

PLASMA-INDUCED MODIFICATIONS OF POLYPROPYLENE TUBES FOR BIOMEDICAL APPLICATIONS

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Doctoral Dissertation
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TUBIC ZA UPORABO V BIOMEDICINI

Doktorska disertacija

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Abstract

In this work, close attention has been devoted to assessing the effects of cold atmospheric pressure plasma treatment on standard polypropylene microcentrifuge tubes. After successful optimization of plasma parameters an automatic plasma treatment device was constructed for repeatable treatment of the tubes' inner surface. Once the effects of plasma treatment on the tubes' inner surface were thoroughly examined and described, the microbiological response of microvesicles and proteins to these surfaces was studied.

Our aim was therefore to optimize plasma treatment parameters for the tubes' inner surface modification, to make such treatment fast, effective, repeatable and applicable in biomedicine. Main hypothesis was, that plasma treatment improves the microvesicle isolation process. Each of the fields involved, plasma technology, polymer sciences and biomedicine, is complex and challenging by itself. Plasma treatment of inner surface of long narrow tubes is almost non-existent in the literature, partially due to difficulties of analyzing curved surfaces and partially due to plasma jet restrictions. Further on, polypropylene is known for its poor functionalization by plasma treatment compared to other polymers like PET for example. It also has to be taken into consideration that polypropylene blends and fillers differ greatly amongst various manufacturers. Three of them were compared in this work. Moreover, plasma treatment has to be homogeneous and repeatable if we tend to use it for biomedical or other delicate applications. This brings us to the last major issue, the repeatability and unknown factors in biomedical experiments. Especially in the microvesicle isolation technique there are numerous variables and the protocol is rather new and still in the process of development and optimization.

Methods used in this work can be divided into three categories, those for plasma characterization, those for polypropylene characterization and those for characterization of biomedical experiments. In the first category, plasma is characterized by optical emission spectroscopy (OES), electric current and voltage measurements. Polypropylene is characterized by water contact angle (WCA) and surface free energy measurements, atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and differential scanning calorimetry (DSC). In the end, for biomedical experiment, flow cytometry, dynamic light scattering (DLS) and atomic force microscopy (AFM) are involved in microvesicle characterization and adsorption measurements by ultraviolet-visible spectrophotometry (UV-Vis) for proteins.

It was found that atmospheric pressure plasma jet can uniformly alter polypropylene tubes' inner surface if the plasma jet parameters and position are chosen appropriately. Further on, large variations were found between tubes made by different manufacturers. It was once again confirmed that plasticware can affect laboratory results in a significant manner. It was also confirmed that microvesicle isolation yields were noticeably improved when plasma-treated tubes were used, even though the process of isolation deviates greatly.

Povzetek

V okviru tega dela smo se posvetili preučevanju učinkov obdelave standardnih mikrocentrifugirnih polipropilenskih tubic s hladno plazmo pri atmosferskem tlaku. Po uspešni optimizaciji plazemske obdelave je sledilo konstruiranje avtomatičnega sistema za ponovljivo plazemsko obdelavo notranjih površin tubic. Ko so bili učinki plazemske obdelave notranjih površin tubic dobro preučeni in popisani, smo se posvetili še študiji mikrobiološkega odziva mikroveziklov in proteinov v kontaktu s temi površinami.

Naš namen je bil, da optimiziramo plazemske parametre za obdelavo notranjih površin polipropilenskih tubic in da naredimo takšno obdelavo karseda hitro, učinkovito, ponovljivo in aplikativno v biomedicini. Vodilna hipoteza je bila, da plazemska obdelava pripomore k izboljšanju izolacije mikroveziklov. Vsako od področij, ki jih v delu združujemo, plazemske tehnologije, znanost o polimerih in biomedicina, je kompleksno in polno izzivov samo po sebi. V literaturi skorajda ni zaslediti plazemske obdelave dolgih ozkih cevi, delno zaradi pri analizi ukrivljenih površin in delno zaradi tehnoloških omejitev takšne obdelave. Polipropilen je znan po svoji slabi plazemski funkcionalizaciji v primerjavi s PET polimerom na primer. Upoštevati je potrebno tudi dejstvo, da različni proizvajalci uporabljajo zelo različne zmesi in polnila za pripravo polipropilena. V tem delu je narejena primerjava med tubicami treh različnih proizvajalcev. Zelo pomembno je tudi, da je plazemska obdelava homogena in ponovljiva, v nasprotnem primeru je ni mogoče uporabiti za biomedicinske ali druge podobno občutljive preizkuse. Tako pridemo do zadnjega velikega izziva, do ponovljivosti in velikega števila neznanih vplivov pri biomedicinskih preizkusih. Pri izolaciji mikroveziklov se pojavlja veliko neznank, ki bi jih bilo potrebno dodatno preučiti, in razmeroma nov protokol, ki se še nadgrajuje in optimizira.

Metode, ki smo jih uporabili v tem delu, lahko razdelimo v tri skupine, metode za karakterizacijo plazme, metode za karakterizacijo polipropilena in metode za karakterizacijo biomedicinskih preizkusov. Za karakterizacijo plazme smo uporabili optično emisijsko spektroskopijo (OES) in meritve električnega toka in napetosti. Polipropilen smo okarakterizirali z meritvami kota vodne kapljice (WCA) in meritvami proste površinske energije, z mikroskopom na atomsko silo (AFM), z rentgensko fotoelektronsko spektroskopijo (XPS) in z diferenčno dinamično kalorimetrijo (DSC). Za konec smo pri biomedicinskih preizkusih z mikrovezikli uporabili še pretočno citometrijo, metodo dinamičnega sipanja svetlobe (DLS) in mikroskop na atomsko silo (AFM) ter v primeru adsorpcije proteinov še ultravijolični spektrofotometer (UV-Vis).

Odkrili smo, da je možno z uporabo hladne plazme pri atmosferskem tlaku enakomerno obdelati notranje površine tubic, če so parametri in položaj plazme izbrani primerno. Velika odstopanja je bilo opaziti med tubicami različnih proizvajalcev. Ponovno je bilo potrjeno, da lahko plastični pribor nezanemarljivo vpliva na končne rezultate laboratorijskih meritev. Potrdili smo tudi, da pri uporabi plazemske obdelane tubice za izolacijo mikroveziklov pride do opaznega izboljšanja ne glede na to, da je sama napaka postopka izolacije zelo velika.

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Abbreviations

TIG	... Tungsten inert gas
DC	... Direct current
AC	... Alternating current
RF	... Radio frequency
MW	... Micro wave
CAP	... Cold atmospheric plasma
DBD	... Dielectric barrier discharge
APPJ	... Atmospheric pressure plasma jet
FE	... Floating electrode
SE	... Single electrode
ROS	... Reactive oxygen species
RNS	... Reactive nitrogen species
UV	... Ultraviolet
DNA	... Deoxyribonucleic acid
RNA	... Ribonucleic acid
AFM	... Atomic force microscopy
XPS	... X-ray photoelectron spectroscopy
MV	... Microvesicle
EV	... Extracellular vesicle
CCV	... Clathrin-coated vesicle
MVE	... Multivesicular endosome
PBS	... Phosphate buffered saline
PP	... Polypropylene
WCA	... Water contact angle
CA	... Contact angle
CCD	... Charge-coupled device
RMS	... Root mean square
DSC	... Differential scanning calorimetry
OES	... Optical emission spectroscopy
DLS	... Dynamic light scattering
BCA	... Bicinchoninic acid
UV-Vis	... Ultraviolet-visible
SLM	... Standard liters per minute
RPM	... Revolutions per minute
2D	... Two dimensional
3D	... Three dimensional
LMWOM	... Low molecular weight oxidized material
PPM	... Parts per million

Symbols

σ	... Surface free energy
S_a	... Average surface roughness
S_q	... Root mean square surface roughness
S_z	... Maximum distance between the highest peak and the lowest valley in defined area
ΔHm	... Normalized melting enthalpy
ΔHm°	... Normalized melting enthalpy for 100 % crystalline polymer
U_{RMS}	... Root mean square voltage
U_{DC}	... Direct current voltage
λ	... Wave length
ρ	... Refractive index of dynamic light scattering
T_m	... DSC melting temperature of polymer
T_c	... DSC crystallization temperature of polymer
U	... Electric voltage
I	... Electric current
P	... Electric power
η	... Efficiency
I_0/I_{tot}	... Ratio between light scatter intensity and laser intensity for DLS
R_h	... Hydrodynamic radius
R_g	... Radius of gyration

Chapter 1

Introduction

1.1 Plasma and Plasma Technologies

1.1.1 What is Plasma

Plasma is the fourth state of matter, other being solid, liquid and gas. It is the last one to be discovered and scientifically described and is currently of high interest to the broad public in research as well as industry. A well-known fact is that matter changes its state according to the energetic states of its molecules. Solid is the energetically lowest state where molecules are interconnected and their relative movement is disabled. Therefore, solid state can be described by shape and volume. After gradually adding energy to solid matter, weaker molecule bonds start to dissociate and molecules gain a certain degree of freedom. At this point we get the liquid state of matter which no longer has a shape but still has a fixed volume. As the amount of energy increases, all bonds between molecules are broken and a gaseous state of matter occurs. Matter no longer has a shape or volume in gaseous state. Knowledge up to this point is considered elementary, a less known fact is that by further adding the energy to gaseous matter, bonds inside molecules or atoms break and electrons are no longer held in their orbitals. This is how we get a free electron and a positively charged ion and consequently an ionized gas, which is electrically conductive. This state is known as the fourth state of matter or short plasma. Plasma has no shape or volume but can be shaped by magnetic fields as it is electrically conductive.

Although the natural phenomenon of plasma was known to scientists for centuries, the first description using the term plasma dates less than a century back. Sir William Crookes was the first to identify it in a discharge tube and describe it as “radiant matter” in 1879 [1]. In 1928, Irving Langmuir introduced the term plasma when he wrote: “Except near the electrodes, where there are sheaths containing very few electrons, the ionized gas contains ions and electrons in about equal numbers so that the resultant space charge is very small. We shall use the name plasma to describe this region containing balanced charges of ions and electrons.” It is believed that Langmuir saw similarities to blood plasma that was known at the time and borrowed the name. Word plasma itself is of ancient Greek origin meaning moldable substance. For a long time, the fields of plasma physics (plasma as the fourth state of matter) and blood plasma (in medicine) had nothing in common and any confusion was impossible. This changed recently when a new field of plasma medicine emerged and started to grow exponentially. Physical plasma has more and more direct and indirect applications in the medical field. In this thesis word plasma refers to the fourth state of matter otherwise the term blood plasma is used.

1.1.2 Various Types of Plasma

As in any other naturally occurring phenomenon scientists got the idea of recreating plasma from nature. The lightning was probably the most interesting natural plasma phenomenon of them all. Due to its complexity many cultures described it as the anger of gods for centuries. Along with the invention of electric energy, scientists got the first chance for mimicking the plasma phenomenon under controlled conditions. From that point on plasmas can instantly be divided into natural and man-made plasmas.

In case of lightning, a highly energetic discharge between the clouds and the ground takes place. Prior to that the charge builds up in the clouds and moisture often lowers the dielectric capability of air to a point where discharge can occur. What we see is actually an ionized tunnel of air where the discharge happened. Not only visible light is emitted but also X-rays and gamma rays. Apart from lightning, natural plasma can also be found in our atmosphere as aurora. Aurora is the name for charged particles travelling from the sun as solar wind, which get trapped by the Earth's magnetic field and release their energy in the form of photons. As the magnetic fields are the strongest at the poles, that is where aurora can be observed. Aurora on the North Pole is called Aurora Borealis or the Northern lights while the one visible from the South Pole is called Aurora Australis or the Southern lights.

Progressing further away from earth into the space we learn that over 99 % of universe is estimated to be in the plasma state of matter [1]. The Sun, solar corona and stars are plasmas so hot that every matter would instantly evaporate and fully ionize, thus turn into plasma state. Even intergalactic medium consists of charged particles emitting visible light. In contrast, the rest of the universe is an empty vacuum with nothing but ionized particles and charged dust particles, again by definition making it a plasma.

As shown above further division of both natural as well as man-made plasmas is needed based on the temperature of particles. On the one hand we have thermal plasmas (lightning, sun, stars) and on the other non-thermal plasmas (aurora). As the name suggests, thermal plasmas are associated with high energy and temperatures of particles. Thermal plasmas are also commonly referred to as equilibrium or fully ionized plasmas, meaning all particles are ionized and have the same temperature (up to 10^8 K). When it comes to man-made plasmas, thermal are the ones used for TIG welding, plasma cutting and similar applications demanding high temperatures. On the contrary, non-thermal plasmas also referred to as non-equilibrium plasmas have a lower particle temperature and are only partially or weakly ionized [2, 3]. Neutral species are abundant amongst charged particles. Temperature of electrons, which are a couple of orders of magnitude smaller than ions, can be extremely high (up to 5×10^4 K) yet it will not affect the overall gas temperature (near ambient or slightly elevated). Consequently, such plasmas enable sensitive materials to be treated without thermal damage or without changing the bulk material properties, only treating the surface. For this reason, non-thermal plasmas are interesting for scientists and researchers for numerous applications in many fields.

Non-thermal plasmas are further divided in low and atmospheric pressure plasma. Low pressure non-equilibrium discharges are initiated and sustained by direct current (DC), radio frequency (RF), or microwave (MW) discharge. These plasma sources operate at low-pressure (inside vacuum chamber) since the breakdown electric field is smaller and the current is more controllable. They can generate a large area uniform plasma with a controlled electron density. Non-equilibrium atmospheric pressure discharges can operate in a wide range of temperatures and many configurations described in the next chapter.

