coli* and rGO functionalities.

The microscopic and zeta potential studies not only demonstrate the difference between the interaction of *E. coli* and rGO in NB and PS media but also suggests the possibility of using rGO for filtration and bacterial removal. Overall, the potential of graphene-based structures for water purification is widely recognized and a number of studies showed the possibility of using graphene and its derivatives in filter elements [19–21], which could employ the formation of conjugates of graphene-based materials and bacteria [22]. In our experiments, filtration of the suspensions of *E. coli*-rGO flocculates nearly completely removed live and dead bacteria from the filtered liquids, demonstrating the possibility of using rGO for the removal and separation of biological contaminants.

2. Materials and Methods

2.1. Synthesis of rGO

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. GO was synthesized as described in our previous work [23], following the general procedure by Marcano et al. [24]. The reduction of GO to rGO was performed using ascorbic acid [25,26]. L-ascorbic acid (500 mg) was added to 250 mL of a GO aqueous dispersion (0.2 mg/mL) under vigorous stirring. The stirring was continued for three days at room temperature, and the reaction progress was monitored by UV-vis spectroscopy. After three days of stirring the color of the mixture changed from light amber to black. The reaction mixture was filtered with ethanol and water, and then dried under vacuum.

Four rGO suspensions in deionized water at concentrations of 0.1, 1, 10 and 100 mg/L were prepared and used in the following fluorescence experiments.

2.2. Materials Characterization

Raman spectroscopy was performed using a DXR Raman microscope (Thermo Fisher Scientific, Waltham, MA, USA) with a 532 nm excitation laser and a 100× objective. SEM of rGO flakes on Si/SiO₂ substrates was performed using a Supra 40 field-emission scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany). SEM images of *E. coli*-rGO aggregates were recorded on a Vega 3 scanning electron microscope (Tescan, Brno, Czech Republic). Atomic force microscopy (AFM) was performed using a SmartSPM 1000 scanning probe microscope (AIST-NT, Novato, CA, USA). For AFM analysis, a droplet of an aqueous rGO suspension was deposited on a Si/SiO₂ substrate and dried in air. X-ray photoelectron spectroscopy (XPS) was performed using a K-Alpha X-ray photoelectron spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a monochromatic Al K α (1486.6 eV) X-ray source.

2.3. *E. coli* Biofluorescence Tests

We used recombinant GFP expressing *E. coli*, which was transformed with pRSET-emGFP plasmid containing an ampicillin-resistant (amp^R) gene using the standard electroporation procedure. The culture was used as a model system to investigate the antibacterial activity of rGO. Similar bacterial tests were widely used in the evaluation of toxicity of nanomaterials [27–29].

Bacteria were grown and tested in nutrient broth (NB) supplemented with ampicillin. 1 mL of overnight culture was grown in NB and then inoculated in 100 mL of fresh NB at 37 °C for 3 h. 50 mL of the culture was concentrated by centrifugation at 5000 g for 5 min. The culture was washed three times using a physiological saline solution (PS; 9 g/L NaCl aqueous solution) and centrifuged at 3000 g for 3 min. Optical density at 660 nm (OD₆₆₀) of the resulting suspension of *E. coli* in PS was approximately 0.13, as measured using a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA).

In a typical fluorescence experiment, 5 μ L of the GFP *E. coli* suspension in PS was mixed with 200 μ L of a culture medium (NB or PS) followed by the addition of 45 μ L of an aqueous suspension of rGO with a concentration of 0.1, 1, 10 or 100 mg/L. After the addition of rGO to GFP *E. coli* suspensions, the behavior of bacteria was monitored by their green fluorescence at 528 nm using a microplate reader with an excitation at 485 nm. The fluorescence of GFP *E. coli* in different media was monitored at 37 °C for 17 h. The presence of rGO, which absorbs light in the entire visible range of spectrum, in the *E. coli* suspensions was taken into account for the correction of the fluorescence values. Each fluorescence kinetics experiment was performed at least 10 times, and the averaged results are presented. The analysis of statistically significant differences within the treated groups was carried out using the analysis of variance (ANOVA) for a single factor with further application of the Tukey's multiple analysis of variance with a family error rate of 0.05.

2.4. Investigation of Interactions of *E. coli* with rGO

Optical microscopy was carried out on a Biolam M-1 microscope (LOMO, St. Petersburg, Russia) using the Ziehl–Neelsen stain. Confocal fluorescent microscopy measurements were performed on a TCS SP8 X CLSM setup (Leica Microsystems, Wetzlar, Germany) using the default settings for GFP (excitation at 488 nm, emission detection in the 500–600 nm range). Zeta potentials of dispersions were measured using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, United Kingdom).

The suspensions for optical photographs were prepared by mixing 0.18 mL of 100 mg/L rGO solution with 0.02 mL of another solution (PS, *E. coli* in PS, dead *E. coli* in PS, NB, *E. coli* in NB, dead *E. coli* in NB). For the preparation of samples of dead *E. coli* in NB and PS, the bacterial suspensions were placed in a boiling water bath for 10 min.

3. Results

3.1. Characterization of rGO

Considering the dependence of the toxicity of graphene-based materials on their characteristics, such as chemical composition, flake size and surface functionalization [15–18], we first performed a detailed materials characterization of rGO, which was used in this study. In general, GO could be reduced to rGO using a variety of chemicals such as hydrazine, hydroxylamine and sodium borohydride [30]. However, GO and related materials are known for their sorption ability [31], and when a toxic chemical, such as hydrazine [24,32], is used to produce rGO, there is a possibility that traces of a reducing agent or its derivative remain in a rGO sample and later affect the results of a nanotoxicity experiment. Therefore, for reducing GO to rGO in this study we specifically chose ascorbic acid [25,26], which is nontoxic and its possible traces were not expected to cause any inhibitory effects in experiments with *E. coli*.

Characterization of rGO deposited on a Si/SiO₂ substrate by scanning electron microscopy (SEM) and atomic force microscopy (AFM) revealed that the flakes had a wide size distribution with their lateral dimensions ranging from about 50 nm to several μm . SEM image in Figure 1a shows several rGO flakes on Si/SiO₂ with the largest flake in the field of view being $\sim 100\ \mu\text{m}$ long. Smaller rGO flakes are shown in the AFM image of another area of the sample in Figure 1b. Figure 1c shows a representative height profile measured across one of the rGO flakes in Figure 1b. The flake has a height of about 1 nm, which is consistent with previous reports for rGO monolayers [33].

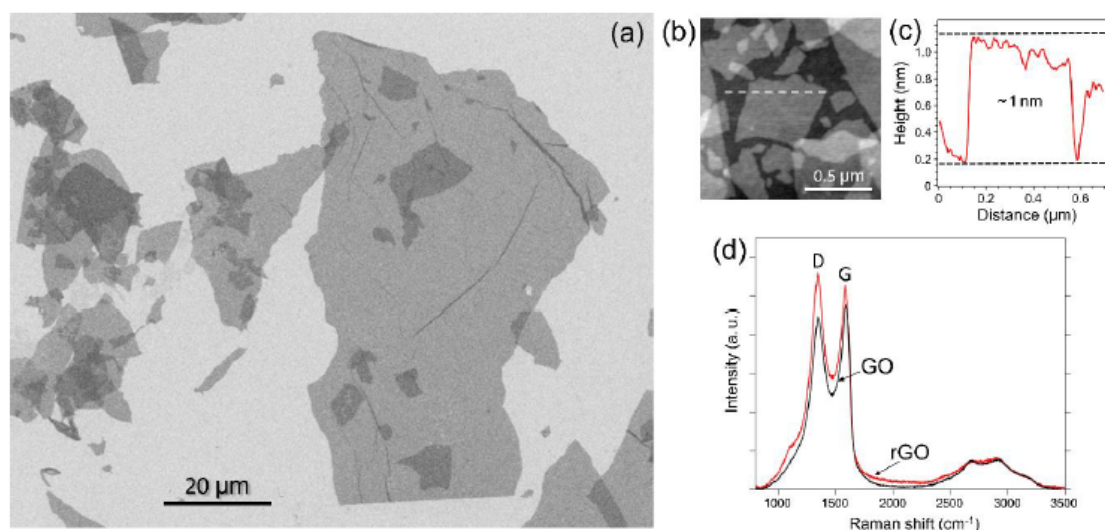


Figure 1. Characterization of graphene oxide (GO) and reduced graphene oxide (rGO). (a) Scanning electron microscopy (SEM) image of rGO flakes on a Si/SiO₂ substrate. (b) Atomic force microscopy (AFM) image of rGO flakes on a Si/SiO₂ substrate. (c) AFM height profile across the rGO flake, which was measured along the dashed line in panel (b). The flake has a height of about 1 nm. (d) Raman spectra of GO and rGO.

The Raman spectra of GO and rGO (Figure 1d) are also consistent with prior literature reports [34–37]. These spectra exhibit two broad peaks at about 1586 and 1345 cm^{-1} , which are known as D and G bands, respectively [38]. It was previously demonstrated that in GO the intensity of the G band is slightly higher than the intensity of the D band, but upon the GO reduction the D band becomes more intense than the G band [34–37]. This previously established trend can also be seen in Figure 1d in the Raman spectra of GO and rGO prepared in this work.

The reduction of GO to rGO was confirmed by X-ray photoelectron spectroscopy (XPS). The survey spectrum of rGO (Figure 2a) shows the presence of carbon (C1s) and oxygen (O1s) and no traces of

surface contamination. The XPS O1s and C1s spectra of rGO and their deconvolutions are shown in Figure 2b,c, respectively. A comparison of XPS C1s spectra of rGO (Figure 2c) and GO (Figure 2d) shows the removal oxygen-containing functionalities in GO upon reduction using ascorbic acid. The XPS C1s core level spectrum of rGO predominantly demonstrates sp^2 hybridized carbon atoms (284.6 eV) with considerably smaller contributions from carbon bonded to different oxygen-containing functional groups, such as hydroxyls (285.6 eV), epoxides (286.7 eV), carbonyls (287.6) and carboxyls (289.5 eV). Deconvolution of the XPS C1s core level spectrum of GO produces four peaks positioned at 284.85 eV, 286.93 eV, 288.53 eV and 290.52 eV, which were assigned to sp^2 carbon, C–O, C=O, and O–C=O, respectively (Figure 2d). The carbon components were assigned according to the reported XPS spectra of GO and rGO samples containing the same oxygen-containing functional groups [34,36,39,40]. The quantitative XPS analysis of the samples shows that the C/O atomic ratio increases from 1.83 in GO to 9.37 in rGO, respectively, which is consistent with the XPS results reported for the rGO produced by GO reduction with ascorbic acid [26]. The fact that some oxygen-containing functionalities still remain in rGO is consistent with its solubility in water and will be important for explaining the results of nanotoxicity and aggregation experiments.

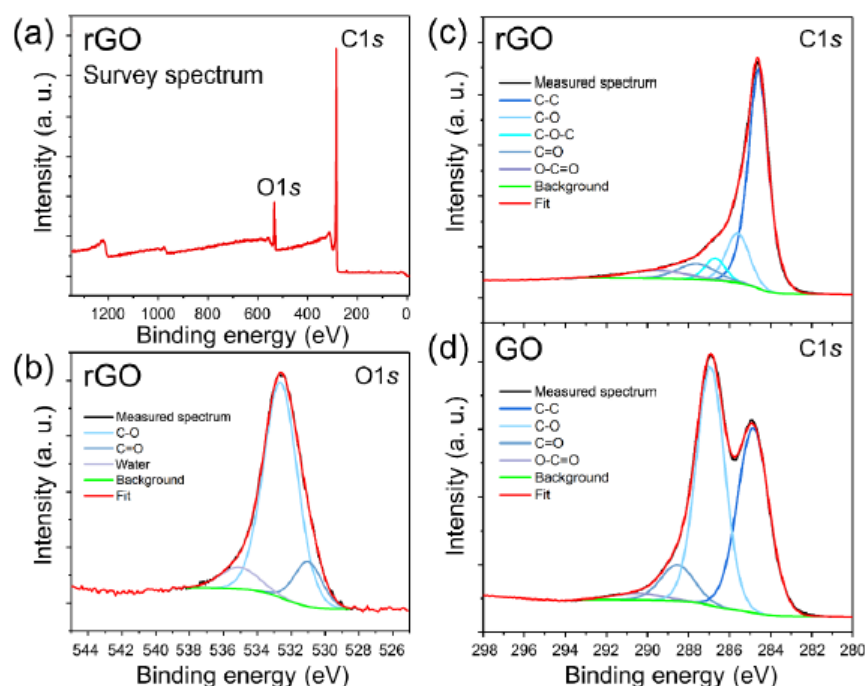


Figure 2. X-ray photoelectron spectroscopy (XPS) characterization of GO and rGO. (a) XPS survey spectrum of rGO. (b) XPS O1s spectrum of rGO. (c) XPS C1s spectrum of rGO. (d) XPS C1s spectrum of GO.

Overall, the results of materials characterization provided in Figures 1 and 2 are consistent with other literature reports on rGO. The rGO material consists of well-exfoliated uniform monolayer flakes (Figure 1a–c) with minor amounts of oxygen-containing functionalities (Figure 2).

3.2. Bacterial Fluorescence Studies

For this study, we used green-fluorescent *E. coli* bacteria, which are shown in optical and confocal microscopy images in Figure 3a. The images were collected simultaneously, and their comparison shows that all bacteria exhibited green fluorescence. The rGO dispersions at different concentrations were added to *E. coli* dispersed in NB and PS culture media, and then the behavior of bacteria was monitored by fluorescence spectroscopy.

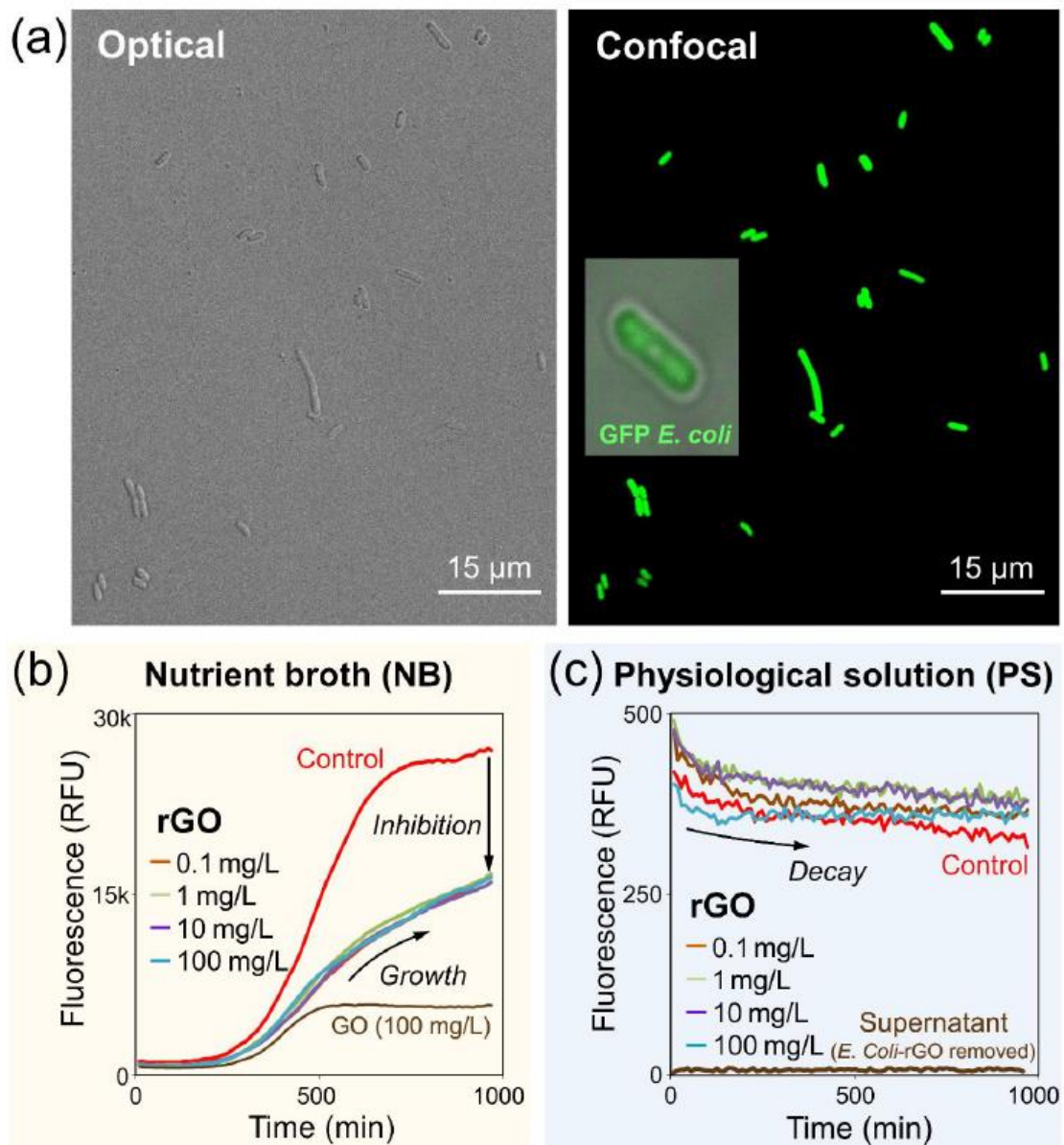


Figure 3. Effect of rGO on *E. coli* bacteria in nutrition broth (NB) and physiological solution (PS) media. (a) Optical (left) and confocal microscopy (right) images of suspensions of green fluorescent protein (GFP) expressing *E. coli* bacteria. The inset in the right panel shows magnified image of an individual *E. coli* by confocal microscopy. (b,c) Fluorescence of GFP *E. coli* bacteria in NB (b) and PS (c) after the addition of rGO at concentrations ranging from 0.1 to 100 mg/L. The red curves in (b,c) show the fluorescence of *E. coli* in control samples, in which the bacteria were measured in NB and PS, respectively, without the addition of rGO. The brown curve in (b) shows fluorescence of GFP *E. coli* bacteria in NB after the addition of 100 mg/L GO solution. The brown curve in (c) shows no fluorescence from the supernatant decanted from the *E. coli*-rGO colloidal solution in PS, suggesting that the bacteria aggregated with the rGO sheets and were removed from solution. The fluorescence in panels (b) and (c) is shown in the same relative fluorescence units (RFU).

Figure 3b shows that in the NB growth medium the fluorescence of *E. coli* significantly decreased (almost 2-fold) for all concentrations of rGO compared to the control sample (red curve) to which the rGO was not added. However, just as in the control experiment, in the presence of rGO the bacteria continued to grow, albeit at lower rates.

Compared to the experiments performed in NB, the dispersions of *E. coli* in PS generally showed much lower fluorescence intensities (Figure 3c), which is associated with the absence of nutrients in PS compared to NB. Also, unlike the case of NB, where the introduction of rGO had a strong inhibitory effect on the bacterial growth, in PS, the administration rGO had little effect on the behavior of GFP *E. coli* (Figure 3c), and then the fluorescence of GFP *E. coli* decreased with time due to the absence of nutrients.

The fact that bacteria remain alive after the addition of rGO can also be verified by optical and confocal microscopy. Figure 4a shows optical (left panel) and confocal (right panel) microscopy images of a droplet of a suspension of rGO flakes (10 mg/L) and *E. coli* bacteria in PS 15 min after mixing. Large flakes of rGO dispersed in PS are visible in the optical image, while the green fluorescence of GFP *E. coli* in the confocal microscopy image shows uniform distribution of bacteria in the droplet. The emission of GFP *E. coli* confirms that the bacteria are alive in the presence of rGO flakes.

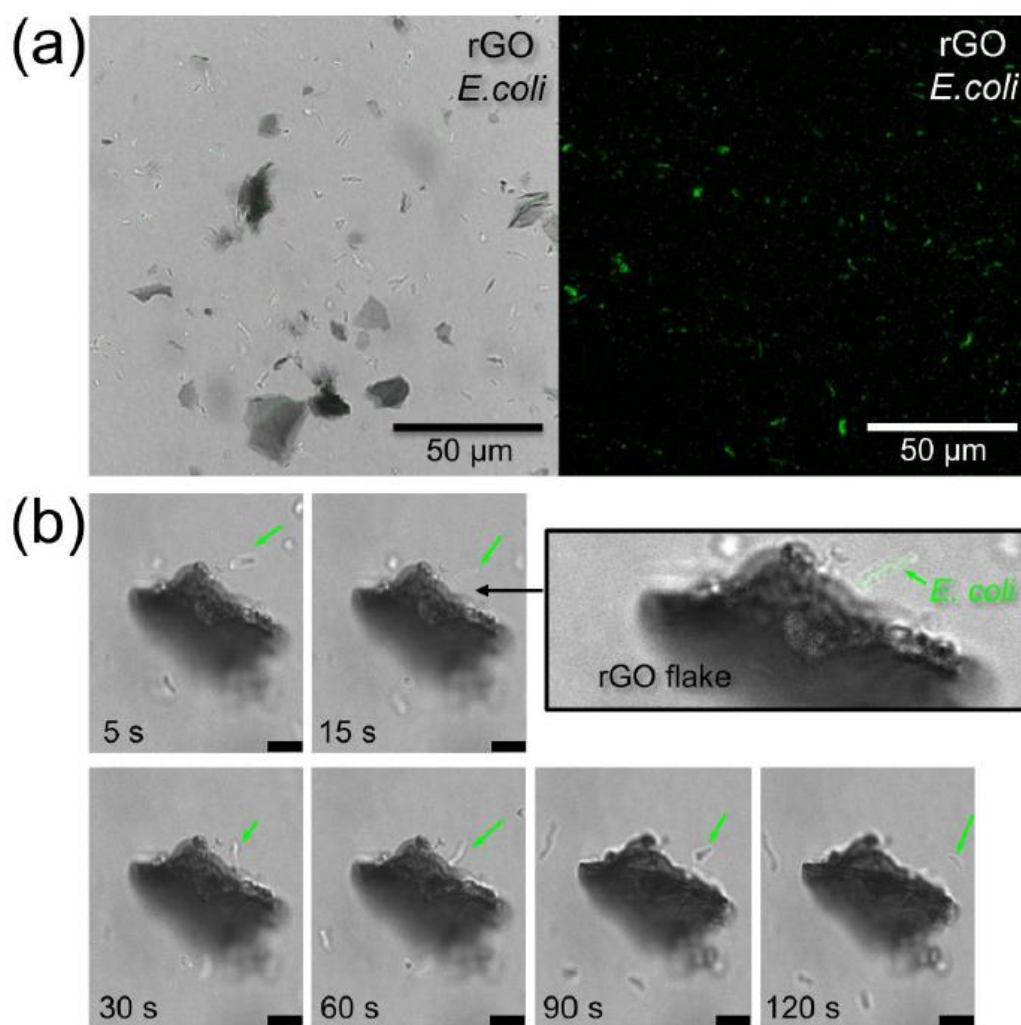


Figure 4. Dynamics of the interaction of rGO and bacteria dispersed in PS. (a) Optical (left) and confocal (right) images of rGO flakes (10 mg/L) and *E. coli* bacteria dispersed in a droplet of PS. Live bacteria emit a green fluorescent signal. (b) A series of confocal microscopy images illustrating the interaction of an individual *E. coli* shown by the green arrows with a rGO flake. Scale bars are 5 μm.

Figure 4b shows a series of confocal microscopy images illustrating the interaction of an individual *E. coli* with a rGO flake. The bacterium approaches the flake and then leaves continuing its green fluorescence, indicating that it remains alive and was not damaged by the contact with the flake.

This observation indicates that a contact with graphene does not necessarily damage the cell membrane and result in bacterial death, as discussed in previous studies [7,41–43]. However, other toxicity mechanisms could manifest in a particular experiment, which could involve adsorbed rGO contaminants [14,44] and a variety of other factors [4,5].

Overall, in both media—NB and PS—the bacteria exhibited qualitatively same behavior with and without rGO, suggesting that the presence of nutrients was a more important factor for bacteria growth than the addition of rGO. Yet, rGO had very different effects on *E. coli* in NB and PS, strongly decreasing the fluorescence of bacteria in NB and showing little effect on it in PS compared to control experiments (Figure 3b,c).

While the observed effects of rGO on *E. coli* in different media are likely the result of a complex interplay of multiple physicochemical phenomena, they could in part be rationalized through the interaction of nutrients (if present) and bacteria with the dispersed rGO sheets. Graphitic structures are known for their sorption ability, and accumulation of the nutrients on dispersed rGO sheets could be the reason for the reduced growth rate of *E. coli* in NB if rGO is introduced (Figure 3b). In this case, small biomolecules present in NB likely saturate the surface of rGO sheets, minimizing the interaction of bacteria with rGO. Conversely, in the PS medium, where no such nutrients are present, the conditions are more favorable for the interaction between bacteria and rGO. The rGO sheets contain a variety of oxygen-containing functionalities, such as hydroxyl, epoxy and carboxyl groups [24,34], which engage in inter- and intra-molecular hydrogen bonding [45]. The cell walls of *E. coli* consist of a variety of biomolecules [46,47] containing similar oxygen functionalities, which form hydrogen bonds as well [48]. Because of the presence of these oxygen functional groups in both rGO and cell membranes, the aggregation of rGO sheets and bacteria mediated via hydrogen bonding is expected. A number of studies have further shown that bacteria can consume oxygen from GO sheets [49,50], which could be the reason for the possible slight activation of *E. coli* in PS in the presence of rGO (Figure 3c).

Previous studies considered numerous factors affecting the toxicity of graphene-based materials toward bacteria, such as flake size [41], surface functionalization [51], number of layers [52], coagulation and dispersity [22]. Our results demonstrate that the nature of the culture medium also plays an important role in nanotoxicity experiments and can substantially affect the conclusions regarding the antibacterial properties of rGO. This conclusion is consistent with the previously reported data for GO [43] and is likely valid for a variety of other graphene-based materials.

3.3. rGO-Bacteria Interactions

In order to substantiate the hypothesis that the difference in the effects of rGO on *E. coli* in NB and PS media is related to adsorption of bacteria on rGO sheets, we performed microscopic and zeta potential studies of rGO-bacteria interactions. These interactions could be easily visualized by mixing a highly concentrated rGO solution (1 g/L) with a bacterial suspension in PS, as shown in Figure 5a. The mixing of two suspensions results in an immediate formation of a stable flocculate structure (Figure 5a,b), which could be dispersed by a sonication but then quickly reforms. Based on the visual observations, similar 1g/L solutions of GO exhibited an even stronger flocculation with *E. coli* in PS producing larger and visibly denser aggregates.

A droplet of an *E. coli*-rGO colloidal solution in PS was deposited on a glass microslide and dried in air for a microscopy analysis. Optical photographs in Figure 5c show that the bacteria are localized on a rGO flake but not on a bare substrate, suggesting strong interaction between bacteria and rGO sheets, which is likely mediated by hydrogen bonding. The bacteria/rGO aggregates were also visualized by SEM (Figure 5d).

Interestingly, no flocculation was observed when rGO suspensions were mixed with suspensions of *E. coli* in NB (Figure 5b), and in the optical and SEM images the rGO flakes and bacteria were observed separately. In principle, the presence of salt in PS could affect the aggregation of graphene-based materials, as was shown in previous studies [53]. However, in the control experiment, we did not

observe any flocculation when rGO was mixed with pure PS without *E. coli* (Figure 5b) suggesting that the presence of bacteria is important for the aggregation of rGO (Figure 5a), and this is not only the effect of the dissolved salt. The conclusion that the salt is not a determining factor for the aggregation of rGO and *E. coli* is also supported by the fact the aggregates formed in suspensions of rGO and bacteria in distilled water.

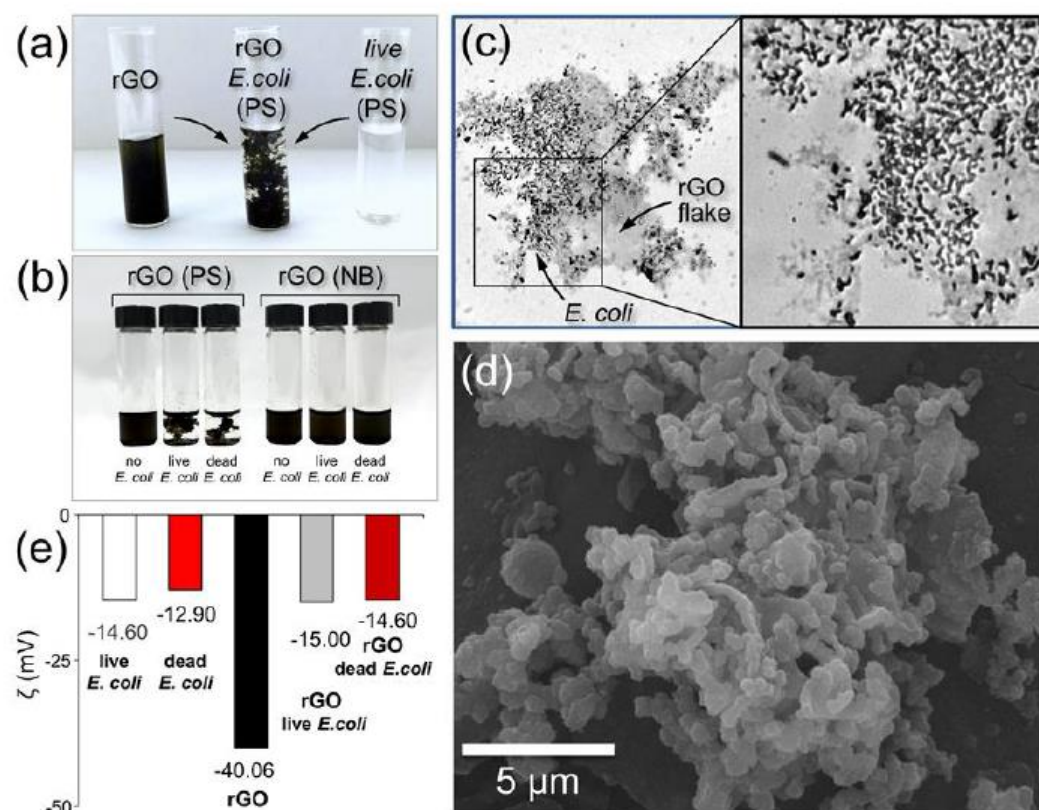


Figure 5. Interactions between rGO and bacteria dispersed in PS. (a) Optical photograph of a 1 g/L rGO aqueous suspension (left), a bacterial suspension in PS (right) and a flocculate structure produced by mixing the rGO and *E. coli* suspensions (middle). (b) Optical photograph of the suspensions produced by mixing rGO solution with (from left to right) PS, rGO solution with alive bacteria in PS, rGO solution with dead bacteria in PS, rGO solution with NB, rGO solution with alive bacteria in NB, rGO solution with dead bacteria in NB. (c) Optical photographs of *E. coli* bacteria localized on a rGO flake. (d) SEM image of *E. coli*-rGO aggregates. (e) Zeta potentials of colloidal suspensions of live and dead *E. coli* bacteria in PS, rGO and mixtures of rGO with live and dead *E. coli*.

Overall, these observations are consistent with the results of fluorescent studies shown in Figure 3b,c. The flocculation is likely caused by the hydrogen bonding between the oxygen-containing groups in rGO sheets and biomolecules comprising the cell walls of *E. coli* bacteria. The flocculation is observed for the bacteria dispersed in PS, while in NB the interaction between the rGO and *E. coli* is inhibited as the surface of rGO sheets is saturated by the nutrient biomolecules. These observations suggest that the inhibition of the GFP *E. coli* fluorescence in NB should be stronger for GO than for rGO considering that the former contains more oxygen-containing functional groups that could interact with nutrients. This is illustrated by the brown curve in Figure 3b for the 100 mg/L GO solution, which shows a considerably lower fluorescence of GFP *E. coli* than the 100 mg/L rGO solution.