1.1.2.1 Cold Atmospheric Pressure Plasma

Cold atmospheric plasma (CAP) is a term that stands for many different configurations of non-equilibrium plasmas operating under ambient conditions. The term cold is used when temperatures are close to room temperatures or a slightly elevated jet far from those of equilibrium plasmas. The main advantage of CAP is the possibility to use them for direct treatment of delicate materials or even living tissues for medical purposes. Dielectric barrier discharge (DBD), DBD-like and atmospheric pressure plasma jet (APPJ) are one of the most commonly used configurations, all consisting of electrical power supply, one or two electrodes and a tube for working gas inlet.

1.1.2.1.1 Dielectric Barrier Discharge

Dielectric barrier discharge (DBD) jets have the highest number of possible configurations. Depending on applications DBD can consist of one or two electrodes as can be seen from Figure 1.1. In case of two electrodes, electrical discharge occurs between them and the gas flow transports excited species in a jet-like manner. On the other hand, when only one electrode is used, a strong magnetic field (Figure 1.1 (b)) or high voltage (Figure 1.1 (d)) is the cause of electrical breakdown of gas. DBD plasma devices can be operated by kHz alternating current (AC) power or by pulsed direct current (DC) power source [4]. In all cases there is no risk of arcing due to dielectric insulation, therefore the treated object can be placed as close to the nozzle as desired. Furthermore, the low power density delivered to a DBD keeps the gas temperature of plasma close to room temperature. These advantages make DBD suitable for treating delicate materials directly. DBD therefore has a great potential in medicine.

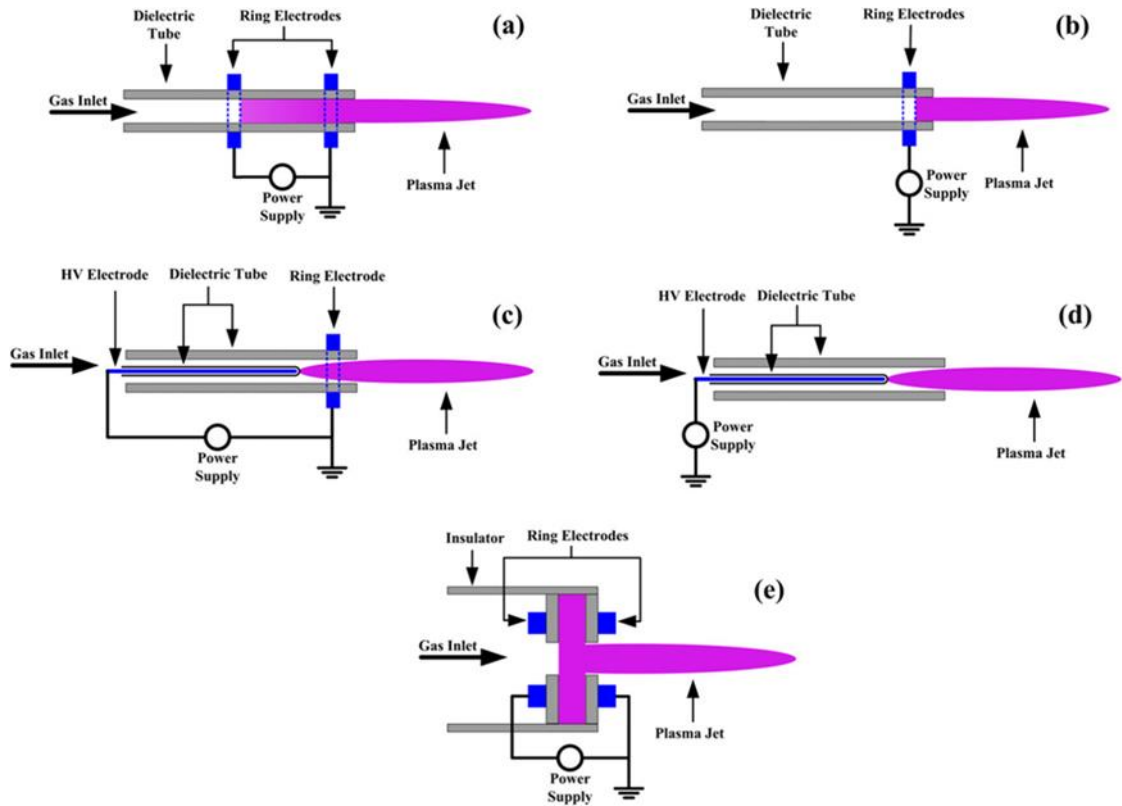


Figure 1.1: Schematics of a DBD plasma jet [5].

1.1.2.1.2 DBD-like Plasma

As the name suggests, DBD-like plasmas have similar configurations as previously described DBD plasma jets. Schematics of DBD-like plasma jets are shown in Figure 1.2. As can be observed from Figure 1.2 (a) and (c), the only difference is that DBD-like plasma has no dielectric tube around the inner electrode. This makes no change in the operation of the jet when plasma plume is not in contact with anything. However, when an object is being treated with a DBD-like plasma jet and the said object is electrically conductive, discharge no longer runs between the electrodes but is diverted between an electrode and conductive object. Object does not have to be a ground conductor but can also remain at floating potential. In this case the name floating electrode dielectric barrier discharge (FE-DBD) often occurs.

DBD-like plasmas can be operated by kHz AC power, by pulsed DC power or by radio frequency (RF) power. The main advantage is that in the case of treating conductive materials that are not too heat or arcing sensitive, more power can be delivered to plasma. In case of medical use, DBD-like plasmas are applicable but less than DBD plasmas where arcing is not an issue.

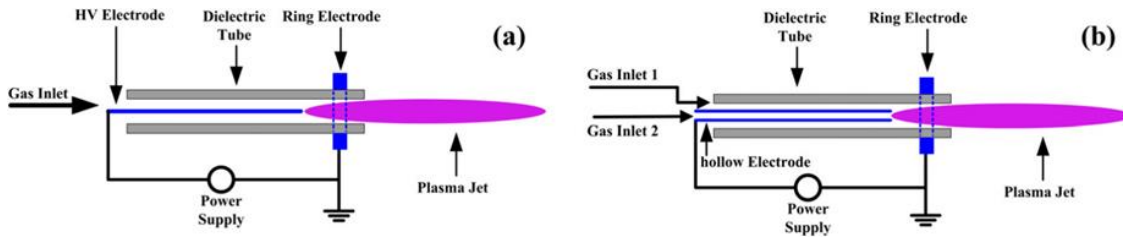


Figure 1.2: Schematics of a DBD-like plasma jet [5].

1.1.2.1.3 Corona Discharge

Corona discharge was the first atmospheric pressure setup to generate non-thermal plasma [6]. It is composed of two electrodes where one is usually planar and the other has a sharp point or edge. Electrodes are connected to a pulsed or continuous DC power source and polarity can be different for different configurations. Asymmetric electrode pair makes the electromagnetic field caused by DC voltage highly inhomogeneous. Highest concentration of electromagnetic field is around the sharp point electrode and this is where gas breakdown occurs creating weakly ionized plasma. This plasma spreads towards the planar electrode expanding over a larger area, as can be seen from Figure 1.3. The use of such corona discharge plasmas is fairly limited due to the two electrodes configuration, risk of arcing and the use of DC voltage with potential harmful effects.

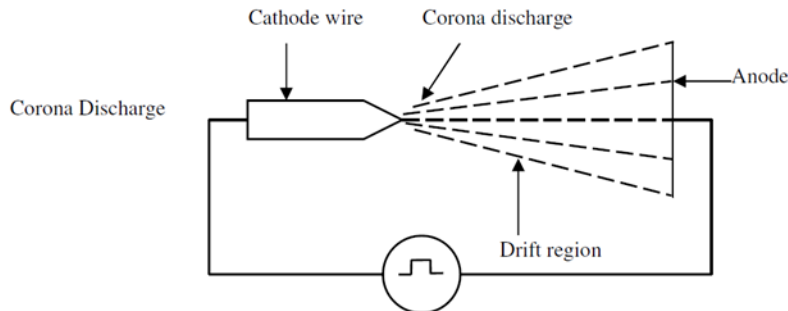


Figure 1.3: Schematics of corona discharge [6].

1.1.2.1.4 Atmospheric Pressure Plasma Jet

Atmospheric pressure plasma jets (APPJ) are also sometimes referred to as single electrode (SE) jets. Their schematics are shown in Figure 1.4 where (a) and (b) are basically simplified DBDs without the outer ring electrode. By removing the ring electrode, the role of dielectric tube is reduced to guiding the gas flow and can even be omitted as shown in Figure 1.4 (c). In this figure, gas flow is guided through hollow electrode. The risk of arcing is present in APPJs and that is why changes in design are needed for APPJ to be used for direct treatment in biomedical applications. In Figure 1.4 (c) a capacitor and resistor with characteristics of about 50 pF and 60 k Ω , respectively, are added into the circuit to prevent arcing [7]. This APPJ configuration is favorable for direct use in medical applications because plasma plume and the hollow electrode itself can be touched without any risk of injury. As previously described jets, APPJs can be operated by kHz AC, DC, pulsed DC or RF power.

For the purpose of research on indirect plasma treatment presented herein, an APPJ presented on Figure 1.4 (a) was used. It was chosen due to its high efficiency when it comes to ionization, due to relatively long visible plasma plume which suggests that plasma device can be further away from the treated sample and due to its design simplicity.

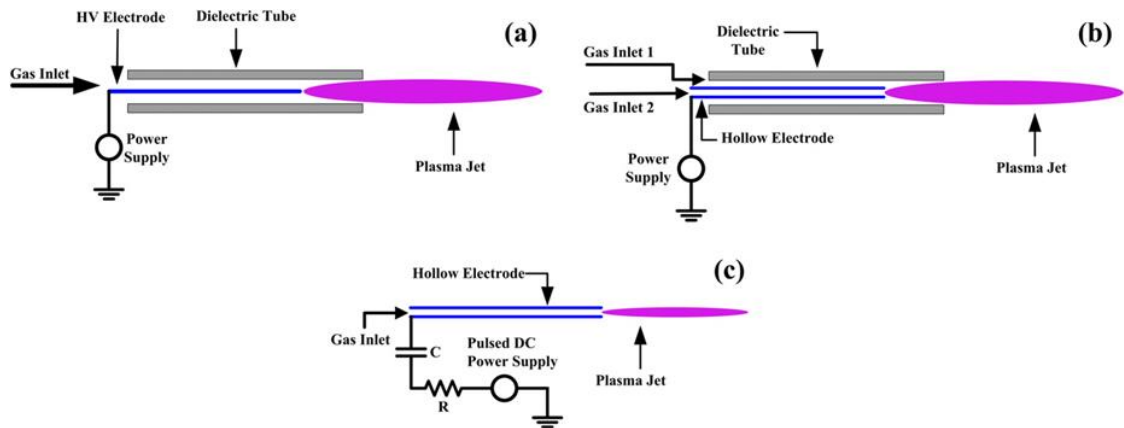


Figure 1.4: Schematic of an APPJ [5].

1.1.3 Plasma Chemistry and Origin of Species

Plasma chemistry is a complex mechanism involving numerous elementary reactions [7–9]. Two main types of reactions are expected, on the one hand homogeneous involving electrons, ions and neutrals in gas phase and on the other hand heterogeneous between plasma species and surrounding surfaces. These typical reactions have been listed in Table 1.1, Table 1.2 and Table 1.3 while their consequences are shown in Figure 1.5. Depending on discharge parameters, surface treated, working gas used and other important factors, thin films can be deposited on the surface, impurities can be removed, and surface can be selectively etched, chemically modified or activated in a fast and ecologically friendly manner. In case of thin film deposition, plasma can be used for cleaning and activating the surface and later for activation of the thin film [10]. Cleaning is performed when there are different oxide layers, protective coatings or similar that has to be removed. Etching can be done selectively (with the purpose of nanopatterning [11]) or unselectively, to obtain nanostructured surface. Activation and functionalization are more of a chemical processes compared to etching which is a combination of chemical and physical process.

Some of the most common plasma species found in the gas phase are ions, electrons and atoms or molecules in neutral or metastable states. Electrons are the driving force of the majority of reactions taking place in the gas phase. They have high kinetic energy and they collide with other species. Collisions result in a change of electric charge and an avalanche of chain reactions. Ions are larger, heavier and slower compared to electrons. They are involved in the most important surface reactions like etching, functionalization and others.

In case of low pressure plasma, gas discharge is confined inside a vessel. Collisions between species and between species and vessel walls are inevitable. Collisions between species can be described as elastic, inelastic or superelastic [8] while the reactions between species and vessel walls are known as surface recombination. Conservation of energy and momentum must be respected in all cases. In case of elastic collision, the momentum is redistributed between particles and total kinetic energy is unchanged. For example, when a light highly energetic electron hits a slower and much heavier atom, only a fraction of its energy is transferred and the collision is elastic due to high differences in mass. If any moving particle strikes another particle with an equal mass that is stationary, it is possible that elastic collision occurs and all of the kinetic energy is transferred. Inelastic collisions differ by loss of initial kinetic energy to internal energy after collision. Momentum is conserved but redistributed. If inelastic collision happens between light and heavy particles, it is possible for lighter particles to lose all of their kinetic energy. Some is transferred in the kinetic energy of heavy particle while the rest is lost to internal energy of both particles. Superelastic collision is the one where there is more kinetic energy after the collision. In this case, internal energy of colliding particles is transformed into kinetic energy while the momentum is still conserved. All surfaces exposed to plasma are subjected to electron impacts and are quickly negatively charged. This results in a boundary layer of positive space charge close to the surface, called the plasma sheath. The result of sheath formation is acceleration of heavy positive ions and slowing down of electron loss. Particles that are nevertheless lost to the surface of plasma vessel wall recombine there and transfer their energy into internal energy of the wall.

At atmospheric pressure, the concentration of particles is much higher and energy dissipation is therefore much faster. In other words, mean free path of charged particles is much smaller. Quenching occurs around the jet and is further increased by turbulent gas flow caused by plasma carrier gas coming into contact with stationary ambient gas. Secondary working gas can be used to protect plasma jet from quenching too fast (Figure 1.4 (b)).

In the experimental part of this work, argon was used as working gas and later on also as shielding gas. The shielding was not done by secondary working gas but with filling up the ambient with argon. The aim was to decrease the quenching effect and prolong the lifetime of plasma activated species so more of them could reach the surface. In the work of France et al. [12] it is suggested that argon plasma is effective at incorporation of oxygen functional groups onto polypropylene surface.

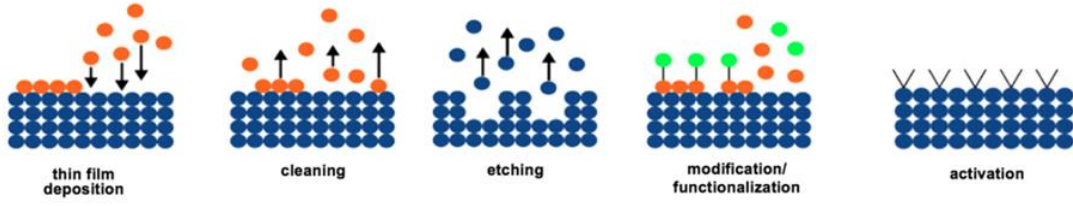


Figure 1.5: Basic categories of plasma chemical interaction with material surfaces [13].

Table 1.1: Plasma surface reactions [8].

Reactions	Description
$AB + C_{\text{solid}} \rightarrow A + BC_{\text{vapour}}$	etching
$AB \rightarrow A + B_{\text{solid}}$	deposition
$e^- + A^+ \rightarrow A$	recombination
$A^* \rightarrow A$	de-excitation
$A^* \rightarrow A + e^-$ (from surface)	secondary emission
$A^+(\text{fast}) \rightarrow A + e^-$ (from surface)	secondary emission

Table 1.2: Plasma gas phase reactions involving electrons [8].

Reactions	Description
$e^- + A \rightarrow A + e^-$	elastic scattering
$e^- + A \rightarrow A^+ + e^- + e^-$	ionization
$e^- + A \rightarrow A^* + e^-$	excitation
$e^- + A^* \rightarrow e^- + A + h\nu$	de-excitation
$e^- + A^* \rightarrow A^+ + e^- + e^-$	two-step ionization
$e^- + AB \rightarrow A + B + e^-$	fragmentation
$A^+ + e^- \rightarrow B + e^-$	dissociative ionization
$A^- + B$	dissociative attachment
$e^- + A^+ + B \rightarrow A + B$	volume recombination

Table 1.3: Plasma gas phase reactions involving ions and neutrals [8].

Reactions	Description
$A^+ + B \rightarrow B^+ + A$ 'resonant' for $B = A$	charge exchange
$A^+ + B \rightarrow B + A^+$	elastic scattering
$A^+ + B \rightarrow A^+ + B^* + e^-$	excitation
$A^+ + B \rightarrow A^+ + B^+ + e^-$	ionization
$A + B^* \rightarrow A^+ + B + e^-$	Penning ionization
$A^+ + BC \rightarrow A^+ + B + C$	fragmentation/dissociation
$e^- + A^+ + B \rightarrow A + B$	volume recombination
$A^\pm + B \rightarrow AB^\pm$	oligomerization
$A + B \rightarrow AB$	oligomerization

temperature under ambient conditions [17]. It has been obvious from the beginning that there cannot be one universal plasma source to fulfil the requirements of all applications. Therefore, multiple plasma sources operating on different principles and with different characteristics were designed. Among them three stand out, atmospheric pressure plasma jet (APPJ) for treatment of small to medium regions, dielectric barrier discharge (DBD) for treatment of larger regions and corona discharge for treatment of narrow cavities. One of the best known commercially available DBDs, that is aiming to standardize the direct plasma treatment in medicine in the kINPen. It is the first plasma source to conform to DIN-SPEC 91315 (German Institute of Standardization), covering general requirements for medical plasma sources and their safe and effective use [18].

Common fields of research using direct plasma treatment in medicine are chronic wound healing [19, 20], tissue engineering and regeneration [21], skin disease treatment, cancer treatment [22, 23] and various dental applications varying from teeth whitening [24] to root canal treatments [25]. All of the above suggest that plasma medicine is a fast growing and highly interdisciplinary field of research, connecting plasma physics, chemistry, biology, medicine and technology. Each of these fields is complex and presents a major challenge, thus combining them can only result in more challenges and complexity. For plasma physics, characterization of plasma parameters and detailed understanding of physical background is of crucial importance to ensure a proper biologic response and a highest possible safety level for patient as well as the doctor using it. The main physical parameters are working gas purity, working gas humidity, constant gas flow, gas and plasma temperature, operating distance and plasma treatment duration. Chemical parameters such as pH value, ion concentration, antioxidative potential, the amount of reactive oxygen and nitrogen species (ROS and RNS) are of importance as well as biological parameters such as cell density, cell to volume ratio and cell culture media [26]. Some of these are presented in Figure 1.7. Parameters concerning the safety of plasma devices, which should be thoroughly tested and eliminated as possible risk factors are:

- Plasma temperature, which is not supposed to exceed the 40 °C threshold tolerable for living tissues. Jets with slightly higher temperatures can still be used but have to be placed further away, not be used locally but move over a larger area shortening the contact time or have an appropriately variable on-off intervals of operation.
- UV radiation, measured by optical emission spectroscopy, highly dependent of on the working conditions and design of plasma device. UV-C (≈ 254 nm) should be avoided as it has a direct impact on DNA while UV-A (320-400 nm) and UV-B (280-320 nm) are not harmful as long as maximal exposition time is taken into account.
- Toxic gases generation, atmospheric pressure plasmas are known to generate reactive oxygen and nitrogen species, especially ozone (O_3), nitrogen monoxide (NO) and nitrogen dioxide (NO_2). NO is a short-lived species that oxidizes into NO_2 instantly. NO_2 and O_3 are both air pollutants with possible carcinogenic effects. The allowed dosage of both is expected not to be exceeded during relatively short therapeutic plasma treatments. Nevertheless, gases emerging from such a medical plasma device should be carefully exhausted to assure the patients' wellbeing and to avoid exposure of medical personnel on the long run.
- Electromagnetic fields caused by plasma sources, every plasma source is operating at a specific frequency and electromagnetic fields occur inevitably. This should be taken into account and precautions should be made to avoid electromagnetic fields inducing electrolysis in the tissue and/or stimulating nerve or muscle cells. One of the best ways to resolve this issue is to use a Faraday cage for the plasma source.
- Electric current, in applications where electric current flows through the body, it should be limited to 25 mA at 100 kHz as well as at 1 MHz. This was set by the International

Commission On Non-Ionizing Radiation Protection (Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields).

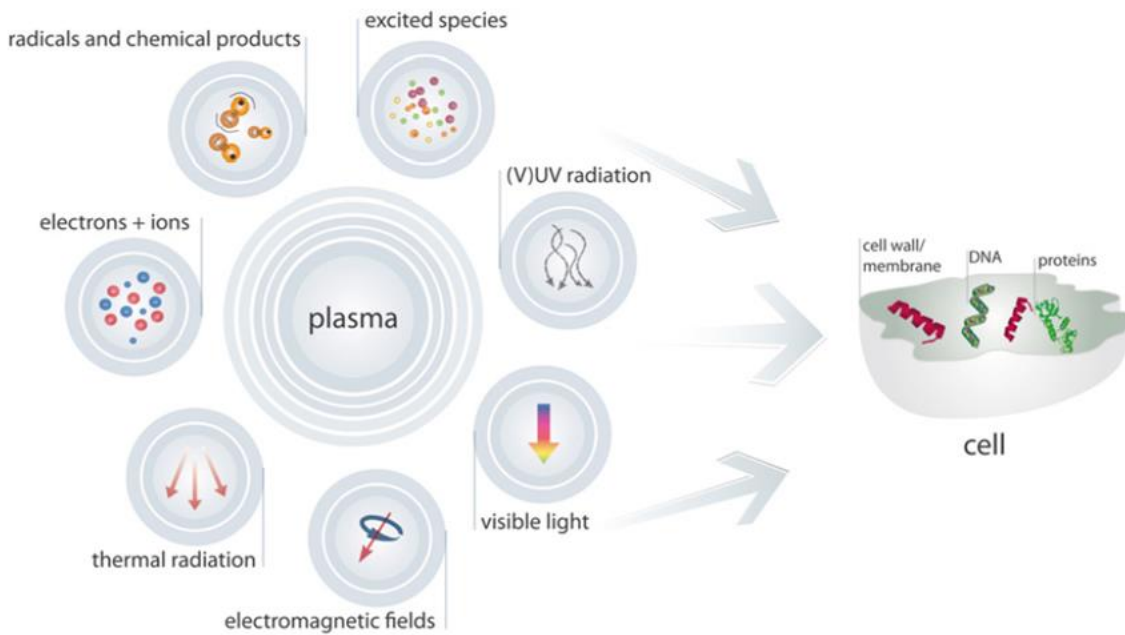


Figure 1.7: Plasma cell interaction, two complex systems [13].

1.2.2 Indirect Plasma Treatment

Indirect plasma treatment in medicine is a huge well-established field of science with numerous applications ranging from improving biocompatibility of implants to decontamination of medical tools. It is called indirect because plasma is not in direct contact with living cells, tissues or organs. Compared to direct plasma treatment, indirect is much more easily applicable and there are less safety requirements to be considered. It is still interdisciplinary but offers every domain to be studied separately.

Materials used in medicine have to be biocompatible. Biocompatibility is the term describing different materials in contact with human body (in the context of present work, especially blood). The body responds to different materials in a different manner and these interactions are often highly important for clinical success of medical treatment. Biocompatibility can be achieved by introducing amino group (NH_2), carboxyl group (COOH), hydroxyl (OH) or other active groups onto material surface by indirect plasma treatment [27]. Appropriate functional groups are incorporated in order to achieve the desired biological response for the desired application. Indirect plasma treatments are often being used for improving biocompatibility of blood vessels, implants and different stents used in modern medicine. Usually oxygen, ammonia, nitrogen, hydrogen or their mixtures are used to provide a certain concentration of NH_2 , COOH , OH on polymer surface. These groups are biocompatible and polar, which means they are active and excited to react with other polar molecules. Other known indirect plasma treatment methods are plasma ion implantation, plasma immersion ion implantation and deposition [28–30], but are not described herein. All of the abovementioned indirect plasma treatments improve surface biocompatibility with bulk material attributes preserved. Aside from surface chemistry, surface charge, surface free energy and surface nanostructure also play an important part

in biocompatibility. Dowling et al. [31] suggest that higher surface roughness increases cell adhesion but has a negative effect on the spreading of cells.

A lot of attention has lately been focused on therapeutic treatments with plasma activated mediums. It was reported that the medium activated by plasma with ultrahigh electron density can specifically target cancer cells and cause their apoptosis [22]. After the medium is treated with plasma, it is introduced to cancer cells or tissues and their proliferation is decreased. Direct plasma treatment has a similar effect but is much more invasive and not applicable in the case of disseminated cancers. The exact mechanism behind this therapeutic effects of plasma-activated medium is currently not known but the results of *in vivo* and *in vitro* experiments are promising.

One of the first uses of indirect plasma treatment in medicine was plasma decontamination and sterilization of medical tools and accessories [32]. With decrease of glass and metal and increase of polymer-made medical equipment the use of heat treatments for sterilization is now out of the question. Alternative sterilization technique appeared in the form of plasma ionized hydrogen peroxide (H_2O_2) which has antimicrobial properties. This sterilization technique is approved for medical use but requires a relatively long time and vacuum equipment as it is not atmospheric pressure based. Furthermore, plasma sterilization is not applicable to all materials and devices used in medicine. Hence there is still room for improvement.

1.2.2.1 Plasma Treated Polymers

Use of polymers has massively increased in the last decades. As their use diversified rapidly, more and more problems occurred [33]. Many industries solved their problems by using the CAP treatment in different forms. CAP is easily applicable and relatively cheap to incorporate in production lines to tailor surface properties of polymers. Lately polymers found new applications in medicine and plasma treatment has again proved to be indispensable. Lots of glassware was replaced by disposable plasticware, causing biocompatibility issues [27], leaching compounds into stored samples [34], surface interaction with delicate samples and similar. CAP is here to change the surface energy, biocompatibility, wettability and morphology making plasticware more cost effective and user friendly.

1.2.2.1.1 Nanoscale Surface Topography

Surface topography of polymers changes with plasma treatment. Reactive species in ionized gas cause chemical and physical surface modifications like etching and ablation. Important role in surface topography changes is played by the crystalline and amorphous phase of which the polymer consists. These two phases have slightly different densities and etch with different rates [35]. The result is increasing of surface roughness in the first stages of plasma treatment. Specific bulb-like (also known as globules [36], spherulitic features [37], etc.) structures occur on the polymer surface and grow until a certain limit is reached. After that the bulbs are presumably etched due to their height and the roughness of the sample surface drops again. For this reason, topography of plasma-treated surfaces needs to be systematically studied. Atomic force microscopy (AFM) is the preferable method for surface topography evaluation. It is also important to state that various manufacturers use diverse raw materials, fillers, manufacturing tools and processes resulting in various surface structures and topography. This has to be taken into consideration when comparing results after plasma treatment. Another important aspect when surface topography results are compared amongst researchers is the area on which the topography was measured. Average surface roughness is decreasing with decreasing surface area of AFM measurements [38, 39].

Therefore, only similar size area roughness can be compared. Areas between $1 \times 1 \mu\text{m}$ and $10 \times 10 \mu\text{m}$ are commonly used in literature.

1.2.2.1.2 Surface Functionalization

A variety of functional groups can be introduced onto polymer surface by plasma treatment. By choosing an appropriate working gas or a mixture of gases one can selectively change surface properties of polymer with its bulk characteristics preserved. Most commonly used gases are inorganic gases like O_2 , H_2 , N_2 , F_2 , He , Ar , CO_2 , SO_2 and NH_3 . Each has its own characteristics influencing plasma parameters and stability. Generally, these gases are divided into those which elevate or lower surface free energy. When plasma-induced dissociation of gases is effective, plasma treatment times are short and provide high intensity of charged plasma species. Hence polymer surfaces are saturated with functional groups rather quickly.