Unlike other studies, we also performed experiments with dead bacteria. Similar flocculate structures were formed when rGO suspensions were mixed with the suspensions of dead bacteria in PS (Figure 5b), which was noted for the first time and does not confirm the opinion about bacterial

biofilms as the basis for the formation of graphene-bacteria conjugates [11,22]. Measurement of the zeta potential of the particles in the rGO suspensions before and after mixing with bacterial cells (alive or dead) showed its decrease from 40 to 14–15 mV (Figure 5e), which indicates the electrostatic nature of the formation of *E. coli*-rGO flocculates.

When we decanted a supernatant from the freshly prepared *E. coli*-rGO colloidal solution in PS, thus removing the dark *E. coli*-rGO flocculates, the visibly clear solution did not exhibit any detectable fluorescence signal (see the brown curve in Figure 3c), suggesting that the bacteria were aggregated with the rGO sheets. Likewise, filtration of the suspensions leads to almost complete removal of bacteria from the filtered liquids, which indicates the total character of the conjugation process and highlights the potential of rGO for filtration and separation of biological contaminants, regardless of whether they include live or dead microorganisms.

4. Discussion

While previous studies showed that the toxic effects associated with rGO depend on a large number of factors, which include the flake size, surface functionalization and concentration among other parameters, here we demonstrate that the type of culture medium is also an important factor that could affect the outcome of a nanotoxicity experiment. We performed experiments involving the same dispersions of rGO and same fluorescent *E. coli* bacteria and found that rGO inhibited the growth of bacteria in a nutrition medium but had little effect on the behavior of *E. coli* in a physiological saline solution. These observations were valid for various concentrations of rGO, which ranged from 0.1 to 100 mg/L.

While the observed effects of rGO on *E. coli* in different media are likely the result of a complex interplay of multiple physicochemical phenomena, they could in part be rationalized through the adsorption of nutrients (if present) and bacteria on the dispersed rGO sheets. The interaction of bacteria and rGO is likely mediated via hydrogen bonding between the biomolecules forming the cell walls of *E. coli* and oxygen-containing groups in rGO sheets. We investigated the interaction of *E. coli* and rGO and found it to also be medium dependent. In physiological saline solution, as well as in distilled water, *E. coli* and rGO instantly aggregate, and microscopy reveals accumulation of bacteria on rGO flakes. Furthermore, this aggregation of rGO and *E. coli* was observed regardless of whether the bacteria were alive or dead. No aggregation was observed in nutrition media. Filtration of the aggregate suspensions led to nearly complete removal of bacteria from filtered liquids, which highlights the potential of rGO for filtration and separation of biological contaminants, regardless of whether they include live or dead microorganisms.

Interestingly, the effect of culture medium on the toxicity of GO toward *E. coli* was also considered in the study by Hui et al., who also attributed the observed phenomena to the molecular adsorption on GO sheets [43]. Although this study focused on GO instead of rGO and operated in a higher concentration range (80–300 mg/mL, which could be related to the lower optical absorption of GO compared to rGO), the observations made for GO [43] and rGO (Figure 3b,c) overall agree with each other. A small decrease in the amount of *E. coli* in saline solutions was observed several hours after the addition of 80 µg/mL GO [43] and for all rGO concentrations tested in this work (Figure 3c). Interestingly, the work by Hui et al. shows that at higher concentrations, such as 200 µg/mL, GO becomes highly toxic to *E. coli* in saline solutions [43]. On the other hand, the present work demonstrates that at lower rGO concentrations down to 0.1 µg/L all tested solutions showed similar fluorescence decays to the control experiment, suggesting that they are likely caused not by the toxic effect of rGO but rather the overall lack of nutrients. Also, in cases of both GO [43] and rGO (Figure 3b), the bacteria grow with the addition of nutrients. While a direct comparison between the GO and rGO experiments should be done cautiously, it appears that the two studies are not contradictory and complement each other by covering different materials and concentration ranges.

In summary, the type of culture medium is shown to strongly affect the antibacterial properties and bacterial filtration ability of rGO in experiments with *E. coli* bacteria and is likely an important factor for consideration in nanotoxicity studies of other graphene-based materials.

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3.5 Effect of GO on Bacterial Cells: Role of the Medium Type and Electrostatic Interactions

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GO is a two-dimensional material, which has outstanding mechanical, electrical, and thermal properties that can facilitate energy transfer in microbial fuel cells. Compared to rGO, the presence of many oxygen groups is expected to enable this material for surface modification strategies. Since oxygen groups can form hydrogen bonds with the cell membrane, we can also expect that this material can be applied as an intermediate layer in our developed process of PE deposition, which would separate a toxic layer of positively charged PEs with the cell membrane. Although the deposition of particles should be relatively straightforward, there are no coherent conclusions about toxicity to bacteria. Accordingly, in many cases, the introduction of GO particles demonstrated a strong inhibiting activity against Gram-negative, while in others against Gram-positive bacteria. Thus, the authors aimed to study the effect of GO particles on cell viability using the *Escherichia coli* TOP 10 cells.

To confirm the composition of GO NPs Raman spectroscopy was applied. By using atomic force microscopy, the authors determined the particle thickness to be 1 nm, which confirmed the sheet structure of regular GO NPs. To analyze viability, the authors incubated bacteria with a suspension of GO particles in both nutrient media and a physiological solution, measuring the intensity of GFP protein over time. To assess differences in viability, GO was added to the media solution in a concentration gradient.

The authors found that in nutrient-rich media, the introduction of GO particles to bacteria demonstrated a higher toxicity (GFP expression was lower 9 times) than in the case of rGO (2 times) at the highest concentration. In the absence of nutrients, a significant decrease of GFP (almost 3 times) was only observed when the highest concentration of rGO was dispersed in the media. Since it was expected that GO can adsorb on the cell surface due to the presence of oxygen groups, which can form hydrogen bonds, the authors studied the interaction of cells with GO particles using a confocal microscope and a particle analyzer. Although GO particles were found to adsorb on the cell surface in physiological solution, such interaction did not cause cell lysis as bacteria preserved fluorescence. In addition, it was demonstrated that the interaction of live bacteria with GO decreased the zeta potential by 14 mV, potentially indicating the effect of electrostatic interaction.

The authors concluded that GO particles are 4.5 times more toxic than rGO in nutrient-rich media, while in physiological solution, viability is reduced only at the highest concentration by 3 times. The confocal images of cells with GO particles as well as a decrease in zeta potential may indicate the electrostatic nature of the interaction. To prepare the surface for the introduction of GO NPs, it is necessary to consider the effect of concentration as well as further explanation of the mechanisms causing toxicity.

The candidate performed toxicity studies, partially prepared confocal microscopic studies and partially wrote the manuscript. The manuscript was prepared in collaboration with all co-authors.



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Effect of GO on bacterial cells: Role of the medium type and electrostatic interactions



Alexander Gusev^{a,b}, Olga Zakharova^{a,b,*}, Inna Vasyukova^a, Dmitry S. Muratov^b, Iaroslav Rybkin^c, Daniil Bratashov^c, Aleš Lapanje^{c,d}, Igor Il'nikh^b, Evgeny Kolesnikov^b, Denis Kuznetsov^b

^a Derzhavin Tambov State University, 33, Internatsionalnaya street, Tambov 392000, Russia

^b National University of Science and Technology MISIS, 4, Lenin avenue, Moscow 119991, Russia

^c Saratov Chernyshevsky State University, 83 Astrakhanskaya Street, Saratov 410012, Russia

^d Jozef Stefan Institute, 39 Jamova, 1000 Ljubljana, Slovenia

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ABSTRACT

Graphene and graphene oxide (GO) both being two-dimensional materials are gaining popularity among researchers as a promising nanomaterial for various medical and biological applications. The aim of this study is to elucidate the influence of nanostructured GO sheets on viability of a model species of gram-negative *E. coli* bacteria transformed with pRSET-emGFP plasmid in in vitro experiments. It was shown that GO at concentrations between 0.0025 and 2.5 g/l in growth medium inhibits growth of bacterial colonies, while in physiological saline solution (PS) this effect decreases dramatically to the point of complete disappearance.

It was shown that in order to obtain a pronounced antibacterial effect one needs to introduce high concentrations of GO into the media (up to 2.5 g/l), which can be important for development of antibacterial materials for biomedical applications.

Some of the obtained data provide clear evidence to electrostatic nature of interaction between bacterial and GO sheets. A number of previous papers suggested the process of biofilms formation by bacteria as the primary reason for aggregation between graphene-like materials and bacterial cells. However, formation of flocculent structures consisting of GO and dead bacteria and accompanied with decrease in zeta-potential of particles in the suspension to 18 mV proves that electrostatic interactions play the major role in aggregation.

The obtained data can be used for employing GO and similar materials in new systems for water-purification from biological contaminants. Besides, our results stress the importance of accounting for the conditions in which goods and coatings containing graphene-like materials as an antibacterial agent are used, as well as unification of the experimental conditions.

1. Introduction

Recent achievements in engineering, medicine and diagnostics are closely connected with the new knowledge and developments in nanotechnologies. Considerable amount of nanoproducts and nanomaterials are now used in everyday life. At the same time, two-dimensional materials such as graphene and graphene oxide (GO) are gaining popularity among researchers as a promising nanomaterial for various medical and biological applications [1–4]. By its structure graphene is the thinnest known form of all the known allotropic modifications of carbon and is the basis for such carbon materials as graphite, nanotubes and fullerenes [5]. Due to unique combination of its physicochemical properties, including high specific surface area and mechanical strength

(it is 200 times stronger than steel), perfect electrical and thermal conductivity, graphene possesses unprecedented mechanical and rheological characteristics [6,7]. The aforementioned allows graphene to become a promising material for biosensors and agents for addressed delivery of drugs and genes [8], tumours visualization systems, etc. [9].

In particular, high specific surface area and flat structure facilitate loading graphene with medicinal drugs in order to use it as a carrier with further bioconjugation [10]. High efficiency of graphene-like structures for anti-tumor drug carriers has been shown on the examples of doxorubicin and camptothecin [11–13].

Besides, modern medicine faces an acute question of antibiotics losing efficiency because of drug resistivity developed by microorganisms [14–17]. Use of graphene-like structures showing high cytotoxicity

* Corresponding author at: Derzhavin Tambov State University, 33, Internatsionalnaya street, Tambov 392000, Russia.

E-mail address: olgazakharova1@mail.ru (O. Zakharova).

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against gram-positive and gram-negative bacteria can be an alternative to antibiotic therapy [18,19]. By the example of *E. coli* and *B. subtilis* it was shown that 1 mg/ml concentration of graphene is effective for inhibiting bacterial growth [20]. Similar results were presented for GO, when 2-hour incubation of *E. coli* in GO solution at the test substance concentration of 85 mg/ml has led to considerable damage to the cell membranes and death of > 90% of the bacterial colony [21]. Liu et al. in their research have noted correlation between the cytotoxic effect and the size of scales, they have observed almost 99% *E. coli* death when treated with large sheets of GO [22]. The antibacterial efficiency reaching almost 100% efficiency has been demonstrated on *L. monocytogenes* and *S. enterica* colonies being incubated with graphene and GO [23] и *P. aeruginosa* [24]. At the same time, neither graphene nor its derivatives display negative effect on cells of humans and other mammals as shown on the example of human epithelial cells A549 [25]. Veerapandian et al. [26] reported, that GO exposed to UV light of 254 nm for 20 min prior to inoculation and culture growth of both gram-positive and gram-negative bacteria increase the cytotoxicity of GO. In [27] authors also showed that UV treatment of GO is beneficial for enhancing the photoluminescence properties.

Development of antibacterial materials and surfaces can be another promising application for graphene-like structures, including creation of composite structures for new medical dressings, endoprosthesis replacement matrices, tissue engineering, etc. [28–33]. Composition of GO with silver nanoparticles serves as a remarkable example of a material with pronounced antibacterial effect due to activity of the both components. In this case GO sheets provide large interaction area with the cell cultures while oxygen-containing groups serve as nanosilver crystallization centers [34–36]. Such composite materials are seen as promising coatings preventing biofilms formation on medical products and on food packaging [37]. Gold nanoparticles when combined with reduced GO also demonstrate antibacterial effect against colonies of *E. coli* (97.47%), *S. aureus* (100%), *B. subtilis* (99.76%) and *P. aeruginosa* (100%), while showing good biocompatibility with human carcinoma cells [38]. Polymer composites based on chitosan, polyvinyl pyrrolidone, polyethylene oxide and GO allow manufacturing super-thin and strong materials for tissue engineering. Experiments on rats show that the aforementioned materials display good biocompatibility, contribute to supporting the characteristic cell morphology on the damaged skin areas and improve wound healing by 33% compared to traditional gauze and cotton dressings [39]. Similar results were obtained during experiments on mice and rabbits where antibacterial effect of graphene-containing dressing materials was noted along with facilitation of the tissue regeneration process [40].

Beside purely medical applications, efficiency of graphene-like structures as a basis for creating biosensors has been shown, including the ones for monitoring the content of individual substances in biological liquids, for detecting contaminants in food and water, etc. For example, the graphene-silver system can absorb antibiotics from water even at low concentration both effectively and with high selectivity [41]. Carbon electrodes coated with electrochemically deposited GO can be used for quick detection of Pb in water and foods even at super-low concentrations (below 1 bln^{-1}) [42]. Viswanathan et al. in their works have shown high efficiency of the developed graphene-based biosensors for monitoring glucose levels in blood [43].

Besides, graphene, its oxides and composite materials based on them are very promising for biotechnological applications as they can be used as antibacterial agents in industrial-scale water purification filters [44–46] or as specialized filters against bacteria [47]. Composite material containing both GO and iron oxide nanoparticles displays very good antibacterial activity against *E. coli* by means of inducing oxidative stress and damaging cell membranes [46]. Porous membranes containing GO and antimicrobial peptide nisin can be effective for filtering and disinfection of water contaminated with methicillin-resistant *S. aureus* [44].

Based on the above, the question of safety of graphene-like

nanomaterials for humans and for the environment has not been clearly decided yet and it is still unclear whether their cytotoxicity will limit their biological and medical applicability.

2. Materials and methods

2.1. Synthesis and characterization of graphene oxide

In our research a sample of GO prepared by means of chemical exfoliation of graphite flakes by the Hummers method has been used [48–50].

Concentrated H_2SO_4 (69 ml) was added to the mixture of graphite flakes (3 g, 1 wt equivalent) with NaNO_3 (3 g, 0.5 wt equivalent), the obtained mixture was cooled down to 0°C . Then KMnO_4 (9 g, 3 wt equivalent) was added slowly, in order to keep the temperature of the reaction mixture below 20°C . After that the reaction mixture was heated up to 35°C and stirred for 30 min, at the same time 138 ml of water was added slowly to it. The reaction temperature was kept at 98°C by heating the mixture for 15 min on an electric heater. After this the heating was switched off and the reaction mixture was cooled down in a water-bath for 10 min. Then 420 ml of water and 3 ml of 30% H_2O_2 were added.

The resulting mixture was run through a sieve (U.S. Sieve Size (WS Tyler, 300_m)) and then filtered through polyester fiber (Carpenter, Co).

The obtained product was centrifuged (for 4 h at 4000 r.p.m.), the supernatant fluid was decanted.

The remaining solid material was rinsed successively with 200 ml of water, 200 ml of 30% HCl and 200 ml of ethanol ($2\times$); after each rinsing cycle the mixture was run through a sieve (U.S. Sieve Size Standard), filtered through polyester fiber and centrifuged (for 4 h at 4000 r.p.m.) while the supernatant fluid was decanted.

The resulting material was coagulated with 200 ml of ether and the obtained suspension was filtered through a PTFE membrane with the pore size of $0.45 \mu\text{m}$. The resulting concentration of GO in the colloidal solution was 25 g/l.

Raman spectra were measured using Thermo DXR Raman microscope with 532 nm laser at 1 mW power through $100\times$ objective.

Atomic force microscopy (AFM) data was done using AIST-NT instrument in semi-contact tapping mode with NTMDT AFM tips. GO suspension (1 mg/ml) was spin coated on silicon wafer at 4000 rpm for 1 min and dried at 100°C for 10 min to obtain thin flakes for analysis.

2.2. GO suspensions

The GO suspensions were prepared with distilled water ($\text{pH} = 7.1 \pm 0.2$). The working suspensions with GO concentrations $2.5 \dots 0.0025 \text{ g/l}$, were prepared by diluting the initial nanomaterial suspension (25 g/l) and stirring with a glass rod for 20 s. After stirring the suspensions were processed in Ultrasonic Cleaner CD-4800 (Codyson, China) for 5 min (70 W, 44 Hz, volume 1.4 l).

2.3. *E. coli* biofluorescence test

We used recombinant Green Fluorescent Protein-Expressing *Escherichia coli* which was transformed with pRSET-emGFP plasmid.

This culture was used as a model system to investigate the antimicrobial activities of GO.

1 ml of overnight culture was grown on nutrient broth (NB, Sigma Aldrich) supplemented with ampicillin (NBamp) and inoculated in 100 ml fresh media NBamp for 3 h at 37°C . 50 ml of culture was concentrated by using centrifugation (5000g for 5 min). The culture was three times washed in 0.9% NaCl and centrifugated at 3000g for 3 min. Optical density (OD660) was approximately 0.13.

To estimate the influence of GO, 5 mkl of obtained cells were diluted in 200 mkl NBamp media and 200 mkl of NaCl and mixed with 45 mkl

of GO with following final concentrations: 0.0025, 0.025, 0.25, 2.5 g/l. The same procedure was used for graphite as for control carbon material. Our measurements were performed on a microplate reader Synergy H1 with measuring of fluorescence of GFP using the excitation at 485, emissions 528 nm. Cell solution was incubated for 17 h at 37 °C. For sterilization, bacterial suspensions were placed in a boiling water bath for 10 min.

2.4. Investigation of interactions of GO and bacteria

Confocal fluorescent microscopy measurements were done using Leica TCS SP8 X CLSM setup. (Leica, Germany) Default settings for GFP were used (excitation line 488 nm, emission detection in 500–600 nm range).

Scanning electron microscopy (SEM) was carried out using a TM-1000 microscope (Hitachi, Japan). It was used suspensions of bacteria based on physiological saline (PS) with the addition of GO in concentrations of 0.25 g/l.

Light microscopy was carried out using a Biolam M-1 microscope (LOMO, Russia) using Ziehl-Neelsen stain.

2.5. Zeta potential analysis

The analysis of zeta potential was obtained sample of graphene oxide, *E. coli* bacteria, and a suspension of GO with bacteria was performed using a Zetasizer Nano ZS device (Malvern Instruments Ltd., England).

3. Theory

Nevertheless, there is a body of research denying antibacterial activity of graphene and graphene-like structures and their biocompatibility. Ruiz et al. in their works show that GO possesses no antibacterial, bacteriostatic and cytotoxic effect towards gram-negative bacteria and mammalian cells. Besides, these authors note that the absence of toxic effects in their studies can be attributed to the high level of purity from any kinds of technological admixtures of GO used in their research [51]. A number of authors also mark the absence of cytotoxic effect of graphene and GO against eukaryotic cells. Moreover, graphene materials were very successfully used as a substrate for cell growth and differentiation [52–54]. A considerable number of research works were carried out in the field of functionalization of graphene-like structures with various substances such as, for example, amine groups [55], carboxylic groups [56], polyethyleneglycol (PEG) [57,58], protein-based structures [59], etc. in order to reduce possible toxic effects against biological objects and increase stability and biocompatibility of the material. Luo et al. in their papers showed the absence of toxic effect of PEG-functionalized GO against *E. coli* and, moreover, they demonstrated increase in bacterial growth which might be used in microbiological equipment [58].

The aim of this study is to explore biological effects of GO on bacteria and the factors affecting it. For this, we synthesized GO, investigated its characteristics and conducted a bacterial test using Green Fluorescent Protein-Expressing *Escherichia coli* Top 10. To investigate the mechanisms of interaction of bacterial cells and GO which are not clear still, we used light and confocal microscopy, SEM, as well as the method of dynamic light scattering.

4. Results and discussions

The synthesized GO (Fig. 1a) was studied by Raman spectroscopy and AFM.

4.1. Raman spectroscopy

As could be seen in Fig. 1 Raman spectra of GO has 4 bands, which

were attributed to D (1338 cm^{-1}), G (1590 cm^{-1}), 2D (2680 cm^{-1}) and D + G (2980 cm^{-1}) vibration modes. Relative intensity of D mode (D/G) is 0.95 and is typical for most GO samples [60,61].

4.2. Atomic force microscopy

AFM image is shown in Fig. 1b. The width of GO flakes varies significantly, but the height is close to 1 nm, which indicates single layer GO.

4.3. Bacterial tests results

As one can observe from bacterial fluorescence intensity during the experiment in a growth media, the values of the studied parameter were much below those in the control group. As a whole, we have discovered a linear effect, i.e. increase in GO concentration resulted in increase in statistically significant time- and dose-dependent ($p < 0.05$) (Fig. 1d) biocidal activity.

The obtained data indicate that GO displays bioactivity and leads to dramatic inhibition of *E. coli* colonies growth in all the studied concentrations. Nevertheless, after 12 h of exposure none of the concentrations has led to complete colony death.

Antibacterial effect of GO against gram-negative bacteria has been confirmed by means of several methods in a number of research works. For example, in [62] exposure to GO in concentrations 0.05...0.15 g/l led to mechanical damage of cell membranes with further cell death. Despite the registered toxic effects, it should be noted that in similar studies high values of GO antibacterial effect were observed at much lower concentrations. In [63] exposure to water suspension of GO in the concentration of 1.25×10^{-4} mg/l has led to complete death of a *E. coli* colony in 2–2.5 h. It is feasible that such a difference observed in the toxicity levels can be explained by variations in GO physicochemical properties and in sensitivity of the evaluation methods used by the research teams.

When PS was used instead of growth media we observed a slight increase in *E. coli* fluorescence intensity in the presence of GO in the concentrations of 0.0025 and 0.025 g/l. Increase in GO concentration resulted in inhibition of the life processes of bacteria (Fig. 1e).

Our data correlates well with the results received by Barbolina et al. [64]. In their work they have shown that in de-ionized water cytotoxic effect on bacteria of GO in the concentration of 1 g/l ranges from very slight to none at all, depending on the purity of GO.

Antibacterial properties of GO were compared with those of initial graphite used for its synthesis. The graphite samples consisted of a combination of chemically uniform but structurally varying allotropic modifications of carbon. The following results were obtained. In a growth media (Fig. 1d) under similar initial conditions 12-hour exposition led to > 45% inhibition of *E. coli* colony growth in every studied sample. It should be noted that in the minimal concentration the toxic activities of GO and graphite (GR) were almost similar while increase in concentration led to considerable difference in the observed colony growth inhibition ability. At graphite concentration of 0.025 g/l bacterial fluorescence intensity decreased by 50.8% compared to the control while the same concentration of GO reduced this parameter by 61.5%. At 0.25 g/l concentration of graphite and GO this parameter decreased by 44.7% and 79.4%, accordingly. At the highest studied GO concentration *E. coli* fluorescence intensity was at its minimum being reduced by 88.4% compared to the control, while exposure to the highest concentration of graphite produced the results almost similar to the ones at 0.25% g/l. It should be also noted that unlike GO, graphite produced no dose-dependent effect.

When PS was used instead of growth media the toxic effect of both graphite and GO changed dramatically (Fig. 1e). Unlike the aforementioned effect of GO in PS, graphite in concentrations of 0.0025...0.25 g/l produced no influence on fluorescence intensity and, accordingly, had no toxic effect on *E. coli*.

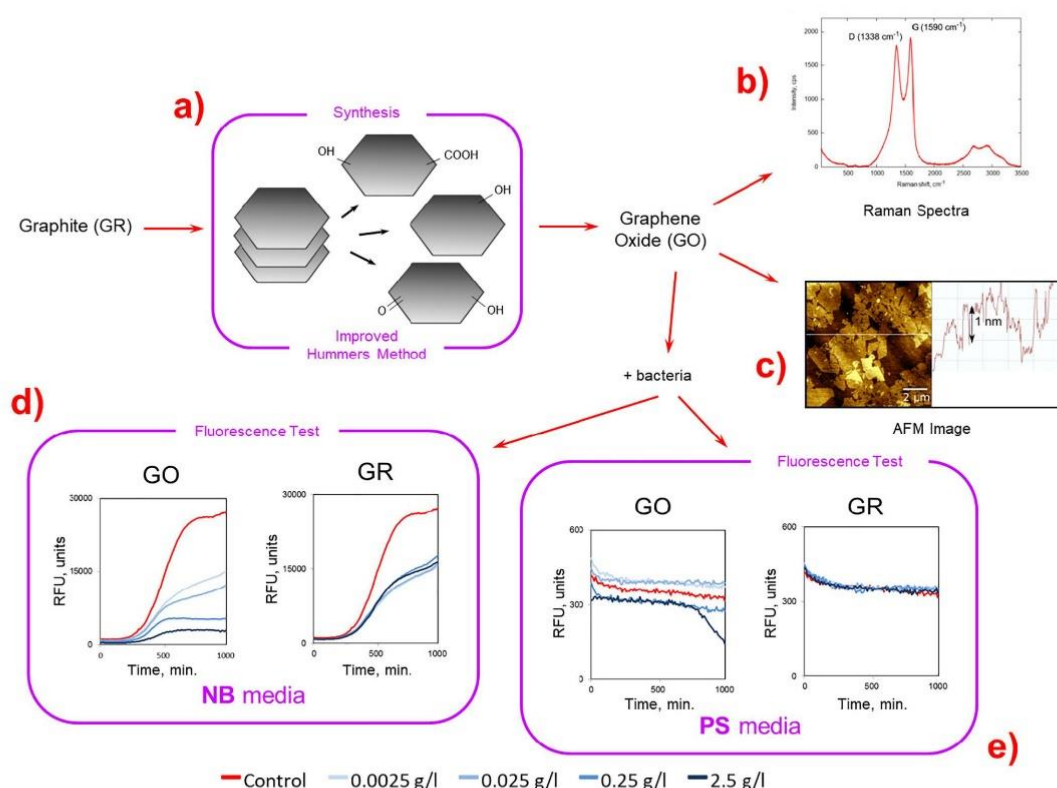


Fig. 1. a) GO synthesis; b) Raman spectra of GO; c) AFM image of GO; d) bacterial fluorescent test results in NB media; e) bacterial fluorescent test results in PS media.

In the process of evaluation of time dependence of the period of exposure to the studied carbon materials the following regularities were discovered. During experiments in growth media with exposure to the minimum studied concentration of 0.0025 g/l the rate of fluorescence intensity growth in time was slightly higher in the GO group than in the graphite (GR) group. And after 8 h exposure this parameter was statistically higher in the GO group. Nevertheless, when the exposure time reaches 10 h all the differences in fluorescence intensity and, consequently, in the *E. coli* colony development became completely negligible. At the GO and GR concentrations of 2.5 g/l precisely the opposite effect was observed, i.e. throughout the whole experiment in the GO group considerable inhibition of colony growth was recorded and by the end of the exposure period the GO and GR groups differed by up to 2.4–4.6 times (Fig. 1d).

When PS was used instead of growth media the toxicity results changed, as we have mentioned previously. During exposure to GO at the concentration of 0.0025 g/l fluorescence intensity was decreasing at a slower rate than during exposure to GR (Fig. 1e), it was verified after the exposure period of 8 h. On the other hand, when the active substance concentration was increased up to 2.5 g/l no considerable difference between the results in the GO and GR groups was observed, though in the GO group fluorescence intensity was decreasing at a slightly higher rate (Fig. 1e). These results support the previously obtained data on higher biological and antibacterial activity of GO compared to other carbon modifications [65].

4.4. GO-bacteria interactions

During the experiment, the interaction of bacteria and nanomaterial was observed. In solution of GO with a concentration 0.25 and 2.5 g/l the formation of a flocculate structure was observed when mixed with a bacterial suspension in PS.

During experiments using growth media no similar results were observed, as well as during all the experiments with graphite. Images obtained via microscopic methods (Fig. 2b–d) show bacterial clusters localized on GO particles surfaces and the bacteria maintain viability which is proved by their green color indicating GFP synthesis (Fig. 2c), these results are consistent with the data obtained by Barbolina et al. [64].

4.5. Zeta-potential analysis

The analysis of results of suspension stability evaluations showed that zeta-potential of the bacteria and GO samples was rather high, amounting to -36.87 and -39.38 mV, correspondingly. It was an indication of considerable electrical stability of the suspensions. While mixing them together led to decrease in this parameter up to -24.93 mV which indicated decrease in the suspension stability (Fig. 2e).

Other nanomaterials also form aggregates with bacteria. For example, Schmidt et al. show in their work the process of agglutination of bacteria with polyvalent nanoparticles [66]. A. Horst et al. [67] show that TiO_2 nanoparticles accumulate on the surface of *Pseudomonas*

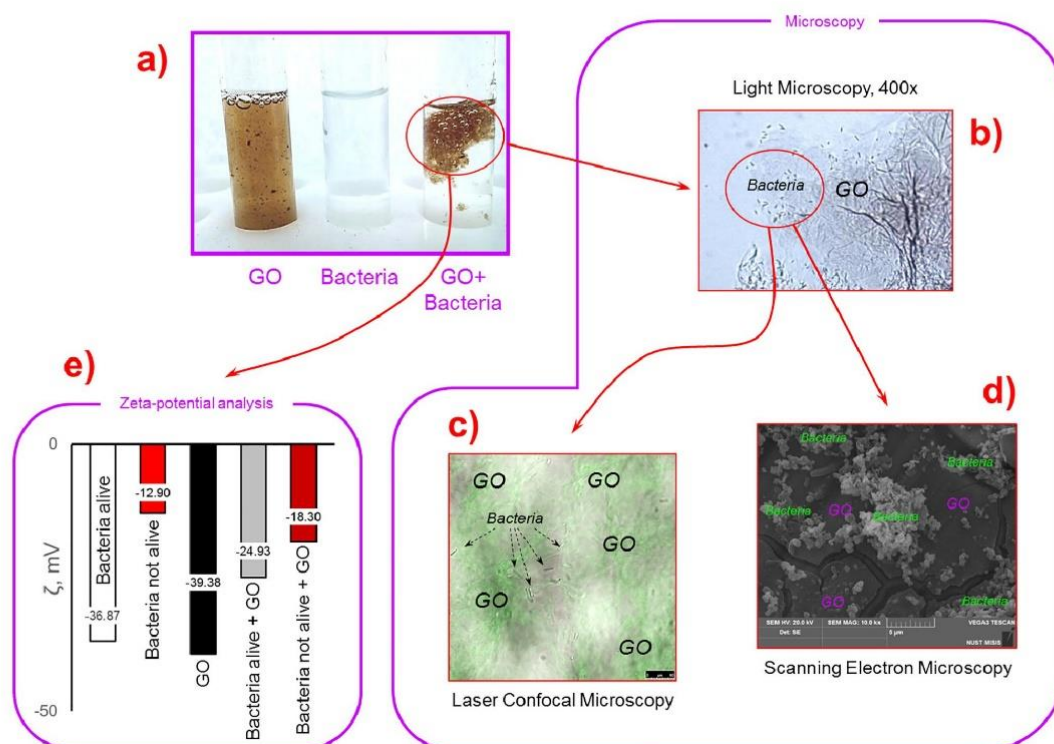


Fig. 2. a) Cuvettes with GO, bacterial suspension and their mixture (in PS); the flocules obtained from the mixture were studied by means of: b) light microscopy - bacteria aggregating on GO sheets were observed; c) confocal laser microscopy - green color of the bacteria indicates their viability; d) scanning electron microscopy - bacterial aggregates on GO scales can be seen; e) zeta-potential calculations - decrease in stability of the GO-bacteria colloidal system containing both live and dead bacterial cells (after treatment in a sterilizer) was observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aeruginosa bacterial cells.