There are always residual atmospheric gases present. In case of low pressure vacuum systems some residual gas is found on reactor walls no matter how low is the pressure and how long the system is being pumped. In case of CAP this effect is much more pronounced as the whole plasma jet is exposed to different gases present in the atmosphere. Atmospheric gases are indirectly excited through plasma species and also react with the polymer surfaces during the treatment. This is sometimes beneficial as the CAP is performing best with inert gases such as helium and argon, but oxygen functional groups are needed on the surface to increase surface free energy. Obvious drawback is that atmospheric gases cannot be controlled, their composition varies and some unwanted functional groups might be formed. For this reason, many research groups use systems with a controlled atmosphere.

1.2.2.1.3 Wettability and Surface Free Energy

Wettability of polymer materials is closely related to their surface free energy, functionalization and morphology [40]. It is one of the fastest and simplest ways of monitoring the impact of plasma treatment on polymer surface. Therefore, it is the most commonly used indicator of plasma treatment effects. The CAP plasma treatment with helium and argon in air results in a higher surface free energy and lower water contact angle, and hence better wettability of polymers [41–43] and enhanced biocompatibility, cell growth, and proliferation. The noble gases are also preferred due to more stable plasma jet operation and ignition of plasma at lower input powers. If fluorine, fluorine containing or similar working gases are used, resulting surface has a lower surface free energy than prior the treatment. This directly results in increased surface contact angles (decreased wettability).

The method is based on placing a droplet on top of the surface and measuring the contact angle between solid, liquid and gas interface. Different size droplets can be used depending on the sample size and roughness but the same size is preferable when results are being compared. Deionized water is most commonly used while diiodomethane, ethylene glycol, thiodiglycol, formamide, ethane-1,2-diol, alpha-bromonaphthalene and other solvents are also often mentioned, especially when an information on surface free energy is wanted. In such case, surface free energy is calculated by one of the proposed theories. Some of these theories require the use of multiple solvents and are based on comparison of contact angles among different solvents with known surface tension. Contact angles of solvents can be measured by a special software, taking a picture of droplet and manually or automatically obtaining the result. Other known techniques involving automated wettability measurement are advancing and receding contact angles [44]. It has been discovered that hysteresis between advancing and receding contact angles is scientifically

important. In the case of advancing contact angle, droplet is filled with liquid at a constant rate and the changes in contact angle are observed simultaneously. Receding is the opposite, after the drop is placed, the liquid is extracted from it at a constant rate while contact angle changes are monitored.

1.2.2.1.4 Ageing of Plasma Treatment Effects

Plasma treatment of surfaces is known to change with time and that phenomenon is called ageing of plasma treatment effects [45]. The surfaces like to return to equilibrium state after they have been subjected to strong non-equilibrium plasma modifications. The ageing can be monitored via contact angle and X-ray photoelectron spectroscopy (XPS) measurements. Two fundamental factors play a crucial role in the process of reverse modification. The first is surface topography and this was shown to remain constant and does not change after plasma treatment. The second factor is surface chemistry, and it is obviously the one to be blamed for the ageing effects. In some cases, it was reported that samples aged in a matter of minutes while in other cases plasma treatment effects are reported to be present after 6 months [46].

1.3 Microvesicles for Medical Diagnostics

1.3.1 Origin of Microvesicles

Microvesicles (MVs) are cell derived particles involved as mediators in intercellular communication and found in all body liquids [47, 48]. MVs contain biological information about the cell of origin in the form of various proteins, lipids, sugars and genetic material (DNA, miRNA). They belong to the group of extracellular vesicles (EVs) which also includes exosomes and apoptotic bodies. The term exosome was first used by Trams et al. [49] and extracellular vesicles were described by Pan et al. [50]. Initially EVs were thought to be a disposal mechanism of unwanted cell material, their importance in cell to cell communication was subsequently discovered. For a number of years, the nomenclature was not unified and a variety of terms was used to describe EVs. The controversy around the size and nomenclature still exists but basic requirements for EVs were set by the International society for extracellular vesicles [51]. The size of exosomes is expected to be between 30 and 150 nm while microvesicles range from 100 to 1000 nm and apoptotic bodies up to 5000 or even 10000 nm. Apart from size they can be separated by their origin. Exosomes are derived from endosomal membrane by inward budding and are released by fusion to the plasma membrane. Microvesicles on the other hand are formed by outward budding and fission of the blood plasma membrane shown in Figure 1.8, where the acronyms CCV and MVE stand for clathrin-coated vesicle and multivesicular endosome, respectively. Apoptotic bodies are far less interesting as they are the result of programmed cell death. Nevertheless, apoptotic bodies cannot be overlooked as they can be mistaken for MVs if not evaluated properly.

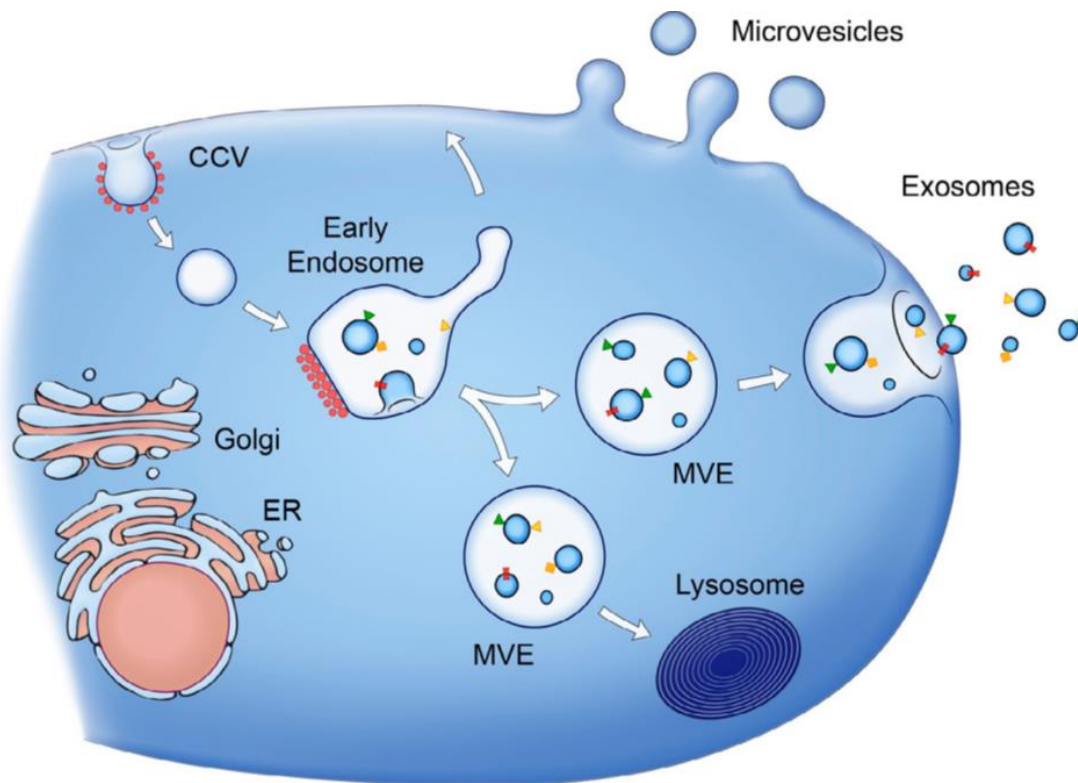


Figure 1.8: Release of MVs and exosomes [52].

1.3.2 Microvesicle Diagnostics and Treatments

Microvesicles are promising biomarkers for some of the most common and widespread diseases [52–54]. Some of these are metastasis of cancer, diabetes, coronary diseases, inflammatory diseases, neurologic diseases, atherosclerosis and other presenting a major health issue for our fast ageing society. The expenses of treating such diseases are high while the success rates are not. The main problem lies in the fact that diseases are diagnosed too late and already mature to the point where drugs can only mediate their effects. Early stage diagnosis is therefore suggested and microvesicles are one of the most promising alternatives at the moment. Due to their involvement in intercellular communication they carry important bioactive molecules such as various proteins, lipids, sugars and genetic material (DNA, miRNA) and play an important role in physiological and pathological pathways inside our body, including disease progressions [55–57]. The first information about disease can be an increased amount of microvesicles found in one of body liquids, preferably vein blood. Furthermore, a lot of information on the cell of origin can be obtained by analyzing the bioactive cargo carried by microvesicles. This real time diagnostic is not only relatively fast and precise but also uninvase and can be performed routinely every time we visit the doctor. The scientific community is working on that and advance was made but standardization of isolation protocols and databases for MV characterization are still not available. It is important to isolate and purify microvesicles carefully as they are not abundant and high concentrations are needed for precise diagnostics. Apart from microvesicles small size, their isolation is impaired by the fact that they are likely to interact with surfaces, especially those of medical tools used for collection, sampling, transport and storage. This results in considerable loss of MVs to surfaces or can cause enough damage to the MVs so that the diagnose precision is poor.

Another promising field of research concerning microvesicles is treatment of patients after they have been diagnosed. The majority of diseases are spread through the body by infected biological material carried by microvesicles. The general idea is therefore to either purify the existing microvesicles or replace them by healthy ones. This approach has given good results, especially in antitumor therapies. Microvesicles were pulsed with tumor antigens in a clinical trial and improvements were observed in some patients [58, 59]. The most important is that proof of concept was successful and such therapeutic treatments are feasible and safe.

1.3.3 Isolation of Microvesicles from Body Liquids

Microvesicles were shown to be present in all body fluids, the most commonly found in literature are MVs isolated from blood plasma, serum, saliva [60], urine, cerebrospinal fluid, breast milk [61], uterine fluid, nasal fluid and amniotic fluid [62]. Among these blood plasma is the best source of MVs. It is necessary to isolate and purify them to get high enough concentrations for further analysis. The isolation processes differ greatly among research groups [63]. The gold standard of MV isolation seems to be the process of centrifugation and ultracentrifugation steps, where supernatant is discarded and pellet resuspended in phosphate buffered saline (PBS) multiple times [64]. MVs are isolated and purified by sedimentation during centrifugation process. At low centrifugal forces, apoptotic bodies and dead cells are eliminated. At medium centrifugal forces debris and larger vesicles are removed. At high centrifugal forces MVs are precipitated into pellet concentrated at the bottom of the centrifugation tube. This pellet can be analyzed as is or can be further washed by PBS and centrifuged to further remove contaminations. Another step that can further improve isolation is sucrose gradient centrifugation which is based on different flotation densities. With sucrose gradient centrifugation exosomes can be separated from microvesicles but not from viruses due to similar densities and sizes. Another technology mentioned in literature is microfiltration [65, 66], microfluidic devices [67] and antibody-coated magnetic beads [68, 69]. Each of these have its own pros and cons. Microfiltration replaces the first centrifugations at low speeds but faces challenges of contamination and trapping of MVs during the process. Microfluidic devices offer new ways of microscale separation with fluid flow being directed by frictional forces, large area-to-volume ratio and low Reynolds numbers keeping the laminar flow. The advantages offered by such devices are small sample sizes, reduced costs and higher throughput. The main disadvantage is that this technology is not yet applicable to larger amounts of samples. Antibody-coated magnetic beads technique works on the principle of binding of microvesicle specific proteins with specific markers to isolate them. This was found to be more efficient than centrifugation and density gradient separation [64] but is again not suitable for larger sample volumes and may cause MVs to lose their functionality.

The aim of herein presented research work is to improve the process of microvesicle isolation from body liquids and help microvesicle diagnostic techniques and therapies to advance. By cold argon plasma treatment of polypropylene tube surfaces that come in contact with microvesicles during centrifugation, we tend to decrease contact surface by nanostructuring and decrease shear stress by increasing surface free energy. We believe this should result in higher microvesicle isolation yields.

Chapter 2

Aims and Hypotheses

The aim of this thesis is to alter surface characteristics of polypropylene tubes' inner surfaces with cold atmospheric pressure argon plasma in order to improve either microvesicle isolation procedure or to improve other biomedical applications associated with proteins. To achieve such improvements, cold atmospheric pressure plasma source must be characterized, material characteristics of tubes must be well known, the interaction between plasma and tube surface must be studied and finally the plasma treatment of long narrow tubes optimized. Further on, it is of crucial importance that plasma treatment is uniform across all inner surface of the tube and the treatment must be perfectly repeatable. The best way to ensure that is to construct an automatic device for treatment of multiple tubes by cold atmospheric pressure plasma. Such a device should therefore be constructed and tested in order for successful commercialization to be possible.

The hypotheses are the following:

- Cold atmospheric pressure argon plasma can be used to modify inner surfaces of polypropylene tubes in order to alter their surface characteristics (like wettability, morphology, surface chemistry etc.) and prevent destructive contact with biomedical samples like microvesicles or proteins.
- Inner surfaces of tubes can be evenly treated by cold atmospheric pressure argon plasma to ensure repeatable characteristics across all surfaces.
- An automatic device for cold atmospheric pressure argon plasma treatment can be designed and optimized to make the treatment of multiple long narrow tubes fast, reliable and repeatable.
- Cold atmospheric pressure plasma-treated polypropylene tubes exhibit superior characteristics in the microvesicle isolation process compared to the untreated ones, resulting in increased microvesicle yields.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Polypropylene Tubes

Polypropylene microcentrifuge tubes are widely used for sample preparation and storage. Polypropylene offers a unique high strength and low weight ratio and is one of the most common polymers. It is also recyclable which makes it even more suitable for mass use. First polypropylene microcentrifuge tubes were made and patented by Eppendorf company in 1963. Sometimes they are referred to as Eppendorf tubes or short “Eppis”. They have revolutionized the laboratory work together with other tools, such as piston pipettes, mixers and centrifuges involved in the microliter system. Today they are considered a golden standard in laboratory work and various manufacturers are producing them worldwide. Most commonly used is a transparent 1.5 mL conical tube with a snap-cap.

Standard polypropylene tubes from various manufacturers were ordered for the purpose of this thesis. Such tubes are a part of the microliter system and are common consumables in all biomedical laboratories. They are transparent and have a volume of 1.5 mL with engraved measuring marks on the side. The typical conical shape of the tubes bottom enables easy handling and storage in specially designed racks. Another feature of these tubes is a snap-cap, a lid that is attached to the tube and is used to close it and prevent sample loss. It is made to close with a carefully designed shape and size tolerance of tube and cap. All of the above makes these tubes suitable for ultracentrifugation, which is a crucial step in microvesicle isolation. Untreated or plasma treated, these tubes are designed for one-time use only.

After preliminary testing, tubes from three manufacturers were chosen on the base of wettability, morphological and chemical differences. To make labelling, tracing and result presentation easier and transparent, tubes were renamed as follows:

- **Sample 1**, AHN Biotechnologie GmbH (Nordhausen, Germany), reference number 3-207-50-0, LOT number P-162150.
- **Sample 2**, Ratiolab GmbH (Dreieich, Germany), article number 5615000, LOT number 16A0993.
- **Sample 3**, Eppendorf GmbH (Hamburg, Germany), model DNA LoBind, catalogue number 022431021, LOT number G174706P.

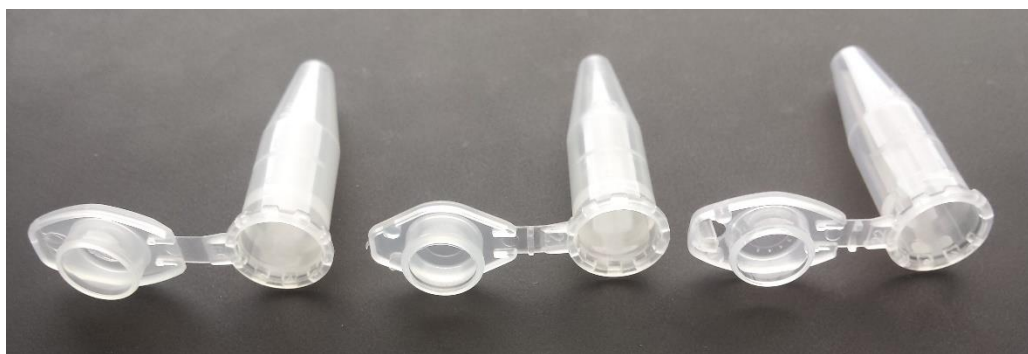


Figure 3.1: From left to right, Samples 1, 2 and 3.

In Figure 3.1 visual differences between tubes can be observed. These are mostly limited to the shape of the cap and the deformable connection it has to the tube. Each tube behaved differently when it was being cut with the scalpel for inner surface analysis. Sample 1 was the hardest to cut through, Sample 2 was the softest and Sample 3 was hard but brittle when cut through. There are also slight differences in weight that can be seen in Table 3.1. This was measured by a scale (Denver Instrument Company, Arvada, USA) with 0.1 mg precision. The same scale was also used to measure tubes after plasma treatment but as expected no weight change was detected.

Table 3.1: Average sample mass.

	Mass (mg)	Standard deviation (mg)
Sample 1	948.0	5.4
Sample 2	1019.3	2.5
Sample 3	1002.0	2.9

3.1.2 Polypropylene Foil

Foil of pure grade polypropylene (PP) was bought to make experiments in preliminary stage faster and easier from the measuring point of view. The foil was transparent, square shaped (30 x 30 cm) and 0.05 mm thick, supplied by GoodFellow Cambridge Ltd. Batch number 300710408 produced on 18 November 2016.

Polypropylene foil was used for preliminary experiments, it was cut into square pieces and rolled inside the tube. After plasma treatment it was removed and flattened. Water contact angles on PP foil were measured on 5 different areas, from top to bottom as shown in Figure 4.10. At this point, tubes were treated in horizontal position in a stationary mode, meaning that the plasma jet was placed at the top of the tube and remained there the whole treatment time.

3.2 Methods

3.2.1 Plasma Treatment of Tubes

Atmospheric pressure plasma jet (APPJ) with a single electrode was used for treatment of PP. It was powered by a commercial 25 kHz high voltage alternating current electrical source made by Voltcraft, model VLP-2403 PRO. Plasma jet output parameters were set to 2.5 kV and 3 mA. The electrode was a 0.1 mm diameter copper wire inside a 60 mm

long quartz tube with an inner diameter of 1.2 mm. Argon was used as working gas and was leaked through previously mentioned quartz tube at 2 slm with a flow controller (Bronkhorst, model MV-304). Argon 4.8 (99.998 % purity, can contain up to 4 ppm of O_2 and 3 ppm of H_2O) was obtained from Messer in a large gas cylinder containing 18 kg of compressed argon. Atmospheric pressure plasma works best with inert gases. Argon was chosen over helium due to its lower price and subsequent higher applicability in industry or mass use in laboratories.

Preliminary testing was done in a stationary mode where the distance between plasma jet and treated sample remained constant. Later on, a dynamic treatment was adopted where plasma jet was mounted on top of the linear stage while the tube was coaxially fixed inside an enclosed container shown in Figure 3.2. The container was prefilled with argon for 30 seconds prior to treatment, to ensure a controlled atmosphere. During the treatment, the plasma jet was travelling in and out of the tube five times, complete treatment time being approximately 15 seconds.

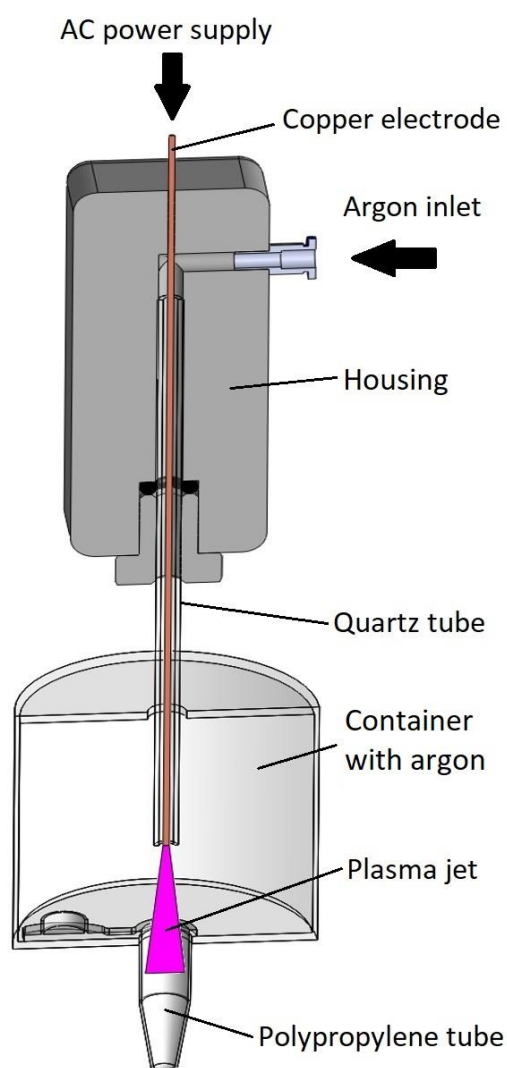


Figure 3.2: Schematics of plasma treatment.

3.2.2 Contact Angle Measurements and Surface Free Energy Calculations

Wettability of inner surfaces of tubes was measured by Advex Instruments See System E equipped with a CCD camera connected to a personal computer (Figure 3.3). For this purpose, plasma-treated tubes were cut in half along the symmetry axis. Afterwards a 2 μL droplet of liquid was placed on the inner tube surface with an Eppendorf pipette and a high resolution image was taken. Each measurement was repeated on at least 6 different spots of the surface and the CAs were determined by the See System 6.3 and 7.0 software with a three-point fitting method. Three different liquids were used for CA measurements to determine surface free energy, each on a fresh sample. The first was demineralized and deionized water, the second was diiodomethane (99 %) from Sigma-Aldrich and the third was ethylene glycol (99.8 %) also from Sigma-Aldrich. All liquids were freshly prepared or opened directly before use and any contamination was carefully avoided. The combination of these three solvents was chosen due to their differences in polar and dispersive component (Table 3.2) on the base of which surface free energy of a solid can be calculated. Acid-base model was used for calculating the surface free energy with See System 6.3 and 7.0 software. Calculations show the value of total surface free energy and its polar and non-polar (dispersive) component together with acid/base polarity (electron-receptor/donor) component. All of them are given in mJ/m^2 units, the symbols and explanations are listed here:

- σ total; is the total surface free energy.
- σ LW; is Lipschitz-van der Waals (non-polar) component of surface free energy.
- σ AB; is Lewis acid-base interaction (polar) component of surface free energy.
- σ +; is acid polarity (electron-acceptor) component of surface free energy.
- σ -; is base polarity (electron-donor) component of surface free energy.

Table 3.2: Liquids used for surface free energy measurements [70].

Name	Formula	Surface free energy (mJ/m^2)				
		Total	Dispersive	Polar	Acid	Base
Water	H_2O	72.8	22.6	50.2	25.5	25.5
Diiodomethane	CH_2I_2	50.8	49.0	1.8	0.01	0.0
Ethylene Glycol	$\text{C}_2\text{H}_6\text{O}_2$	48.8	32.8	16.0	3.0	30.1

Sample curvature was neglected based on the small size of droplet and comparative nature of results (all samples have the same curvature). It should be kept in mind that due to curvature these results are not directly comparable to other researches where flat samples are used. At least 6 measurements were performed for each untreated and treated tube to minimize the statistical error. CAs on treated samples were measured within one hour after plasma treatment as that is also the time in which they are used for microvesicle isolation.



Figure 3.3: WCA equipment and measurements.

3.2.3 Atomic Force Microscopy

Changes in surface morphology and topography of polypropylene tubes were analyzed by atomic force microscopy (AFM, Solver PRO, NT-MDT, Russia) in tapping mode in air (Figure 3.4). Morphology stands for qualitative evaluation of surface shape whereas topography refers to quantitative feature dimensions such as roughness. The AFM device used is shown in Figure 3.4. Samples were cut in small pieces (approximately 3×3 mm), fixed on an aluminum holder and inner surface was scanned by a standard Si cantilever with a force constant of 22 N/m and at a resonance frequency of 325 kHz. Cantilevers' tip radius was 10 nm, the tip length was 95 μm and the scan rate was set at 1.3 Hz. Every measurement was repeated at least 5 times. On several occasions average surface roughness was measured on three different positions along the tube; at the top, middle and bottom of the tubular part with constant diameter, to confirm that plasma treatment is even. AFM measurements could not be performed for the lower, conical part of the tube due to large sample curvature.



Figure 3.4: AFM device and support systems.

3.2.3.1 2 and 3 Dimensional Morphology

Morphological features were closely examined from 2D and 3D AFM images taken on 10 x 10, 5 x 5, 2 x 2 and 1 x 1 μm^2 areas of untreated and plasma-treated tubes. On the untreated inner surfaces of tubes we can observe traces from the manufacturing process, like tool marks and random irregularities. On the other hand, etching effects can be seen on plasma-treated tubes, which is highly time dependent. The Nova AFM software was used for image processing, sample curvature was undone by second order polynomial fitting function for all images.

3.2.3.2 Surface Roughness (Topography)

Multiple surface roughness parameters were measured from representative images on 10 x 10, 5 x 5, 2 x 2 and 1 x 1 μm^2 areas with the Nova AFM software. The AFM device is using the cantilever to scan profile line after line and then interpolate points between lines to connect them into a surface. This makes surface roughness measurements highly precise compared to profilometers. There are many ways of expressing the surface roughness, one of the most popular are average (arithmetical mean deviation) surface roughness, root mean square (RMS) surface roughness and the average distance between highest peak and lowest valley, presented below:

- S_a ; average surface roughness

$$S_a = \frac{1}{A} \iint_A |Z(x, y)| \, dx dy \quad (3.1)$$

- S_q ; RMS surface roughness

$$S_q = \sqrt{\frac{1}{A} \iint_A Z^2(x, y) \, dx dy} \quad (3.2)$$

- S_z ; maximum distance between the highest peak and the lowest valley in defined area

$$S_z = \max(Z(x, y)) - \min(Z(x, y)) \quad (3.3)$$

3.2.3.3 Microvesicle Detection

In order to confirm the presence and assess the quality of microvesicles in isolates from flow cytometry (Chapter 3.2.10), AFM analysis was performed. Due to their small size and delicate nature, microvesicles are a challenge for most techniques. Since the roughness of tube inner surfaces was much higher than the average microvesicle size, it was impossible to analyze them directly on tube inner walls. It was decided that after centrifugation in different tubes microvesicles should be put on “mica”, which is a silicate mineral with perfect hexagonal sheet-like arranged atoms and can easily be split into multiple flat layers. The main advantage is that such freshly split surface is perfectly flat and clean and therefore offers a superior platform for AFM measurements of nano-sized biological samples. The preparation of samples for AFM consisted of:

- Fixation of mica onto AFM sample holder by two-side adhesive tape.
- Removal of upper layer of mica by adhesive tape to ensure microvesicles are placed on a fresh, clean and undamaged layer.
- Carefully pouring the isolate with microvesicles on mica, at this point there was approximately 10 μL of each sample left after flow cytometry and DLS analysis.
- Letting the isolate incubate with mica surface for 30 minutes.
- Gently wash with deionized water to remove weakly attached microvesicles.
- Analyze the surface 30 minutes after washing to prevent the formation of artefacts from drying of the microvesicles.