Change in zeta-potential of suspensions consisting of GO and dead bacteria (after treatment using boiling water bath) to -18.3 mV indicates electrostatic nature of formation of GO-bacterial cells aggregates (Fig. 2e). This was shown for the first time ever and it corresponds well to the fact that when growth media was used instead of PS no flocculi formation was observed. It can be explained by difference in the media degree of ionization. The obtained results correlate well with the data on aggregation properties of graphene-like materials in high saline media [68] and do not support the common view that formation of biofilms by bacteria is the reason of graphene-like materials aggregation [47,68,69].

5. Conclusions

Thus, our experiments have shown that water-based suspension of GO when added to bacterial culture medium display a strong antibacterial effect if compared with graphite. Furthermore, increase in concentration of the studied substance leads to increase in the difference in toxic effect values. In PS presence of GO in concentrations from 0.0025 to 0.025 g/l has a stimulating effect on *E. coli*, while in concentrations above 0.25 g/l GO displays antibacterial effect. Apparently, this dependence of GO antibacterial properties on the employed medium is the reason for conflicting results obtained by different researchers.

Correlation between the toxicity level of a carbon-based material against *E. coli* as a model for gram-negative bacteria and the material

structure has been established on the example of GO and GR. Nevertheless, it should be noted that strong antibacterial effect requires high concentrations of GO which consideration can be important for development of antibacterial coatings for biomedical applications.

Our team has received data indicating the electrostatic nature of interactions between bacterial cells and GO sheets. A number of previous papers suggested the process of biofilms formation by bacteria as the primary reason for aggregation between graphene-like materials and bacterial cells. However, formation of flocculent structures consisting of GO and dead bacteria and accompanied with decrease in zeta-potential of particles in the suspension to 18 mV proves that electrostatic interactions play the major role in aggregation.

The obtained data can be used for employing graphene-like materials in new systems for water-purification from biological contaminants.

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A. Gusev et al.

Materials Science & Engineering C 99 (2019) 275–281

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3.6 *In vivo* Capture of Bacterial Cells by Remote Guiding

Rybkin, I., Pinyaev, S., Sindeeva, O., German, S., Koblar, M., Pyataev, N., ... Lapanje, A. (2021). *In vivo* capture of bacterial cells by remote guiding. *BioRxiv*. <https://doi.org/10.1101/2021.08.06.455395>. The manuscript is in the BioRxiv repository and is under final revision for submission to: Small

Since the formation of very precise cellular aggregates is difficult due to their low yields, we have developed a solution that allows the aggregates to be enriched using only the magnetic properties of the NPs when they are introduced into the coatings. The cells were coated in a labeled PE capsule with dye active in a far red spectrum. Magnetic NPs were introduced between PE layers to enable magnetic capture. Using Scanning Electron Microscopy (SEM), the authors confirmed the presence of metal NPs within the PE layers. Moreover, using TLM it was shown that the coated cells have prolonged the lag phase, which would allow the authors to gradually control the release of bacteria from the capsules over time.

In order to prepare an appropriate case, which would also demonstrate the possible application of the developed method, we aimed to make delivery systems in living organisms using magnetized bacterial cells. Although such a system differs from technological setups, the organism conditions allowed us to test the robustness of our solution.

From a biological point of view, several types of bacteria have been found to be effective in mitigating the development of cancer, as they can be selectively cytotoxic, can enhance the immune response, remain sensitive and detectable for external regulation. However, the introduction of native bacteria remains unsafe due to the high immunogenicity of the bacterial surface and the general distribution of bacteria in the body, which increases the risk of sepsis. To improve the control and safety of the spread of bacteria, as well as to facilitate detection, the authors developed and applied a method that allows remote control and detection of bacterial cells using a magnetic field in a living organism.

All coated cells were injected into live mice with or without a magnet. All mice survived the cell injection and were alive until the end of the experiment. Using a biofluorescence imaging system, we analyzed the fluorescence distribution of cells within the mice. The analysis of the obtained images showed that the introduction of a magnet increased the concentration of cells by more than 5 times throughout the entire experiment as compared with the group in which the magnet was absent. In general, there was no difference in the distribution of coated cells between the magnetic and control groups in the organs of the mice, since high concentrations of bacteria were used to obtain a sufficient signal. All cells were collected mainly in either the lungs or the kidneys of the animals. It was also found that mice develop brighter signals over time, and the increase in signal occurs 1.3 times faster in the group of magnets. However, the overall intensity remains almost half of the value of the control group.

Summarizing all the findings, the authors have prepared a new method for obtaining magnetically guided bacterial cells, which are easily detectable in the far red spectrum. It was shown that for high delivery efficiency, the concentration of cells increased by more than 5 times, while all animals remained alive during the experiments. With such a simple method, it is possible to significantly expand the repertoire of strains for cancer treatment and control over the properties of cells to develop safer bacterial therapies. Since the environment of blood resembles a liquid environment where the biofilms are naturally present, it showed us the ability to orient cells in a living organism merely using a magnetic field.

The candidate's contribution includes development of a method for the cell coating, image analysis, statistical analysis, preparation of figures and partial writing of the manuscript.

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***In vivo* capture of bacterial cells by remote guiding**

Iaroslav Rybkin^{1,2,3,4}, *Sergey Pinyaev*⁵, *Olga Sindeeva*^{3,6}, *Sergey German*^{3,6}, *Maja Koblar*^{2,4},
*Nikolay Pyataev*⁵, *Miran Čeh*², *Dmitry Gorin*^{3,6}, *Gleb Sukhorukov*^{3,7}, *Aleš Lapanje*^{2*}

1 Helmholtz-Zentrum Dresden-Rossendorf, Permoserstrasse 15, Leipzig 04318, Germany

2 Jožef Stefan Institute, Jamova cesta 39, Ljubljana 1000, Slovenia

3 Saratov State University, Astrakhanskaya Ulitsa, 83, Saratov 410012, Russian Federation

4 Jožef Stefan International Postgraduate School, Jamova cesta 39, Ljubljana 1000, Slovenia

5 National Research Ogerev Mordovia State University, Bolshevistskaya Ulitsa 68, Saransk 430005, Russian Federation

6 Skolkovo Institute of Science and Technology, Center for Photonics and Quantum Materials, 3 Nobel Street, Skolkovo 143026, Russian Federation

7 Queen Mary University of London, Mile End Rd, Bethnal Green, London E1 4NS, UK
E-mail: ales.lapanje@ijs.si

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Recently, it has been shown that several bacterial strains can be very efficient in cancer treatment since they possess many important properties such as self-targeting, ease of detection, sensing and toxicity against tumors. However, there are only a few relevant “candidates” for such an approach, as targeting and detection one of the biggest challenges as well as there are many limitations in the use of genetic approaches. Here, it is proposed the solution that enables surface modification of alive bacterial cells without interfering with their genetic material and potentially reduces their toxic side effect. By the electrostatic interaction fluorescently labeled polyelectrolytes (PEs) and magnetite nanoparticles (NPs) were deposited on the bacterial cell surface to control the cell growth, distribution and detection of bacteria. According to the results obtained *in vivo*, by the magnet entrapment of the modified bacteria the local concentration of the cells was increased more than 5 times, keeping the high

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concentrations even when the magnet is removed. Since the PEs create a strong barrier, *in vitro* it was shown that the division time of the cells can be regulated for better immune presentation.

1. Introduction

Using a top-down engineering approach, the ideal cancer therapy can be envisioned as micro or nano programmable “robot devices” that (i) specifically target tumors preferentially by self-propelling, (ii) are selectively cytotoxic only to cancer cells and (iii) responsive to external signals, which can transfer information about the local environment and will enable remote guiding.^[1] Specific targeting is one of the most important properties since it would allow the use of toxic substances without systemic effects. In addition, self-propulsion independently to the blood flow enables penetration into tumor regions that are otherwise inaccessible to the passive systemic deliverable therapies. According to above mentioned criteria for ideal anticancer treatment, bacterial cells seem to share many of these characteristics and in fact, there are genera, which have been shown in laboratory experiments as well as in clinical tests (Phase I)^[2] to preferentially accumulate in tumors and lyse cancerous cells, including *Salmonella*,^[3] *Escherichia*,^[4] *Clostridium*,^[5,6] and *Bifidobacterium*,^[7] *Caulobacter*,^[8] *Listeria*,^[9,10] *Proteus*,^[11] and *Streptococcus*.^[12] Although there are very encouraging reports of anticancer bacterial therapy treatment, the current approach is far from the ideal cancer therapy since (i) in many cases native bacteria must be genetically modified at least to ensure safe use, (ii) specificity of delivery must be increased and (iii) it is needed to remotely monitor distribution of the bacterial cells. Currently it is known that the cells used in bacterial therapy are not directed specifically to the tumorous mass, but the accumulation is a passive process where bacterial propagation is only enabled

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within the locally deprived immunity,^[13] in contrast to the other tissues where bacteria are suppressed by strong immune responses of the host.

In addition, bacteria and their cellular components are also extremely potent and strong immune stimulators with endo- and exotoxins where their systemic delivery increases the risk of sepsis as well as development of toxic syndrome, since the only available control of their propagation in such approaches is by using antibiotics.^[14] Due to the above mentioned drawbacks, the bacterial therapy cannot be extended to the wide range of bacteria although some might have very potent toxins, e.g. *Pseudomonas* strains,^[15] since they cannot be directed to the tumor cells or because of very low scores on the above mentioned characteristics of the ideal bacterial agent that could be used in the approach.

Having in mind these drawbacks, especially, to increase repertoire of bacteria that can be used in treatments by the specific delivery to the site, monitoring their distribution and controlling their growth, here we propose the robust solution that solves these drawbacks without introducing genetic manipulations methods.

Since the bacterial cells are negatively charged, they can act as a core for deposition of PE layers on their surfaces using a Layer-by-Layer (LbL) approach, to form a tailor-made core-shell structure. Deposition of the PE on the cell surface can add new modalities to the bacteria such as survivability in the harsh conditions or improved adhesive properties.^[16] In addition, it can bring new physiological activities as increased specific binding to cells, or it can expand the spectrum of action of the cells using modified PEs with fluorescent dyes, enzymes or ligands (e.g. antibodies),^[17–19] as well as potentially allow them to get detected in the body. For the remote targeting of the bacteria, inside the layers it can also be added different sorts of NPs, which could be used for the controlled delivery as shown in delivery systems using nano- and microparticles.^[20] It is expected that such shells on the one hand separate the surface of the cell from the immune system and on the other, due to the shell strength, they

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can control the division rate^[21] and therefore, invasiveness of the delivered bacterial growth. Accordingly, our main aim in the study was to test an idea to guide, detect and monitor distribution and growth of bacteria within the mammal body in order to make safer anticancer or perhaps, also other therapies using bacteria, as well as to broaden the spectra of possible use of different bacterial strains. In relation to that, by using the *in vivo* experiments on the mouse model more specific aims were: (i) to remotely guide bacterial cells by using magnetic field, (ii) monitoring the distribution of bacterial cells inside the mammal body and (iii) controlling division rate of the selected strain.

2. Results

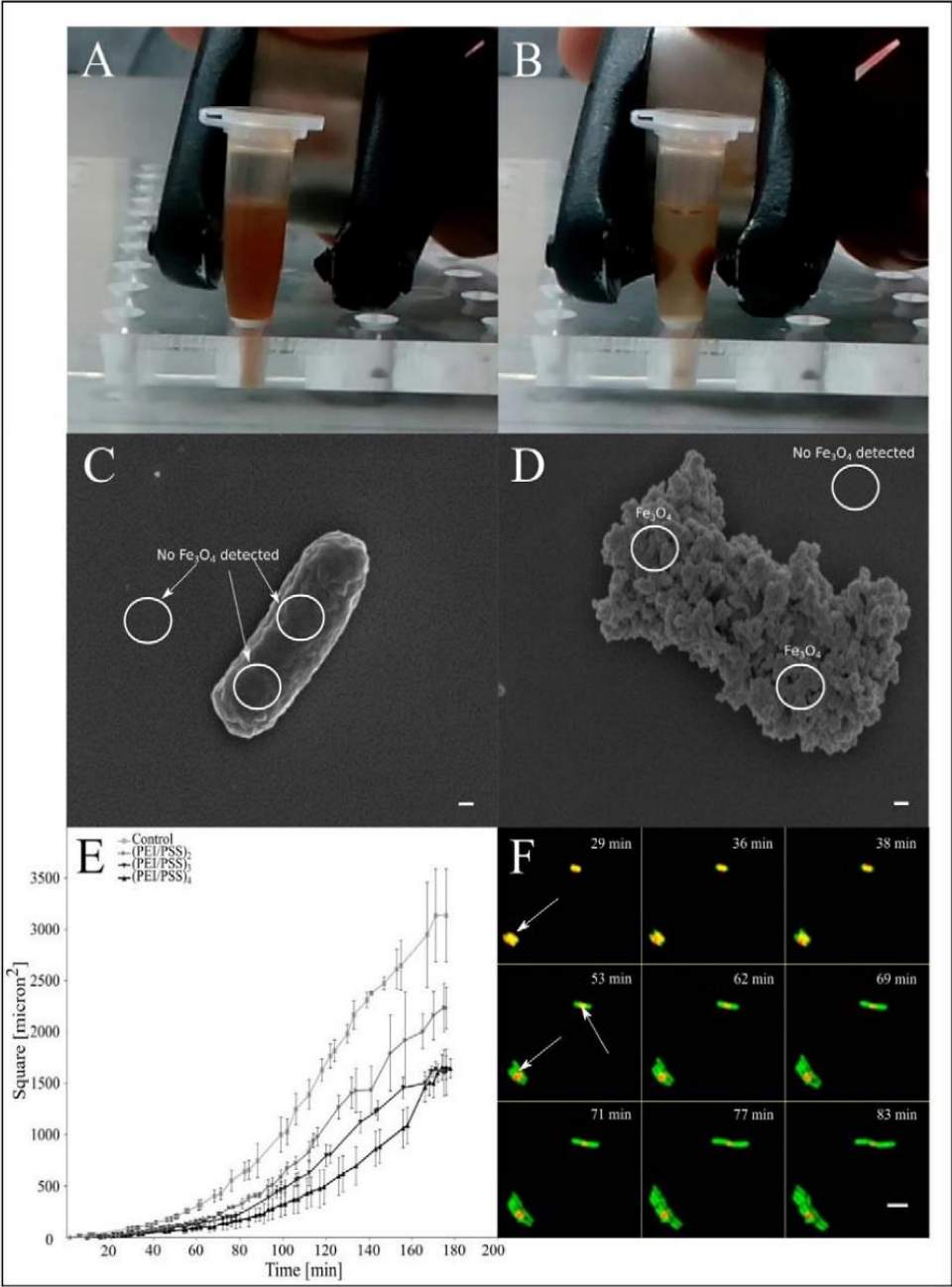
The surface of the bacterial cells was efficiently functionalized by the method of electrostatic attachment of the PEs labeled with fluorescent dye and the following consequent deposition of the magnetic NPs (**Figure 1A and 1B**). In order to prepare Scanning Electron Microscopy (SEM) images of bacteria coated with PE and magnetic NPs it was found that just air drying is one of the most convenient and relevant approaches, since the chemical fixatives interfered with the structure of the shells, causing many artefacts such as aggregation or release of the cellular compounds (data not shown). To confirm the presence of iron NPs on the surface of the coated cells, we applied a combination of SEM and energy-dispersive X-ray spectroscopy (EDS) techniques (**Supplementary Table 1**). As a result of the coating, the magnetite NPs adsorbed on the polycation-treated bacteria provided a dense layer on the entire cell surface (**Figure 1C and 1D**).

By time-lapse confocal microscopy (TLCM), we showed that the entrapped cells stay alive inside the formed PE layers. We determined that the most appropriate for the injection amount of layer was 4 bilayers deposited on the cells, which enabled us the highest possible

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fluorescence level, slower division rates of the cells and at the same time, keeping the aggregation of the cells during the LbL procedure as low as possible. After incubation *in vitro*, bacteria started escaping from the shells by breaking the capsule at the distal positions, leaving parts of PE capsule, which surrounded the escaping cells (Figure 1F). On the single cell level, we determined that the growth rate was proportional to the number of the PE layers (Figure 1E) deposited on the bacterial surface, where the slowest growth was observed for the cells entrapped in 8 layers. Although there was no toxicity, all the coated cells demonstrated on average 1.4 times longer lag phase than untreated cells. The cells coated in our procedure showed that they can be released from the capsules in 50 minutes after incubation in the most optimal conditions.

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Figure 1. *In vitro* magnet entrapment of the *E. coli* cells coated by polyethylenimine(PEI)/poly(styrene)sulfonate (PSS)/PEI/Fe₃O₄ (A, B). When a magnet is applied, movement occurs instantly. Additionally, presence/absence of nanoparticles was proved by SEM (EDS), where intact cells did not have any particles, scale bar = 100 nm (C), while the modified cells had magnetite only on the surface (D). Effects of entrapment of cells in layers of polyelectrolytes on growth (E) of the population of the single bacterial cells. Escape of the cells from the LbL layers when entrapped in PE layers (F) red is LbL shell indicated with arrows, green is GFP producing cells, overlap regions are yellowish, scale bar = 5 μm.

After administration of the electrostatically functionalized bacterial cells (EFBC) into the bloodstream of mice, we observed differences in distribution of cells that were dependent on the presence of the permanent magnet. Under exposure to the magnetic field, spreading of the cells in the organism from the place of injection was directed toward the paw with a magnet with the following accumulation of the bacterial cells, forming bright areas in the paw where permanent magnet was placed (**Figure 2 A**) after a certain time. In contrast, within other parts of the body the signal became 2,4 times on average weaker than in the paw. In control groups, the EFBC were mainly distributed in the abdomen forming dense shining areas, whereas in the intact paw the signals were weaker 1.8 times than in other parts of the body (**Figure 2 B**). Throughout all experiments animals remained alive. By the magnet entrapment we were able to significantly increase concentrations of the cells more than 5 times in the paw region than in the control group ($P < 0.001$) (**Figure 2C**). However, although the magnet efficiently attracted bacterial cells, we determined that just a fraction of cells (up to 4.64%±0.14%) was accumulated in the paw, while the overall distribution of free circulating cells remained the same in both groups. After the magnet was removed, we observed that the signal from

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the exposed paw was 5.8 times higher than in unexposed paw over the remaining half an hour ($P < 0.001$).

The fluorescent signal recorded from other parts of the body was increasing over time (Figure 2E). Throughout the whole time of the experiment we observed on average 1.3 times higher increase of the signal intensities from the body in the magnet group than in control ($P < 0.05$, see also **Supplementary Table 2**). Although the increment of signal in the group of animals exposed to the permanent magnet was observed, the signal intensity from the rest of the body was 1.8 times lower than the control at the end of the experiment ($P < 0.001$).

After animals were dissected we analyzed the fluorescent signals from the vital organs, which were 1.1 times significantly higher just in the liver within the magnet exposed group than in the non-exposed group of animals ($P < 0.05$), (Figure 2D). The results of paired statistical comparison between organs showed that the highest amount of the cells was accumulated in kidney and lungs (not significantly different, $P > 0.05$) with the following series of the reduction of the signal in liver>spleen>paw ($P < 0.05$ in inter pairs, Figure 2F).

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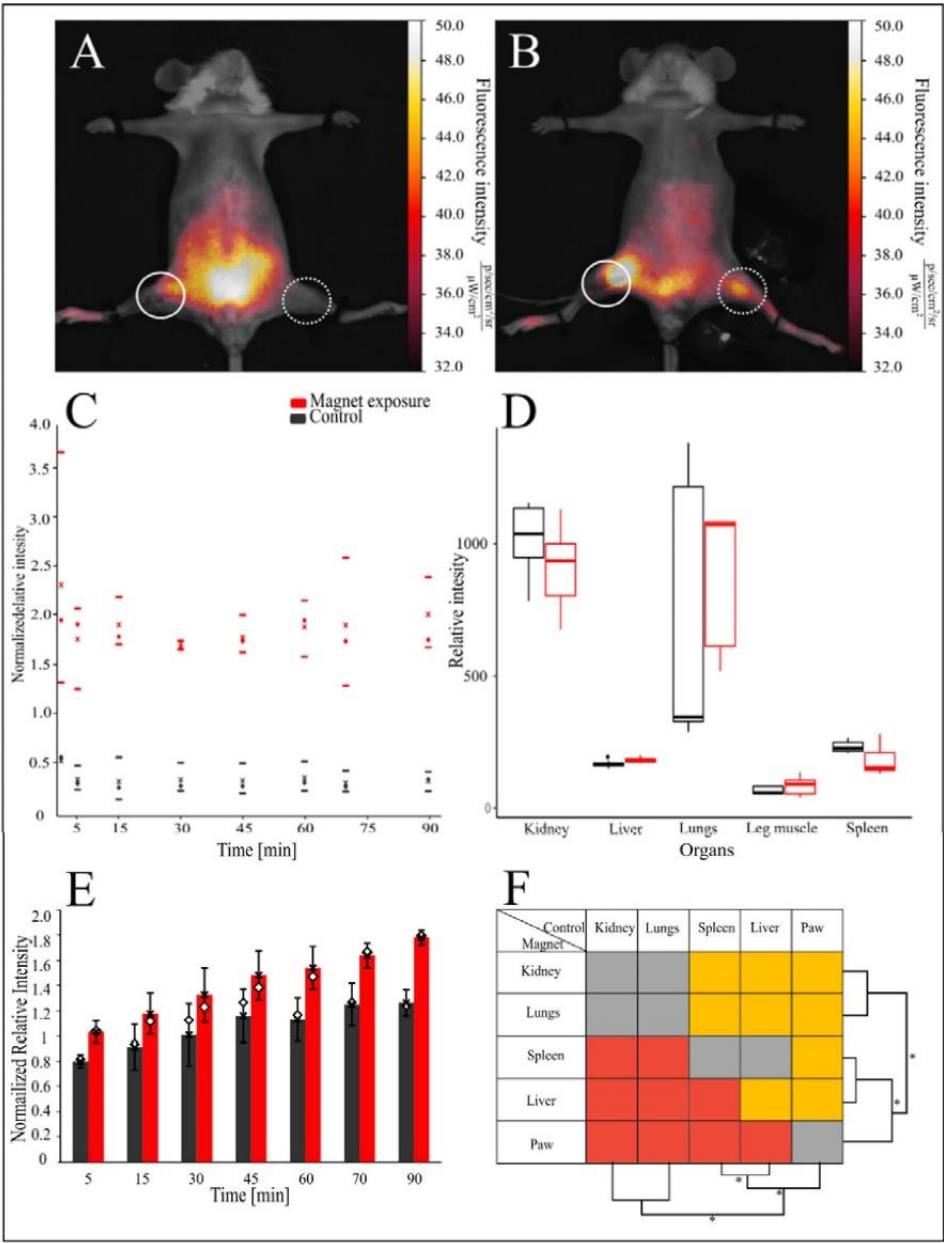


Figure 2. Distribution of the bacterial cells modified with magnetite nanoparticles and Cy-7 labeled PEI in the mice. The bacteria were detected in control mouse (A) and exposed under magnet mouse (B), respectively, dash circles indicate place of injection, solid circles – place

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of cell collecting. Relative fluorescence intensity of the signal in the paw under magnet exposure and normal distribution by blood flow was chosen to compare the accumulation of cells (C), where - Median, $\bar{x}$ - average, upper and lower borders are 25th to 75th percentile respectively. Normalized relative fluorescence was also assessed in the organs of animals (D). Introduction of bacteria led to increase of relative fluorescence intensities in mice bodies over time (E), where - Median, $\bar{x}$ - average and statistical significance of the distributed bacteria between organs (F), red – signal of the magnet group is lower than control, gray-not significant, orange – higher, respectively.

3. Discussion

For the first time here we showed a proof of a new concept that a method of surface modification of bacterial cells by electrostatic deposition of PEs can increase the specificity of delivery of the EFBC to the site of an action by remote guiding more than 5 times (see Figure 2C). The specificity of our solution also exceeded more than 2 times over using microparticles of similar sizes, which could be attributed to a 4 times stronger permanent magnet used in the experiments.^[22] We expect that specificity of targeting could be improved even more since the deposited PEs have various amino or carboxyl groups that can enable adding specific receptors, proteins or antibodies that can target the tumor cells when bacteria are placed in the local environment of the cancers by the magnetic field.^[18,23,24] Since it was needed to gain a sufficient signal, it was injected a high dose of the EFBC ($8 \cdot 10^8$ CFU). As a result, there were many free circulating cells left, where we were able to capture only a small fraction of bacteria (up to 4.6%) in the magnet paw (see Figure 2 B), while the rest of the cells later passively accumulated in organs (see Figure 2 D). Therefore, if we want to increase the

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efficiency of targeting, the concentrations of cells should be lowered and optimized according to the power of the magnet source and vasculature capacity that would hold attached biomass. On a basis of safe concentrations known from literature that can be introduced to an organism, vasculature capacity and distribution of cells within the body determined as collected signals in our experiments, we can use at least 1000 times less bacteria to obtain similar results without extensive circulation of bacterial cells elsewhere in the body.^[2,25]

Although in our experiments we did not use different modifications of the PE capsules, from the data obtained from the literature we can predict that the approach shown here can be further improved by using methods of modifications of the PE shell. One important condition using bacterial therapy is making bacteria stealth to the immune system. We can envision several mechanisms that would allow to gradually control the engagement of the immune system rather than obtaining a septic shock: (i) the PE capsules can hide bacterial membrane and toxic lipopolysaccharides (LPS) compounds from direct exposure, (ii) capsules can control the release of the cells as they create a strong barrier and (iii) specific coatings can increase stealth circulation. Accordingly, the cell wall of bacteria is composed of LPS, muramyl dipeptides and lipoteichoic acid, which are well known to be toxic components for the immune system.^[14] When the native bacteria are administered in high quantities, it is expected to have a strong inflammation with a high immune response followed by a septic shock. If we introduce native bacteria as high as 10^9 CFU to BALB/c mice it should induce 50% lethality.^[25] However, although a high dose of bacteria ($8 \cdot 10^8$ CFU) was used, no lethal cases were observed during our experiments. We suppose that such a high viability can be a result of the presence of the PE shells, which could smoothen the immune response of mice by shielding the LPS surface from direct contact with the immune cells. Specifically, the shielding was shown to be achieved for various inanimate PE capsules when they were introduced to an organism.^[26,27] Moreover, the potential systemic immune response could be

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prevented if we control the portion of the released EFBC that come in direct contact with the immune system. Based on our experiments performed *in vitro* (Figure 1E, 1F), it is shown that the bacteria stay alive being encapsulated. However, they need a longer time that is proportional to the capsule thickness to eventually escape. Therefore, introduction of bacteria with different thickness of the coating would enable gradual release of bacteria from shell and modulation of the intensiveness of the response of the immune system as only a particular portion of cells would release from the capsules at a certain time.

Eventually, the coated bacteria can be recognized by immunity via opsonization, which inevitably will engage activity of macrophages. Thus, to increase the stealth time of the capsules, specific coatings like polyethylene glycol (PEG) or polysaccharides could be used as they reduce hydrophobicity and surface charge, which are primal initiators of opsonization.^[28]

Using the PE assembly can also help to overcome limitations of bacteria that lack targeting ability as it enables formation of the coatings responsive to remote guiding. In combination with controlled time of LPS introduction to the immune system and specific targeting, it can lead to a very high, but local immune response, which in return reduces the overall toxic effect. Besides, we expect that the cell growth will mostly occur in tumor hypoxic regions, since there are the most favorable growth conditions due to suppression of immunity.^[29,30]

Bacteria can become even more potent by introduction of genetic elements for the expression of cytokines,^[29] cytotoxic agents^[31] that additionally would contribute to fighting tumors or become more detectable by production of fluorescent dyes.^[32] However, we do not limit the application of this approach only to mitigate the cancer development as it allows us to use the coated bacteria as adjuvant systems of vaccines. Accordingly, if the parts of the bacterial cell envelopes can enhance the immune response,^[14,33] we can expect even higher elevation of the immunity if the whole bacterium is presented as a coated capsule but not exaggerating

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immune activity to engage the septic shock. In addition, the use of encapsulation can increase adherence of the probiotic bacteria^[16] as well as their adjuvant activity.^[34,35]

In our experiment we also analyzed the overall fluorescent intensities (Figure 2 E), which was unexpectedly gradually increasing during the duration of the experiment. This phenomenon we can speculate that occurred at least due two reasons: (i) as a result of breaching of the shell integrity caused by either due to the bacterial cell division, or interaction with host organism, e.g. phagocytosis, eventually releasing labeled PEs or (ii) dissemination of bacterial cells through the body leading to saturation of the capillaries within the skin tissue that is covering the body surface resulting in a higher detectable signal. We observed a significant change of intensity after 15 min for the group of animals exposed to the magnet and 70 min for control ($P < 0.01$), which could not be attributed to the cell division as it would take at least 40 min under the best conditions to divide. Even, if the cells would be uptaken by the macrophages, killing of 40-70% of biomass (depends on type of cells and expression profile), it would take at least 2h with the following digestion and core, bacterial cell, decomposition for other 6 h.^[36] Also, we excluded the technical error, based on an improper set of the intensity scale, that might result in the signal increase. Hence, these discrepancies of different signal enhancement cannot be explained by shell degradation at least before the first division occurs. Here, we assume that the main reason could be related to a slower retention of saturation of the capillaries by the magnet compared to the control. From the literature it is known that the total surface area of capillaries (2000-27000 mm²) is much greater than the surface arterial vasculature (70 to 160 mm²) of mice.^[37] In contrast, blood volume in capillaries is much smaller in comparison to arteries and veins (5% against 15% and 64% respectively) as well as blood flow is not high.^[38] We suppose that in the long term, the number of freely circulating bacteria will be reduced by filling all capillaries and organs, depleting cells from blood of arteries and veins. The cells will increase the detectable surface signal being stuck near the

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skin surface region. Since the capillaries are highly branched, small differences in the concentration of the cells retained by the magnet, eventually might cause slower saturation of capillaries, till the magnet is removed and held leftovers of bacteria further fill the rest of capillaries. As we did not prolong the experiment, some amount of the cells could be still held in place of the magnet, not reaching control values and as a result the overall intensity was much lower. In addition, retention of the cells by the magnet over time might cause formation of the soft aggregates, which will not be able to fill capillaries due to an excessive size. Eventually, at the end of the experiment, the cells were mainly distributed in kidney and lungs, similar as reported for uncoated bacteria^[30] or silica particles of micron size.^[39] From literature it is also known that nanosized particles tend to be accumulated in spleen, lungs, liver and kidney.^[40–42] Since the vascular volumes of the organs as well as blood flow have the highest values in lungs and kidney,^[43] it could explain the fact that bacteria were mostly accumulated in these organs with the following reduction to liver, spleen and paw. Therefore, for successful delivery, it has to be taken into account that difference in dissemination from this perspective can be considered as vasculature, blood flow as well as size dependent.