3.2.4 Tensile Strength Measurements

Tensile strength of polymerized mixture inside polypropylene tube was measured to get an insight into surface modification of inner tube surfaces (uniform nanostructuring and chemical modification). The assumption was that higher and more uniform roughness should result in higher contact surface between polymerized material and tube and therefore in higher tensile strength. Moreover, newly formed functional groups also enable chemical bonding between monolith and tube surface. This experiment was conducted only on Sample 1 type tubes, untreated and plasma treated (2, 3, 5, 10, 15 and 20-times). Each treatment and measurement was repeated in 3 parallels.

The first step after plasma treatment was polymerization. Polymerization mixture consisted of glycidyl methacrylate as monomer, ethylene-glycol dimethacrylate as crosslinker, porogens cyclohexanol and dodecanol as pore forming agents and initiator benzoyl peroxide [71]. In the center of polymerized material, a hook bolt (with a diameter of 4.5 mm and 35 mm long) was immersed extending throughout the mixture. Bolt dimensions allowed the vial to be closed before polymerization and also fixing the bolt in its position and using it for tensile strength measurements later on. Polymerization temperature was 68 °C to provide fast polymerization reaction [72] and in this way to benefit from the presence of active species on inner tube surface formed by plasma treatment. Polymerization was allowed to proceed overnight in water bath. Tubes were cooled to room temperature and the cover was opened afterwards.

The second step consisted of measuring mechanical properties, tensile strength and strain at brake, using a calibrated measuring device Zwick Roller Z100 (Zwick Roller AG, Ulm, Germany). The tube was mounted onto the holder that enabled pulling of polymerized material by inserted hook bolt, serving as anchorage (Figure 3.5). The preload was achieved with the speed of 0.5 mm/min and was 0.5 N. The testing procedure was set at the speed of 1 mm/min and ended when the pulling force decreased to 80 % of the maximum achieved force. The tests were performed on 3 parallels and the mean values were reported. The temperature during measurements was 23 °C and the relative humidity 50 %.



Figure 3.5: Tensile strength measuring setup.

3.2.5 X-Ray Photoelectron Spectroscopy

Inner surface functionalization of plasma-treated polypropylene tubes was determined by X-ray photoelectron spectroscopy (TFA XPS, Physical Electronics, Munich, Germany) shown in Figure 3.6. Freshly plasma-treated tubes were cut in smaller pieces (3x3 mm) and put inside XPS on a holder containing 4 samples at the time, within an hour after treatment. The samples were excited with monochromatic Al $K\alpha_{1,2}$ radiation at 1486.6 eV over an area with a diameter of 400 μm and approximately 5 μm in depth. Minimal depth is desirable because plasma is known to modify only the top surface layer and preserves bulk material characteristics unchanged. Photoelectrons were detected with a hemispherical analyzer positioned at an angle of 45° with respect to the normal of the sample surface. XPS survey spectra were measured at a pass energy of 187 eV using an energy step of 0.4 eV, whereas high-resolution spectra were measured at a pass energy of 23.5 eV using an energy step of 0.1 eV. An additional electron gun was used for surface neutralization during XPS measurements. All spectra were referenced to the main C1s peak of the carbon atoms, which was assigned a value of 284.8 eV. Survey spectra was taken for carbon peak for every sample. The measured spectra were analyzed using MultiPak v8.1c software (Ulvac-Phi Inc., Kanagawa, Japan, 2006) from Physical Electronics.



Figure 3.6: XPS device.

3.2.6 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed on untreated and plasma-treated tube samples by DSC821 Mettler Toledo (Columbus, Ohio, USA). DSC analysis requires the sample to have small mass and to be flat. Therefore, the flat part of the tube between the cap and tube body was used. In case of plasma jet treatment, the said part was treated statically for 15 seconds, under argon atmosphere with the jet-to-sample distance being 10 mm. Melting and crystallization behavior of polypropylene tubes were observed at heating and cooling rates of 10 °C/min between 20 °C and 180 °C. The sample was under nitrogen atmosphere during the analysis and nitrogen flow rate was 50 mL/min. Melting enthalpy was obtained from the melting peak area, while the polypropylene crystalline fraction was calculated by dividing the normalized melting enthalpy (ΔH_m) by fusion energy of 100 % crystalline PP acquired from literature ($\Delta H_m^\circ (100\% \text{ crystalline PP}) = 207.1 \text{ J/g}$ [73]). All data was processed with a STAR SW 14.0 software (Mettler Toledo, Columbus, Ohio, USA).

$$\% \text{ Crystallinity} = \frac{\Delta H_m}{\Delta H_m (100\% \text{ crystalline})} \times 100\% \quad (3.4)$$

3.2.7 Optical Emission Spectroscopy of Plasma Jet

Optical emission spectroscopy (OES) was measured with Avantes AvaSpec-3648 FIBES Optic Spectrometer, which is based on the AvaBench-75 symmetrical Czerny-Turner design with 3648 pixel CCD Detector Array. The spectrometer measured light was in the wavelength range from 200 to 1100 nm with the resolution of 0.5 nm. The integration time can be set from 10 μ s to 10 min.

A collimator lens was mounted perpendicular to the plasma jet and approximately 2 mm away from it with its axis in the same plane Figure 3.7. The lens was connected to the spectrometer via optical fiber. The integration time was set to 500 ms. Optical emission spectra were measured twice. First, 30 days after the last use of the plasma jet treatment device and second, the next day to analyze how long does it take for the plasma jet to start operating in steady state. In other words, warm-up period of plasma jet was being assessed. Results were compared to those in literature and are similar [74].

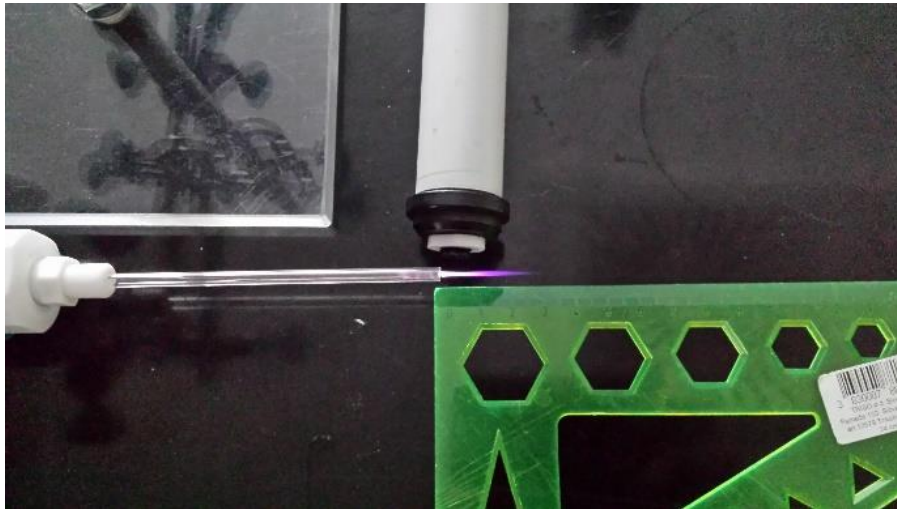


Figure 3.7: Optical emission spectroscopy measurements.

3.2.8 Electrical Characteristics of Plasma Jet

Electrical characterization is one of the crucial steps to standardization of plasma sources and the only way of making the results reproducible and comparable [75, 76]. Electrical characteristics of cold atmospheric pressure plasma jet described in this thesis were measured by a Pearson current monitor model 8590C (Figure 3.8, Table 3.3) and Tektronix P6015A high-voltage probe (Figure 3.9, Table 3.4). Voltage probe was mounted on the beginning of the electrode and the current monitor was positioned around the copper electrode.

The signal from both probes was sent to the oscilloscope (LeCroy WaveRunner HRO 66Zi). Text files with voltage and current waveforms were afterwards exported and processed with OriginPro 8.5 software for graphical presentation.

Table 3.3: Characteristics of Pearson current monitor model 8590C.

Sensitivity	1.0 V/A $\pm$ 1 %
Output resistance	50 Ω
Maximum peak current	500 A
Maximum rms current	5 A
Droop rate	0.7 % / ms
Usable rise time	2,5 ns
Current time product	0.002 As maximum
Low frequency 3 dB point	1.0 kHz
High frequency 3 dB point	150 MHz
Operating temperature	0 to 65 °C



Figure 3.8: Pearson current monitor model 8590C.

Table 3.4: Characteristics of Tektronix P6015A high-voltage probe.

Temperature range	0 to 35 °C
U_{RMS}	14 kV
U_{DC}	14 kV
Input resistance	100 M Ω $\pm$ 2 %
Input capacitance	$\leq$ 3 pF
Temperature coefficient of DC attenuation	0.006 % per °C
Voltage coefficient of DC attenuation	0.018 % per kV
Delay time for 3 m long cable	14.7 ns

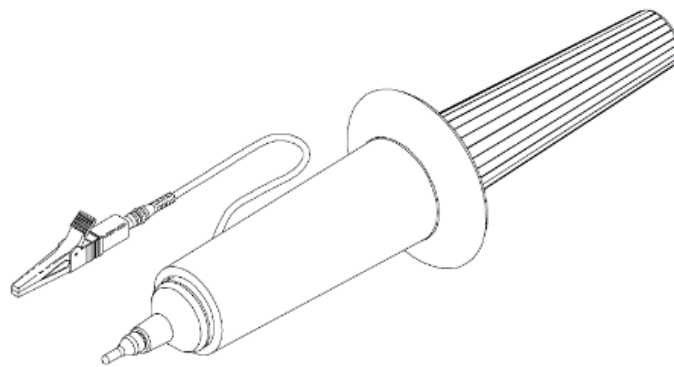


Figure 3.9: Tektronix P6015A high-voltage probe.

3.2.9 Isolation of Microvesicles

The study of microvesicle isolation was approved by the Slovenian National Medical Ethics Committee (n. 117/02/10) and it conformed to the ethical principles given in the Declaration of Helsinki. Samples of whole blood were acquired from 12 and 9 consenting healthy adults, in the first and second experiment, respectively. The subjects were asked to fast for 12 hours before blood collection to prevent false results. Each subject donated 3 mL of whole blood, extracted with a 70 mm long 21-gauge needle with inner radius 0.4 mm (Microlance, Becton Dickinson, CA, USA) by venipuncture into two 2.7 mL vacutubes containing 270 μ L 0.109 mol/L trisodium citrate (BD Vacutainers, Becton Dickinson, CA, USA) and was gently mixed by turning the tube upside down. Prior to sampling, tubes were pre-warmed to 37 °C and samples were kept in thermoblocks to minimize activation of platelets due to thermal stress [77, 78]. Centrifugation of the samples started within 15 minutes after acquisition of the first sample.

In order to separate the cells from blood plasma, the samples were centrifuged at 1550 x g for 20 minutes in a Centric 400/R centrifuge (Tehtnica Železniki, Slovenia) using an RS 4/100 swing-out rotor. Glass collection tubes were used for the low centrifugation. Centrifuge was also preheated to 37 °C, human body temperature, to prevent blood from clotting. After 20 minutes of low centrifugation blood plasma was separated and was floating on top of the rest of the blood. Blood plasma was removed slowly using a tip with a wide opening and placed in a 4 mL glass tube (Figure 3.10). Some subjects had more blood plasma than others and in some cases there was barely enough of sample for analysis.

After homogenizing blood plasma on vortex at 1200 RPM for 5 seconds, each sample was divided into 6 parts, 200 μL each for further centrifugation in 3 different kinds of plasma-treated tubes and the same 3 untreated ones for comparison. Tubes were previously treated at the laboratory of Department of surface engineering and optoelectronics and transported to the Laboratory of clinical biophysics within an hour after plasma treatment. The samples were then centrifuged at $17570 \times g$ for 5 minutes in a Centric 200/R centrifuge (Tehtnica Železniki, Slovenia) with an RA24 angle rotor. The supernatant (160 μL) was discarded and the pellet (40 μL) resuspended in 160 μL citrated phosphate-buffered saline (PBS, pH 7.39 at room temperature). The samples were centrifuged again at $17570 \times g$ for 5 minutes, the supernatant (160 μL) discarded and the pellet (40 μL) resuspended in 60 μL of citrated phosphate-buffered saline. Described process is a slightly modified process described by Diamant et al. [79], used in many other studies [80–83].

The complexity of the entire isolation process and the delicate nature of microvesicles result in high deviation of isolation results. Even the slightest digression from the protocol can result in high error and incomparable results. A lot of work was done on optimization. The place where blood is collected is important and the size of the collection needle as well. It was shown that shear stress inside the collection needle can cause an ex-vivo formation of microvesicles, but is still clinically relevant when the same protocol is respected for all samples [84].



Figure 3.10: Preparation of samples during microvesicle isolation.

3.2.10 Flow Cytometry

Concentration of microvesicles in isolates was determined by MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) flow cytometer with 405 nm, 488 nm and 640 nm air cooled lasers (Figure 3.11). The MACSQuantify™ (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) software version 2.4 was used for data acquisition and analysis of the results. 25 μL of sample was measured. The presence of residual cells and MVs was determined by forward and side scatter parameters. The region of events corresponding to MVs contained more than 90 % of events in all density plots. The flow count of fluorospheres (10 μm , Beckmann Coulter) at a known concentration ($1 \times 10^6/\text{mL}$) was used to determine the concentration of nanoparticles. At least 3800 events were recorded in each sample analysis. The size of the particles in the sample was estimated by the mean intensity of the forward scattered light. The background was measured using pure phosphate-buffered saline (PBS) prior to other measurements and resulted in 2 events per μL , which is negligible.



Figure 3.11: Flow cytometer.

Equation (3.5) was used for calculating the improvement in microvesicle isolation of each plasma-treated tube against its untreated version. In this equation the untreated tube represents 100 %, but only one type of tubes using microvesicles isolated from one subject can be directly compared by this equation.

$$\text{Improvement \%} = \left(\frac{\text{MVs plasma treated tube}}{\text{MVs untreated tube}} - 1 \right) \times 100 \% \quad (3.5)$$

3.2.11 Dynamic Light Scattering

Dynamic light scattering (DLS) is a method used to verify the presence of microvesicles in the solution by determining their size and distribution profile. Particles of different sizes have specific light scatter intensity, but in case when larger particles like microvesicles are present, the method is less sensitive for smaller particles. This is beneficial due to a large amount of smaller proteins and debris being isolated together with larger microvesicles, which in this case fall below the detection limit. Therefore, it is not necessary to know the size and amount of microvesicles in advance to obtain a precise result. The result is presented with refractive index shown in Table 3.5 that gives the information on size and composition of prevailing large particle population.