4. Conclusions

Summing up, we designed a solution for improving bacterial therapy by using the method of electrostatic deposition. We prepared the magnetically responsive bacterial cells, which were detectable in the far red spectrum. Using the permanent magnet allowed us to increase the specificity of targeting more than 5 times compared to the control group. It was found that introduction of a high dose of the coated cells did not lead to animal death throughout the whole experiment. As the polyelectrolyte layers were deposited on the cells, it resulted in the controlled time of the cell division, which might be used for gradual introduction of the coated bacteria to the immunity. Such an approach of encapsulation opens new possibilities

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for using and controlling various microorganisms and makes new concepts in drug delivery as well as in preparation of adjuvants in vaccines.

5. Materials and methods

5.1 Bacteria strains and growth conditions

In our experimental simulation of targeted bacterial therapy we used cells of non motile *Escherichia coli* TOP 10 strain (F⁻ mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (ara leu) 7697 galU galK rpsL (Str^R) endA1 nupG), containing pRSET-emGFP plasmid. The plasmid contains the T7 promoter region upstream of the emGFP reporter gene and Ap^R cassette, which enabled us to observe cells by confocal microscope and to control bacterial contaminants by amending ampicillin in media, respectively. The transformants were cultivated at 37 °C on nutrient agar plates (Sigma-Aldrich) supplemented with ampicillin (100 μ g mL⁻¹, Sigma-Aldrich) (NAamp). From the overnight culture, we transferred 1 mL into the 100 mL fresh medium and incubated until obtaining the optical densities appropriate for conducting the particular experiments. All liquid cultures were incubated by shaking on a rotary shaker at 37 °C and 150 rpm.

5.2 Preparation of PEs for layer by layer encapsulation

We used the negatively charged sodium poly(styrene sulfonate) (PSS, MW=70 kDa) and the positively charged poly(ethyleneimine) PEs (PEI, MW=600 kDa), both purchased from Sigma-Aldrich, for cell encapsulation based on the electrostatic principle.^[44] The solutions of PEs in MQ water (2.5mg mL⁻¹, pH 7 adjusted by NaOH or HCl) were prepared by

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solubilizing PEs firstly by stirring and then by the sonication (35kHz, 100W, 15min). PEI was stained with Cyanine7 (Cy7, Lumiprobe, USA) according to protocol of NHS ester labeling of amino biomolecules.^[45] Briefly, the Cy7 (9 mg in 9 mL of DMSO) solution was added to PEI solution (2.5 mg mL⁻¹ in 40 mL of water with pH 8.4) in 50 mL tube and incubated for 4 h at room temperature under constant stirring conditions. To remove the residual dye after labeling the solution was dialyzed for 3 days using a dialysis tube (Orange Scientific) with nominal molecular weight limits between 12 and 14 kDa and titrated up to pH 7. All the PEs were sterilized by filtration using 0.2 µm sterile filters.

5.3 Encapsulation procedure

Prior to the procedure of encapsulation of cells in PE layers, bacterial cells were grown at 37 °C by shaking at 150rpm until reaching OD₆₀₀ = 0.2 measured in a 200 µL volume on a microtiter plate. The obtained culture of such a densities of bacterial cells were further concentrated by centrifugation of the 50 mL of bacterial culture at 5000 g for 6 min and then washed three times by the centrifugation (5000g for 5 min) and finally resuspended in 30 mL of 0.9 % NaCl. The cells were coated in layers of PEI/PSS/PEI/magnetite-NP/(PEI/PSS)₂ by the following procedure. Firstly, positively charged PEI layer was deposited by adding 0.25% solution of PEI in MQ water (pH=7, adjusted with HCl) to the washed and concentrated cells of final OD₆₀₀=1.2 in 1:1 v/v ratio with PEI and incubated at room temperature for 5 minutes. Unattached PEI was washed out from the suspension by centrifugation at 900 g for 2 min. The obtained pellet was 2 times washed by the gentle pouring of 1mL of 0.9% NaCl not resuspending the pellet. The PEI coated cells were washed in 0.9% NaCl solution and then the second negatively charged PSS layer was deposited (pH 7 adjusted with NaOH) using the same procedure as for PEI except the cells were centrifuged at 1500 g for 3 min. After

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addition of another PEI layer the layer of magnetite NPs was added. The magnetite NPs (12 nm average size), were synthesized according to German et al. (2013).^[46] The NPs were incubated with the cells for 5 minutes (1 mg mL⁻¹ particles, 1:1 v/v particles to cells). The excess of magnetite particles was washed out by centrifugation at 1500 g for 3 min. The coated cells were collected by the magnet, additionally washed 2 times by pipetting and then resuspended in 0.9% NaCl solution. After magnetite, the layers of PEI-Cy 7/PSS were deposited in the same way as there were deposited in the first layers of PE on the surface of the bacteria. Finally, we obtained 8 layers of the PEs on the bacterial cells.

5.4 SEM and EDS analysis

For obtaining the best possible images using SEM visualization, samples were prepared using different protocols: (i) air drying at room temperature without any fixatives, (ii) the freeze drying procedure and (iii) chemical fixation with drying at 40 °C. For normal drying the samples of the coated and control cells (intact, free of magnetic NPs) were suspended in MQ water and placed on silicon wafers to be dried at room temperature for 72 hours. For the freeze drying, the cells were resuspended in MQ and pre-frozen in liquid nitrogen. Further, the samples were put in the freeze dryer (Christ Gamma 1-16 LSC Plus) and left for 72 h at -50 °C. For chemical fixation the cells were prepared by the following procedure.^[47] Briefly, the cells were fixed with 0.5% glutaraldehyde and 1% formaldehyde in a 0.1 M cacodylate buffer 0.1 M and transferred on a clean glass. After washing with cacodylate, 1% osmium tetroxide was added and left for 1 h. After all, samples were rinsed with water and incubated for 5 min in gradient dilutions of ethanol (30%, 50%, 70%, 96%), replaced with hexamethyldisilazane and left for drying overnight at 40 °C. All the samples from different protocols were mounted on SEM stubs, platinum-sputtered and observed in high vacuum

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SEM (Jeol JSM-7600F) with a field emission gun, at low voltages, to observe the structure of the cell. For EDS silicon drift detector (SDD) from Oxford (S Max, 20mm²) was used. To excite the K α a higher excitation voltage was applied.

5.5 Time-lapse confocal microscopy (TLCM)

To enable the precise measurement using the TLCM approach we needed to distribute cells on a thin planar surface and provide them with appropriate growth conditions for a few hours of observations. Accordingly, we prepared a chamber for the cell incubation as described previously.^[21,48] Briefly, the nutrient agar was poured inside a cut of 1cm x 3 cm of a double-sided tape attached to a clean glass. Before solidifying the cover slip glass was carefully deposited to make a smooth NA layer. The chamber was left for 10 min 4 °C to form a solid NA matrix. The non attached slide glass was then removed, the protective layer of the double sided tape was pilled off and then 4 μ l of culture (final OD₆₀₀ approx. 0.4) was evenly distributed over the exposed NA surface. At the end we sealed the chamber with the cover slip by attaching it on the sticky surface of the frame enclosing the NA.

The chamber with the cells was equilibrated at 37 °C for 15 min and transferred to the confocal microscope (Leica TCS SP8X confocal laser scanning microscope equipped with temperature control system cube and box) that was thermostated at 37 °C. The TLCM was performed at 1000x magnification using an objective lens (HCX PL APO 100x/1.44 OIL) immersed in immersion oil. We observed cell growth and division using the excitation at 489, emissions 525/25 and 750 842/33 with 850-900V and 750-800V gains for cmGFP and Cy7 fluorescence, respectively. The morphology of cells and amount of non-fluorescent ones was observed using white light and condenser as the objective lens.

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5.6 Analysis of the TLCM micrographs

To determine the growth properties of the entrapped cells in PE layers we analyzed images using Fiji software.^[49] In prior to the experiment we firstly selected fields under the microscope occupied by similar amounts of cells. From the obtained fluorescent images forming hyperstack we deleted those that were of low contrast or out of the focus. The prepared hyperstacks of the images were then converted to the stack of binary images using the "make binary" plugin. On such prepared images we measured the surface area of cells at the consecutive time points by using the "analyze particles" plugin to determine temporal changes of the cell biomass. The experiments were performed in triplicates and the t-test was used for statistical comparison of the amount of cell biomass per time per group of cells with different numbers of PE layers.

5.7 *In vivo* biodistribution

All experiments were performed according to the relevant institutional (National Research Ogarev Mordovia State University, Russia) and international regulations of the Geneva Convention of 1985 (International Guiding Principles for Biomedical Research Involving Animals). Animal ethics clearance was approved by the ethical committee (protocol № 50 from 29.05.2017). The biodistribution of the encapsulated bacterial cells was investigated in 2 groups of 3 BALB/c female mice 6-8 weeks old with weight distribution 20–25 g. The intra-arterial injections of suspension of bacteria were performed on immobilized anesthetized mice. For general anesthesia Zoletil (60 mg kg⁻¹, Virbac SA, Carros, France) and Rometar (and 10 mg kg⁻¹, Spofa, Czech Republic) mixture was used via intraperitoneal injection. The intra-arterial injection was performed through the polyethylene catheter (PE-10 tip, Scientific Commodities INC., Lake Havasu City, Arizona), which was introduced into

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the right femoral artery. During the implantation the catheter was filled with isotonic sodium chloride solution. The bacterial cells were resuspended in sodium chloride solution of approximate $OD_{600} = 1$ (measured in a 200 μ l of microtiter plate). The volume of bacterial suspension administered in the mouse was 200 μ l.

For all animals, the bacterial cells were injected in the right femoral artery and collected in the left paw by placing the permanent magnet NdFeB (1400 mT remnant magnetization, diameter - 50 mm, height - 20 mm) for 60 min, which was then removed. The control group of animals was treated in the same way except permanent magnet was not used.

5.8 In Vivo fluorescent imaging

We measured distribution of Cy7 labeled bacteria using IVIS® Lumina imaging system (Xenogen Corp.) with a filter set (excitation, 710-760 nm; emission, 810-875 nm). All the fluorescence images were acquired with 0.2 s exposure. The intensities of fluorescent signals on the obtained pictures were also analyzed in Fiji software.^[49] All images were converted to the gray-scale signal using the "RGB to luminance" plugin. Prior to analysis, all the signals were normalized according to the initial base level. The gray-scaled signals from the paw were normalized to the signals from the abdominal area and intensities were measured over the area with the resolution of 400 pxl. Intensities of organs were measured for the whole area of a particular one and normalized by the weight of a single organ. Absolute intensity of every mouse was measured according to the same procedure, except the rectangular frame was used to calculate change in fluorescence. All experiments were performed using 3 animals per tested group. Nonparametric Mann Whitney two-tailed and Kruskal-Wallis tests were used for statistical comparison of signals obtained from different body parts as well as from different organs when animals were sacrificed and dissected.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4

Discussion and Summary

The present work contributes to the scientific knowledge on: (i) the preparation of methods for confinement of cellular biomass based on electrostatic interaction, (ii) evaluation of the toxicity of selected PE coatings on various bacterial strains and (iii) the studies of cell growth of the bacteria confined in PE coatings. We also demonstrated how the difference in cell exposure to the PEs and NPs affects the toxicity assessment, making the results inconsistent between studies. In particular, there have been made several particular contributions such as: (i) the preparation of the method for the deposition of PEs, avoiding toxicity and excessive aggregation, (ii) the study of the physiological parameters of the cells confined in PE coatings (i.e. growth rate, GFP expression and microcolony formation), (iii) the effects of PEs and NPs on viability of bacteria and differences in toxicity between various strains, and (iv) the preparation of concepts for the method application.

According to our study (Section 3.1), first, we could get separately coated cells, which allowed us to study the effects of PE coatings on the physiology of cells. Secondly, we were the first to demonstrate that PE confinement affects the physiology of bacteria by prolonging the lag phase and increasing nonspecific transcription. The delay in the growth could be attributed to: (i) a decrease in cell viability, (ii) a reduced diffusion, or (iii) the strength of the PE coating, which depends on the type of the PEs, their branching and numbers as well as on the ionic strength of the medium. In contrast to other studies (Balkundi et al., 2009; Franz et al., 2010; Jonas et al., 2018), we did not observe any reduction in cell viability since the tested strain could tolerate a positively charged coating and was viable under the microscope. The decrease in diffusion could be associated with (i) biological aspects of cells (i.e. production of appendages), (ii) aggregation of coated bacteria, and (iii) low permeability of the PE coatings.

Since we optimized the procedure to carefully coat individual cells and the growth rate of the cells coated in a pair of PE layers was not different from the control, we did not observe a significant decrease in diffusion properties, which could lower the viability. In particular, we chose the most appropriate growth stage to minimize the presence of cell appendages to efficiently coat bacteria not causing aggregation, where aggregation can be the major cause of lowered diffusion. The efficiency of this approach was confirmed by the fact that the polydispersity index of coated cells had lower values than that of cells coated according to the previously developed protocols. Additionally, due to the diversity of the structures of the cell envelopes (e.g. flagella, pili, EPS, Gram architecture), we also expect that softness parameters must be determined on a case-by-case basis. As a result, we expect that such parameters as concentration of PEs, centrifugal force, type of PEs and number of layers, concentration of cells and their growth phase should be optimized for each specific strain in order to achieve the best possible coating.

A decrease in the diffusion of PE capsules should also not occur, since several studies of the activity of the encapsulated enzymes in capsules with similar thickness showed that

enzymatic substrates larger than 6 kDa, such as short peptides and dextran molecules (1 kDa in our case), freely pass through the capsule (Antipov et al., 2002; Qiao, Galvosas, Adalsteinsson, Schönhoff, & Callaghan, 2005). Since our coating of 4 PE bilayers does not obviously reduce diffusion, each PE bilayer significantly increases the strength of the coatings (Vinogradova, 2004). Therefore, we concluded that cell growth was counteracted by a force, which was correlated with the number of PE layers. We observed that growth delay was significantly correlated with the number of layers, while the choice of PEs or media conditions was the same throughout the experiment. As a result, the addition of each PE bilayer increased the time of the onset of the first cell division and exponential phase, confirming the first hypothesis that the capsules create a force opposing cell growth and its division, resulting in the prolonged generation time.

Since we assumed that bacteria can lose the anabolic regulation over time due to strong confinement, the effect of a confinement on gaining cell mass was investigated. By increasing the thickness of the PE capsules, we observed an extreme case, where the division process of certain bacteria was stalled, causing a disproportionate increase in cell size (more than 2 times) as well as an increase in protein production (more than 5 times). The most similar published results, which we tried to compare, were the effect of hydrostatic pressure on physiology. It was reported that exposure of cells to elevated hydrostatic pressure showed inhibition of cell division by preventing the formation of septum (blockade of *ftsZ* protein), (Bartlett, 2002; Ishii et al., 2004) or increasing their size (Yayanos & Pollard, 1969). However, in our experimental setup, we did not interfere with the diffusion of metabolites or nutrients inside and outside the cell envelope, as well as with the solubility of gases. Therefore, it allowed us to attribute the disproportion of cell size only to the mechanical force of confinement, which could cause the constant engagement of the anabolic metabolism of cells. Since bacteria were not present in the nutrient-deficient conditions, this should engage the accumulation of free phospholipids that inhibit the activity of Guanosine Pentaphosphate (ppGpp), which prevents cytoplasm from exceeding the cell capacity by suppressing transcription. Therefore, inhibition of ppGpp should lead to an increase in the production of cellular components, which can indirectly increase the concentration of GFP due to the production of (i) more plasmids or (ii) more genomic DNA, resulting in an increase in the number of free available RNA polymerases, which can lead to a decrease in the specificity of RNA polymerases transcription. Since chromosomes are synthesized faster than division occurs (Klumpp & Hwa, 2008), it results in the disproportion of gene copy numbers and proteins, especially if the cells cannot divide due to constraint. The stalling division, which prevents dilution of intracellular proteins, including RNA polymerases, should even more decrease the specificity of transcription and increase leakage, resulting in an increased GFP signal. From this point of view, it can be expected that GFP can be increased by free available RNA polymerases that bind to GFP gene copies or consequently bind to the same GFP gene.

Although the obtained results confirmed the second hypothesis that the leakage expression of genetically produced constructs will be increased in confined cells, the specific mechanisms require further study. In particular, since cells were observed to produce large amounts of proteins, transcriptome studies are needed to determine changes of RNA synthesis of confined cells. Also, it is further needed to determine changes that may lead to an increase in the amount of phospholipids, chromosomes and plasmids.

Since, in many cases, the introduction of PEs exhibited controversial results in viability between different Gram architectures (Carmona-Ribeiro & de Melo Carrasco, 2013; Gibney et al., 2012; Jain et al., 2014) or even within one Gram architecture (Carmona-Ribeiro & de Melo Carrasco, 2013; E. Kenawy et al., 2002), we examined what conditions might contribute to controversial outcomes (Section 3.2). For the first instance, our study demonstrated that toxicity is mainly a factor of exposure since in classical MIC tests when

PEs were constantly present in solution, we observed similar inhibition of both strains, despite the difference in susceptibility. However, there was no toxicity observed for the resistant strain when PEs were introduced by the LbL manner. Since PEs are supposed to be bactericidal due to a positive charge, we can expect that the most obvious contribution of toxicity is reached when the cell surface is fully coated. Therefore, we might expect that MIC tests will not provide obvious reasons for toxicity, as PEs spread in the medium could decrease the availability of nutrients due to electrostatic interaction or increase the osmolarity of the solution. However, the contribution of these conditions, which additionally promote the toxicity, requires further study.

The data obtained from STED microscopy confirmed that the effect of a single layer of the four selected PEs is strain-dependent since the toxicity and penetration of PEs was observed only in the cells of *P. stutzeri*. Although we proposed that *E. coli* has more mechanisms for cationic antimicrobial resistance than *P. stutzeri*, it would require performing transcriptomic studies to determine the contribution of specific genes. Since particular genes might be the major cause of resistance, several knockout models should be prepared. It is assumed that toxicity is mainly caused by an excess of positive charge (Carmona-Ribeiro & de Melo Carrasco, 2013; Timofeeva & Kleshcheva, 2011). By decreasing the concentration, we reduced the amount of the positive charge, which significantly improved viability. According to our results, we confirmed the third hypothesis that, on the one hand, the type of cells determines the most appropriate PEs for coating, while the major contribution to toxicity is made by the total positive charge of PEs.

Although we did not see any statistically significant differences between PEs of different molecular weights deposited by the LbL manner, we do not exclude that lower molecular weights can be toxic as previously shown in conventional tests (Carmona-Ribeiro & de Melo Carrasco, 2013; Timofeeva & Kleshcheva, 2011) since the shortest PAH had the greatest ability to penetrate the cell wall. Since most biotechnological processes for production of cell biomass occur in the liquid phase, we suggest using PEI PEs for LbL coatings, as PEs that contain primary amines were found to be more toxic in our case and in other studies (Palermo, Lee, Ramamoorthy, & Kuroda, 2011). However, it is clear that further research is needed to understand the reasons for this prevalence of binding.

Since we developed a method appropriate for entrapment of individual cells without compromising their viability, we have also prepared the proof of concept of the artificial formation of living biofilm (Section 3.3). Many strains were found to be susceptible to the effect of PEs and a decrease in electrophoretic mobility showed better viability, we reduced the linear charge densities by introducing acetyl groups, which significantly improved the viability of bacteria. We expect that the prepared solution can be useful as it allows us to achieve the optimal ratio of viability to surface charge for each bacterial strain. Since only certain cells can form a biofilm due to surface repulsion (Adair et al., 2001; Hori & Matsumoto, 2010; Palmer et al., 2007), coating either bacteria or an inanimate surface with PEs, allowed the cells to attach and to form a biofilm. Initial attachment of cells over several hours resulted in the surface population by bacteria, even for biofilm non-forming strains. From a physical point of view, the interaction of bacteria with the surface is equilibrated with repulsion caused by electrostatic forces and attraction caused by Van der Waals forces. Such interplay leads to the formation of energy minima, where the cell can attach irreversibly (primary minimum) or may be in a transitional stage, which is equilibrated by repulsion (secondary minimum). Since in our system we reduce the energy barrier caused by electrostatic forces by changing the charge of bacterial cells, it helps the cells to promote irreversible attachment, which should be corresponded to a primary energy minimum. Thus, we have confirmed the fourth hypothesis that increased time and stronger

interaction with the surface can induce more efficient biofilm formation of biofilm forming and biofilm non-forming bacteria.

Since we could stimulate cell growth, it was tested whether an artificially deposited probiotic strain could mitigate the growth of pathogenic cells. Although we clearly observed that artificial biofilm further controls surface population by inhibition of pathogenic cells, additional studies are needed to explain the mechanisms that are involved in growth inhibition. As surfaces become corroded (Rijavec et al., 2019) or contaminated by pathogenic microbes (Chaoui, Mhand, Mellouki, & Rhallabi, 2019; O'May, Jacobsen, Stickler, Mobley, & Shirliff, 2008) it can be expected that the primal deposition of modified live bacteria can cause a controllable population of the surface only with beneficial bacteria. In addition, we can expect that electrostatic attachment of cells can also prevent their detachment in wastewater treatment systems as well as improve bioaugmentation (Horemans et al., 2017).

To investigate the ambiguities in toxicity caused by NPs before using them in the LbL manner, we analyzed the effect of rGO and GO on the viability of bacteria (Sections 3.4 and 3.5, respectively). Accordingly, in all cases, we did not observe any membrane disruptions as it was proposed for Gram-negative bacteria (Tang et al., 2013) since the cells preserved the integrity and GFP signal under confocal microscopy and spectrophotometric studies for both types of particles. However, the intensity of GFP decreased when cells were exposed to rGO and decreased even stronger when GO was used at high concentrations in the medium, indicating the interference of NP with cell growth. We can expect that the interaction of GO particles with cells can be mediated via hydrogen bonds (Pulingam et al., 2019). While the interaction of rGO particles with cells can be mediated via hydrophobic interaction (Tan et al., 2019). However, both interactions might be compensated by electrostatic repulsion (Romero-Vargas Castrillón et al., 2015). Since no GFP leakage was observed, it makes other mechanisms as oxidative stress or just the effect of graphene contaminants as acids that can oxidize the membrane (Barbolina et al., 2016) more prominent to cause the cell damage rather than direct membrane damage. In particular, oxidative stress can occur when the oxygen groups of graphene NPs come into contact with the cell membrane (especially for GO) oxidizing it. However, our tests did not include the effect of GO or rGO when they are introduced in the LbL manner, although in the demonstrated cases in the LbL manner there was no inhibition (L. He et al., 2021; H. Luo et al., 2018; Yang et al., 2012). Thus, it is necessary to further investigate the reasons for the cell growth inhibition and the effect of NP in PE capsules.

Since the yield of cell aggregates may be low due to the laboriousness of the work, we developed a method for the deposition of paramagnetic NPs to enrich the cell aggregates merely using a magnetic field. In order to demonstrate a relevant case, which would allow us to solve problems related to moving cell biomass, we chose biomedical application of the coated cells. The bacterial cells have been shown to be potential anticancer agents as they are toxic to tumors, can induce immunity and are self-propulsive (Forbes, 2010). Since current strategies of bacterial action are based on the passive accumulation of bacteria in tumor regions due to the most favorable conditions (Toso et al., 2002), it does not allow to further develop a solution for cancer treatment using bacteria. Therefore, we prepared a case for remote guiding and detection of bacteria using magnetic NP and fluorescently labeled PEs deposited onto the bacterial surface (Section 3.6). Since the specificity of magnet targeting of bacterial cells was 5 times higher than non-magnet exposure, we confirmed the fifth hypothesis that by surface manipulation, we expect to remotely control the position of cells in a tumor tissue being not dependent merely on the ability of bacteria to colonize the tumor region. Most importantly, during the experiment, we did not observe strong side effects such as septic shock, which could be caused by excessive amounts of bacteria (Ali, Jabir, Al-Shmgani, Sulaiman, & Sadoon, 2018). Thus, we envision that under

conditions of controlled release, bacteria can further be used for development of adjuvant components of vaccines, not involving genetic modification. However, additional studies are needed to prove the safety of introducing bacteria, reduce the distribution of bacteria and optimize initial quantity. In our demonstrated case of the proof of concept, it will be needed to find the optimal strain for suppressing tumor growth and to test the antitumor activity in an *in vivo* system. Due to the presence of bacteria in the organism, it is necessary to extend their circulation time in the body and also to make the immune response smoother. To prolong stealth circulation, the surface of bacterial cells can be coated with polysaccharides or PEs that reduce the overall charge and prevent capture by immune cells similarly, as shown for inanimate particles (Yoo, Chambers, & Mitragotri, 2010). Such shells are expected to become an intermediate layer between the surface and the immune system, shielding the toxic parts of the membrane and reducing the capsule charge, which should prevent further opsonization. As a result, it will make the immune adaptation smoother. The use of electrostatic interaction can also increase adherence to the vasculature due to altering the cell surface charge and significantly improve viability of bacteria if tumors are present in regions with harsh conditions since PE capsules were shown to protect cells in bile conditions (Anselmo et al., 2016).

In summary, the most important steps to make multicellular structures are: (i) the mitigation of PE toxicity for bacteria, (ii) the preparation of tailor-made coatings for each of the cell types, and (iii) the adjustment of the physiological parameters of bacteria depending on the composition of the consortia using PE coatings. Toxicity can be reduced by decreasing the PE concentration or its linear charge density. In order to uniformly coat bacteria, each strain requires determination of softness properties and optimization of the protocol for PE deposition. If complex consortia need to be created, the growth rates and metabolic conditions of each microbe must be considered. To increase the number of multicellular aggregates, paramagnetic NPs can be introduced between PE layers of aggregates and separated from the rest of biomass using a magnetic field. Additionally, the surface of the aggregates can be functionalized with different NPs to add new modalities such as mechanical stability, conductivity, catalytic activity and others.

Chapter 5

Conclusions

The main goal of our work was (i) to develop a method for the PE deposition on different bacterial cells and (ii) to study biological responses caused by changing the cell surface properties at a single cell and at population levels. To achieve the main goal: (i) we prepared a method of the PE deposition and (ii) we studied the effect of physical constraint of PE coatings on cell growth. The related hypotheses were that an increase in the number of PE layers would prolong the generation time and increase the leakage expression of genetic constructs due to the constant engagement of anabolic metabolism. Accordingly, at a single cell level, we found that the duration of the lag phase and the generation time of coated bacteria were proportional to the number of deposited layers. In extreme cases, the division was stalled, while leakage of GFP expression and the size of coated cells increased by factors 5 and 2, respectively.

Since in our preliminary experiments the toxicity of PE was strain-dependent, in the third goal we aimed to determine the difference in toxicity between different species with the same cell wall architecture. Thus, the third hypothesis was that the cell type would determine which PE with its physio-chemical properties would be the most suitable to preserve viability, while the major contribution is the total charge of the PEs. After deposition of PEs, they were mostly present inside *P. stutzeri* cells or on the surface of *E. coli* cells. This led to a decrease in the viability of *P. stutzeri* cells and the same viability of *E. coli* cells. In *P. stutzeri*, the PE toxicity depended on the type of exposure. In particular, PEs containing primary amines were more toxic in liquid media and less toxic on solid media. However, a decrease in positive charge of PE increased the viability of cells. For the convenience of coating, we also applied the acetylation of PEs, which reduced the positive charge of the molecules. In addition to the investigation of the ambiguities in toxicity, we have identified a decrease in growth using GO and rGO particles, while the reasons for the decrease require further study.

In the fourth goal we aimed to study the effect of the cell surface modification by PEs on the longevity of cell attachment, development of microcolonies and changes in its shape. Accordingly, the fourth hypothesis was that the increased time and stronger interaction with the surface can induce more efficient biofilm formation of biofilm forming bacteria as well as it can facilitate attachment and biofilm formation of biofilm forming and non-forming bacteria. The introduction of positively charged PEs significantly prolonged the longevity of attachment of both biofilm forming and biofilm non-forming species. As a result, we demonstrated the case of constructing an artificial biofilm by attaching probiotic microbes, which prevented further growth of a potentially pathogenic strain. At the population level, the coated cells were found to form more spherical colonies depending on the thickness of the coatings.

The fifth goal was related to the development of a method that makes cells responsive to magnetic fields in order to facilitate manipulation with cells during aggregate preparation. To test this system, we placed cells in a living organism, which also helped to improve guiding of bacteria. The related hypothesis was that by surface manipulation we expected to remotely control the position of bacteria in the tumor tissue being not dependent merely on the bacterial ability to colonize the tumor region. The results showed that by using magnetic field the specificity of targeting can be increased more than 5 times even after the magnet is removed. Since the environment of blood resembles a liquid environment where biofilms are naturally present, it showed us the ability to orient cells in a living organism merely using a magnetic field.

In summary, the modification of the bacterial surface using electrostatic attachment of PE layers for preparing multicellular aggregates requires: (i) reducing PE toxicity for bacteria, (ii) preparing tailor-made coatings for each of the cell types depending on the properties of the cell surface, and (iii) regulating the physiological parameters of bacteria depending on the composition of the consortium using PE coatings. It has been found that many cells are susceptible to positively charged PEs even within the same Gram architecture, which requires a reduction in toxicity either by decreasing the PE concentration or its linear charge density. In order to uniformly coat a single cell, it is necessary to determine the softness properties of each strain and to optimize the protocol for PE deposition by choosing the optimal type of PE, its concentration, number of layers and conditions for washing as well as careful selection of the growth phase and number of cells. If complex consortium is needed to be created, the growth rates and metabolic conditions of each microbe must be considered to keep cell biomass together. The growth rates are expected to be regulated by the strength of PE capsules, which will define the time of the first division. To keep various bacteria attached on an inanimate surface, the latter can be additionally modified with positively charged PEs, which will prolong the attachment of newborn cells. For increasing the number of multicellular aggregates, the cell surface can be functionalized with paramagnetic NPs introduced between PE layers of the aggregates and separated from the rest of biomass using a magnetic field. In addition, the surface of the aggregates can be functionalized with different NPs to add new modalities such as mechanical stability, conductivity, catalytic activity and others. However, the toxicity of these NPs and methods for reducing toxicity must be considered on a case-by-case basis.

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Other Publications

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Biography

Iaroslav Rybkin, born 14 April 1994 in the Saratov region, finished school with honours in 2011. From 2011 to 2015 he was studying at Saratov State University at the Faculty of Chemical Engineering, where he began his research work on catalysis. He successfully obtained a diploma degree in chemical engineering with honours. In 2015 he joined the group of Prof. Dr. Aleš Lapanje at Saratov State University, where he was preparing a master thesis in material science on the topic of altering surface properties of bacterial cells as well as preparing the background for future PhD work. During his master studies, he received a special grant for young scientists for making his own project. Throughout the whole period, he participated in several international and regional conferences, published an article, prepared a patent, finished a degree with honours and got an honorary certificate for his contribution to the development of science at the university. In 2017, Dr. Aleš Lapanje granted him a PhD position at the Jožef Stefan Institute, where he has been studying and preparing his PhD thesis at the Laboratory for Colloid Biology of the Department of Environmental Sciences. His research work is dedicated to changing the cell surface of bacteria for their spatial organization, which allows to obtain metabolically diverse communities that produce or degrade specific compounds. Until the present time, he was involved in different European projects to solve various tasks as a researcher, became an author and co-author of 9 original scientific articles and 1 patent, and also participated in international and student conferences, publishing a total of 17 abstracts. Since 2019, he has been collaborating with HZDR in Germany, doing research on the joint project. In 2020, he was chosen as one of 5 young scientists in ecology representing his country at the BRICS Young Scientists Forum 2020.