Measurements were performed on a system for Dynamic and Static Light Scatter (3D DLS SLS) cross-correlation spectrometer (LS Instruments GmbH, Fribourg, Switzerland) that uses a 20 mV He-Ne laser Uniphase JDL 1145P, with wave length of $\lambda = 632.8$ nm and an attenuator (Thorlabs Inc., Redwood City (CA), USA). The software used for data processing was 3D DLS spectrometer v6.6.2.34. The entire system is shown in Figure 3.12. Microvesicle samples left over from flow cytometry were used (known MV concentration). 75 μL of sample was diluted with PBS to a total volume of 500 μL after which the solution was filtered through a low bind protein filter with a pore diameter of 0.45 μm .

Table 3.5: Explanation of DLS results.

Typical shapes of particles	Refractive index ρ
Rigid rod	>2
Random ball (polymer chains)	1.5-1.8 (2)
Hollow ball (microvesicles and exosomes)	1.0
Rigid homogeneous ball	0.78
Microgel (mass in center)	~ 0.6



Figure 3.12: DLS measuring station.

3.2.12 Protein Adsorption

Protein adsorption to tube surfaces was measured in order to better understand interactions with biological samples. Calf serum was used as a protein-rich medium, containing a platelet of various proteins. Proteins like albumin for example are also present in blood plasma and in the process of microvesicle isolation and could therefore shed some light on how plasma treatment affects surface interactions.

The bicinchoninic acid (BCA) assay also known as Smith assay was used for determining the total concentration of protein in a solution. Experiments were performed with a set of Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). Assays were performed in quadruplets. The entire methodology is described in Micro BCA Protein Assay Kit manual and in literature [85–88].

Two different ultraviolet-visible (UV-Vis) spectrophotometers were used for protein adsorption measurements. The first was Infinite M200 PRO (Tecan Group Ltd., Mannedorf, Switzerland) and the second one was Cary 300 UV-Vis (Agilent, Santa Clara, USA).

Chapter 4

Results and Discussion

This chapter is divided into four chronological steps. It starts with characterization and optimization of plasma parameters after which an automatic plasma jet device is designed and constructed. Once this device is constructed, it is used for serial treatment of polypropylene tubes that are further analyzed by all accessible techniques. In the last step, after precise tube characterization, these tubes are used in biological experiments with microvesicles and proteins to evaluate the plasma treatment effects and try to correlate plasma modifications of polypropylene to biological response.

4.1 Characterization and Optimization of Plasma Parameters

The optimization process started with the polypropylene foil, preliminary analysis on Sample 1 only and continued on all samples. The overview of polypropylene samples, plasma treatment and analyzing techniques used is presented in Table 4.1. The numbers under plasma treatment column represent times of dynamic plasma treatment with plasma jet moving in and out of the tube. Static plasma treatment of tubes gave poor results and was discarded early on.

Table 4.1: Overview of performed analysis on polypropylene.

Material	Plasma treatment	Analyzing method
PP foil	2, 3, 5, 10, 15, 20	WCA
	2, 5, 20	AFM
	5 (argon atmosphere)	WCA
Sample 1	2, 3, 5, 10, 15, 20	Tensile strength and strain
	2, 5, 20	XPS (survey and high res.)
Sample 1, 2 and 3	5 (argon atmosphere)	WCA Up-Down
		WCA Top-Bottom
		AFM Top-Bottom
		XPS Top-Bottom

4.1.1 Gas Flow Optimization

One of the crucial factors for the atmospheric pressure plasma jet ignition and propagation is gas flow. Argon was chosen, as most of atmospheric plasma jets including the one used in this thesis work best with inert gases and argon has the best price-performance ratio. To observe the effect of argon flow changes, measurements were performed at five values

of gas flow, from 1 to 5 standard liters per minute. These are also the boundary conditions concerning gas flow of plasma jet used. At higher or lower gas flow rates the plasma jet is either not ignited or does not work in a stable operating regime. The results are presented in Table 4.2. Efficiency was calculated using Equations (4.1), (4.2) and (4.3). Efficiency does not change drastically but there is an observable maximum at 2 slm of argon flow, which coincides with visible observations of plasma jet being the longest and most stable at this gas flow rate.

Table 4.2: U/I input against output for variable argon flow.

Input settings				Output measurements			Efficiency (η)
Ar (slm)	U (V)	I (A)	P _{in} (W)	U _{rms} (kV)	I _{rms} (mA)	P _{out} (W)	P _{out} /P _{in} ratio
1	12	0.56	6.72	2.867	1.432	4.11	61.1
2	12	0.56	6.72	2.905	1.461	4.24	63.2
3	12	0.56	6.72	2.868	1.420	4.07	60.6
4	12	0.56	6.72	2.868	1.391	3.99	59.4
5	12	0.56	6.72	2.867	1.408	4.04	60.1

$$P_{in} = U \times I \quad (4.1)$$

$$P_{out} = U_{rms} \times I_{rms} \quad (4.2)$$

$$\eta = \frac{P_{out}}{P_{in}} \times 100 \% \quad (4.3)$$

Considering the highest efficiency, visual observations of plasma jet and low gas consumption, gas flow of 2 slm of argon was chosen as optimal and was used in all subsequent experiments.

4.1.2 Plasma Power Optimization

First of all, minimum and maximum boundary conditions of plasma jet had to be found. The threshold for plasma to ignite was 4.1 V of input voltage while the maximum conditioned by power source was 15 V. The input voltage was increased by 1 V for each measurement and all the results are collected in Table 4.3. The efficiency of output power against the input was around 60 %, maximum efficiency was reached between 10 and 12 V and decreased with increasing voltage. Argon flow was kept at 2 standard liters per minute, as this was found to be optimal in the previous chapter.

Table 4.3: U/I input against output for constant argon flow.

Input settings				Output measurements			Efficiency
U (V)	I (A)	Ar (slm)	P _{in} (W)	U _{rms} (kV)	I _{rms} (mA)	P _{out} (W)	P _{out} /P _{in} ratio
4.1	0.21	2	0.86	1.051	0.503	0.53	61.4
5	0.25	2	1.25	1.289	0.574	0.74	59.2
6	0.29	2	1.74	1.557	0.656	1.02	58.7
7	0.34	2	2.38	1.818	0.775	1.41	59.2
8	0.39	2	3.12	2.072	0.891	1.85	59.2
9	0.45	2	4.05	2.295	1.091	2.50	61.8
10	0.49	2	4.90	2.519	1.248	3.14	64.2
11	0.53	2	5.83	2.721	1.366	3.72	63.8
12	0.56	2	6.72	2.905	1.461	4.24	63.2
13	0.60	2	7.80	3.082	1.562	4.81	61.7
14	0.64	2	8.96	3.245	1.623	5.27	58.8
15	0.70	2	10.50	3.397	1.685	5.72	54.5

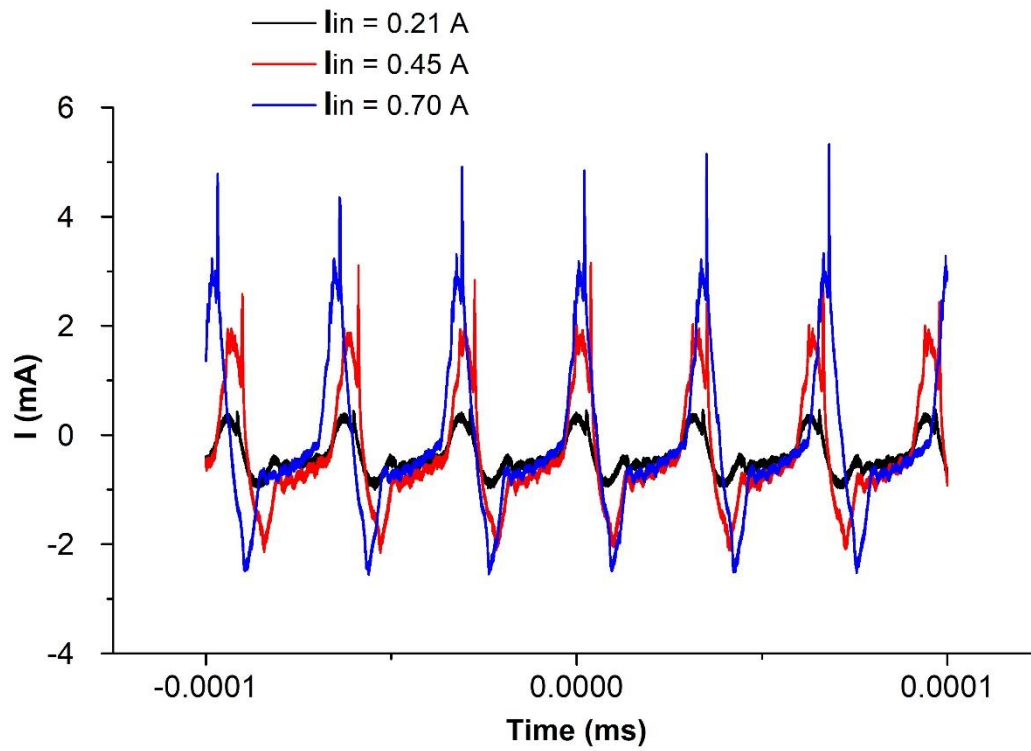


Figure 4.1: Comparison of output current.

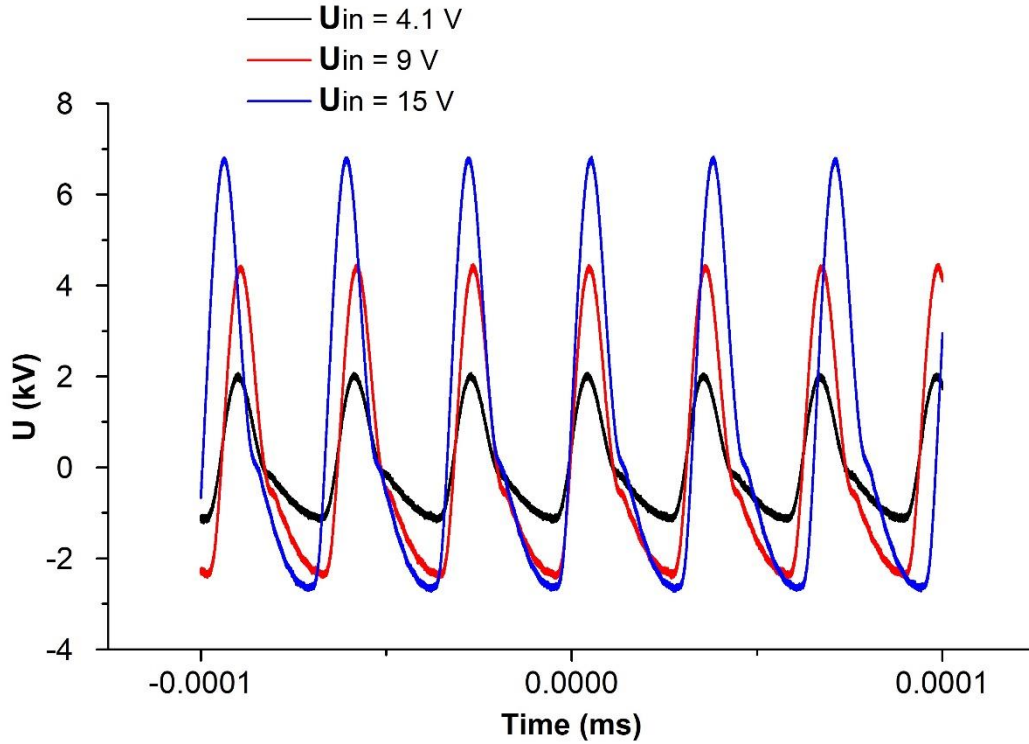


Figure 4.2: Comparison of output voltage.

Three different measurements (minimum, medium and maximum input values) are presented in Figure 4.1 and Figure 4.2. At higher input voltage the frequency slightly drops. At maximum input settings the signal has a slightly lower frequency. There also seems to be a minimum on the negative side of the signal at around -2.5 kV for medium as well as maximum input voltage.

It can be seen from Figure 5.1, Figure 5.2 and Figure 5.3 in Appendix A that the waveforms are not sinusoidal but a combination of multiple sine functions. The voltage and current are therefore multiple harmonics signals.

According to Table 4.3, the highest input parameters with the highest efficiency were chosen to make plasma treatment as fast and efficient as possible. These parameters were input voltage of 12 V and input current of 0.56 A. These input settings were used in all subsequent experiments.

4.1.3 Plasma Treatment Time Optimization

4.1.3.1 Water Contact Angles on Polypropylene Foil

To optimize plasma treatment time, water contact angle measurements were used as the first and fastest indication. PP foil was put inside a tube and plasma jet was moving in and out of the tube for 2, 3, 5, 10, 15 or 20-times in horizontal position. The results are presented in Figure 4.3. The drop of water contact angle values after plasma treatment is expected and is associated with functionalization of polymer surface with oxygen functional groups. These polar functional groups increase the surface energy and decrease contact angles especially for polar liquids, like water. Literature reports about water contact angles between 103 and 87 degrees for untreated PP foils made by different manufacturers [36,

38, 41, 45, 89–92], with one exception where 120 degree water contact angle is reported by Cheng et al., [93]. The same literature reports about a variety of water contact angle measurement results after atmospheric pressure plasma treatments. These are ranging from 24 to 82 degrees, but the majority is reported to be between 42 and 63 degrees. These results are strongly dependent on the plasma jet configurations, power source, time of treatment, PP foil used and other factors making results hard to compare.

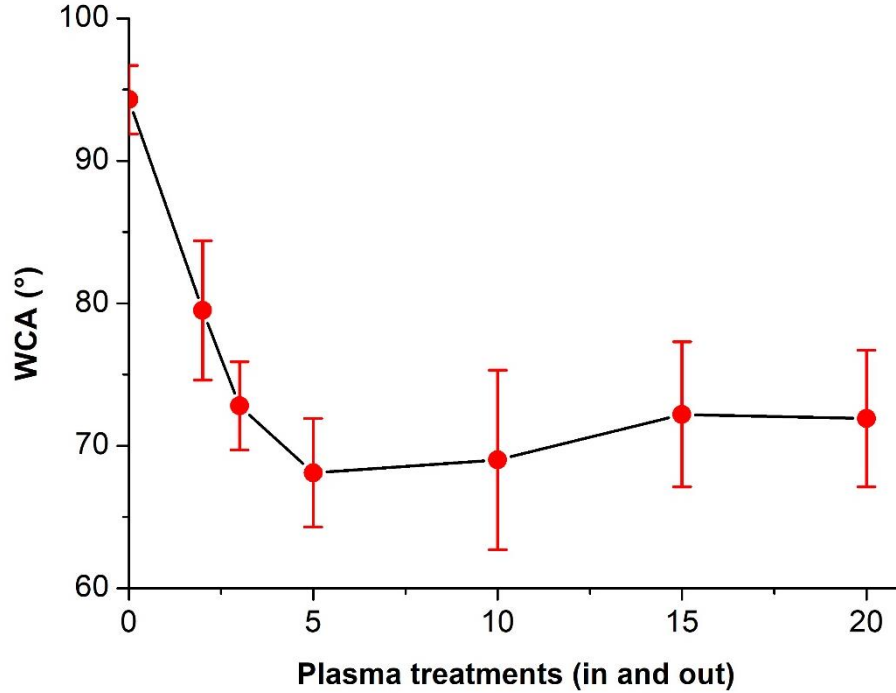


Figure 4.3: WCA on PP foil depending on the time of plasma treatments.

Best results according to water contact angle measurements were achieved by 5-times plasma treatment, as that is both the treatment time with the lowest water contact angle and one of the shortest treatment times. Similar water contact angles are achieved also with longer plasma jet treatment repetitions but to confirm the said time as the optimal plasma treatment time, AFM, XPS, and tensile strength measurements were performed in the following chapters.

4.1.3.2 Surface Morphology of Polypropylene Foil

The morphology of PP foil was studied further on to observe etching effects on the surface. The untreated foil is rather flat (Figure 4.4), while plasma-treated foils exhibit grain-like etched structures. These structures are much smaller and more evenly arranged in the case of 5-time plasma-treated foil (Figure 4.5) compared to 20-time plasma-treated foil (Figure 4.6). The 2-time plasma-treated sample was also examined but did not show enough morphological changes to be distinguished from the untreated one without a doubt. It is believed that etching and functionalization are dominant factors in the first step of the morphology change. Later on, the thermal stress on the surface might be increased due to collisions of highly energetic plasma species with the polymer surface, causing local melting of the polymer.

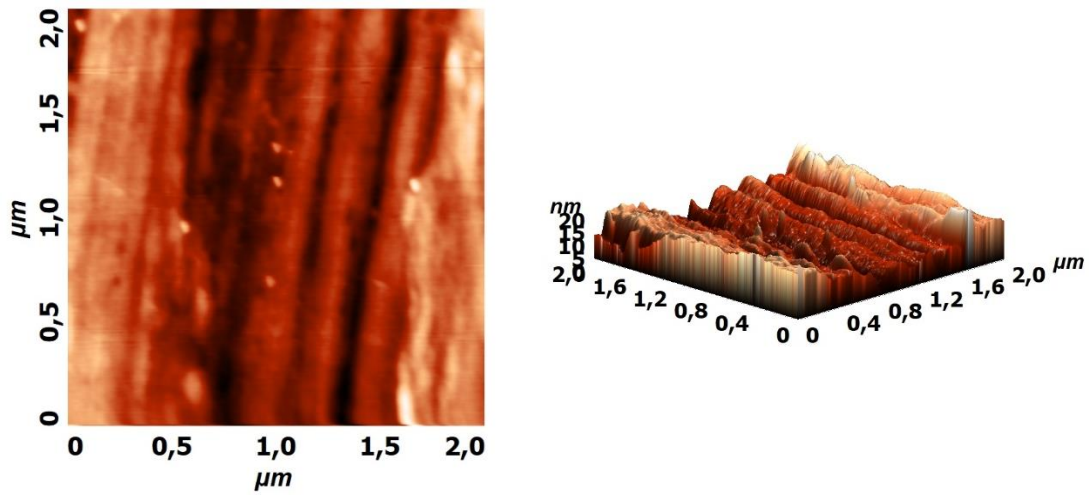


Figure 4.4: AFM images of untreated PP foil.

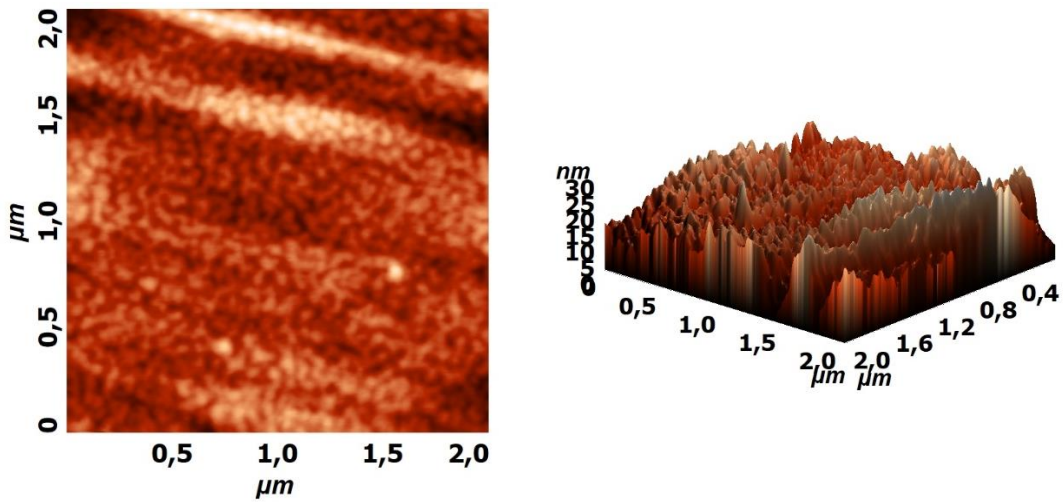


Figure 4.5: AFM images of 5-times plasma-treated PP foil.

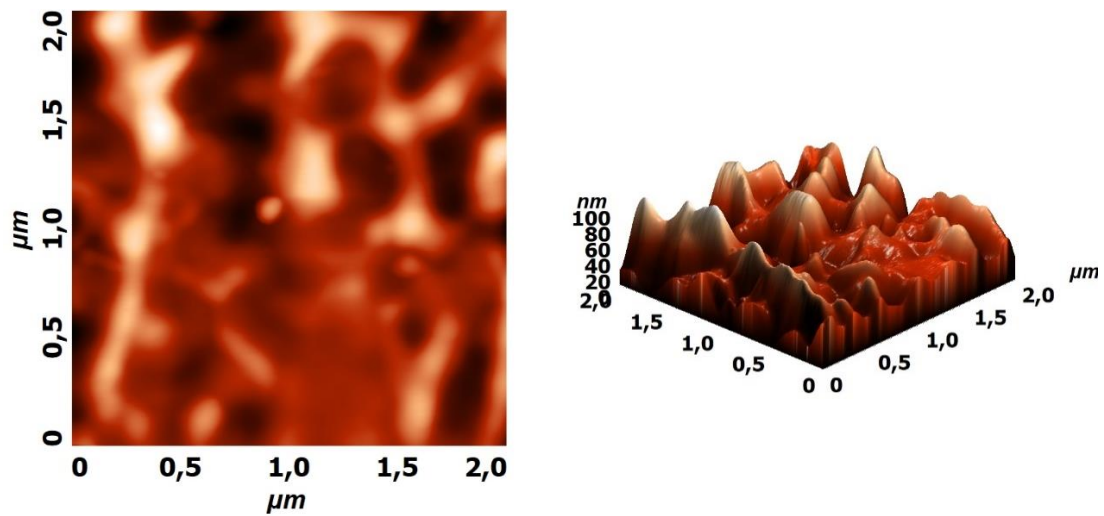


Figure 4.6: AFM images of 20-times plasma-treated PP foil.

The 5-time plasma treatment seems to give more uniform results than 20-time plasma treatment, according to AFM images on PP foil. The main factor in case of morphology is even surface structure and presumably finer nanostructure to decrease contact area between polymer surface and biological samples. The morphological results obtained on foil should be similar to those on tubes, to some extent.

4.1.3.3 X-ray Photoelectron Spectroscopy

Three dynamic treatments were performed on the PP tube (Sample 1), one for 2-time, one for 5-time and another one for 20-time repetitions with plasma jet moving in and out of the tube. Static plasma jet treatment was also examined with plasma jet mounted 10 mm away from the tube and plasma ignited for 90 seconds. Survey spectra of statically plasma-treated tube exhibited an increase of 7 atomic percent of oxygen on the surface, which is less than in the case of dynamic treatments for shorter times where 15 atomic percent of oxygen can be reached after 15 seconds.

In Table 4.4 the results of XPS analysis are presented. As can be observed, the oxygen content increases for 2-time treated tube but the saturation is not reached until 5-time treatment. Another detail that can be observed from Table 4.4 is that shorter plasma jet treatment (5-times) is actually equal or slightly better compared to the longer one (20-times) as there is less nitrogen and more oxygen present on the surface.

In Figure 4.7 a comparison of XPS survey spectra for untreated PP foil and 5-times treated foil is made. As can be seen only oxygen peak increases dramatically while the rest of spectra stays unchanged. This is desirable as the oxygen content on the surface means more oxygen functional groups, increasing surface energy and making the surface more hydrophilic.

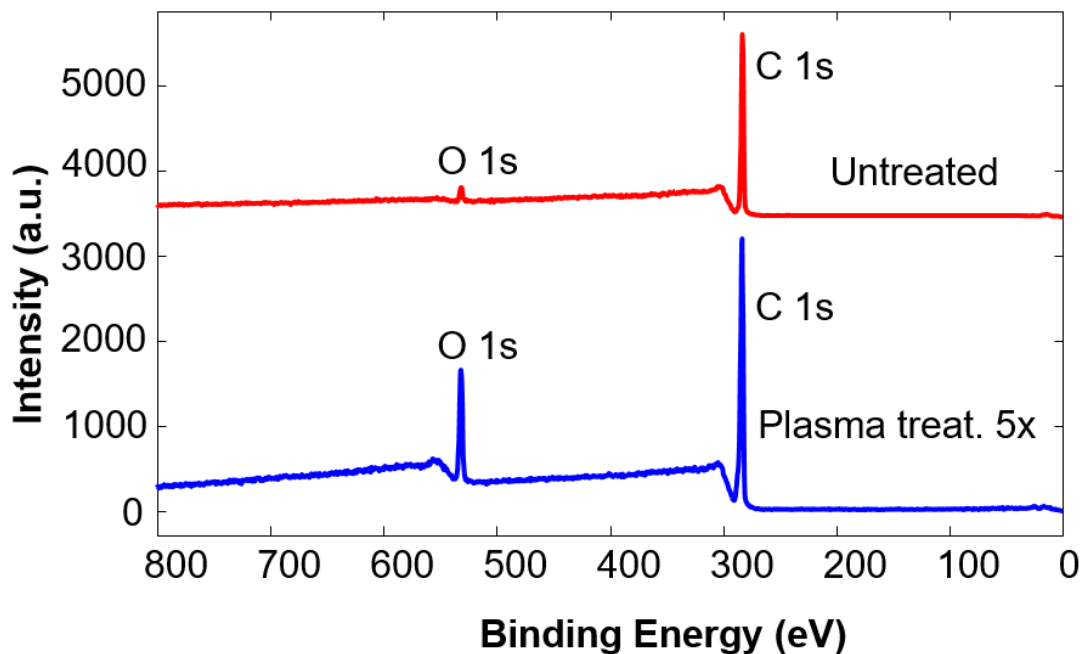


Figure 4.7: XPS survey spectra of untreated and 5-times plasma-treated PP foil.

Novel oxygen functional groups on the surface were further analyzed by high resolution XPS spectra of C 1s. The relative concentration of each chemical component in C 1s high resolution peak was determined by deconvolution of experimental spectra using Gaussian-

Lorentzian curves. As seen in Figure 5.4 (Appendix B), the C 1s spectrum of untreated foil has the first peak at 285.0 eV, which corresponds to C-C and C-H bonds of polypropylene, a small peak at 286.5 eV corresponding to C-O groups, minor peaks at 287.8 eV corresponding to C=O and O-C-O and at 289.2 eV corresponding to O-C=O. The C 1s spectra of 5 and 20-time plasma-treated foil on the other hand consist of broad shoulder at high binding energy side which is due to incorporation of novel oxygen functional groups (Figure 5.5 and Figure 5.6, Appendix B). However, differences between 5- and 20-time plasma-treated foil can be observed from C 1s spectra, as 5-time treated foil exhibits more pronounced peaks corresponding to C-O as well as C=O and O-C-O functional groups. The percentage of each functional groups determined from high resolution C 1s spectra is presented in Table 4.4. It can be seen from these results that the relative concentration of C-C bonds decreases on the plasma-treated surface on account of incorporation of oxygen species, such as alcohols, carbonyls and carboxylic acid.

Table 4.4: Concentration of elements obtained from XPS survey spectra and deconvolution of high resolution carbon spectra.

	C (%)	O (%)	N (%)	C-C (%)	C-O (%)	C=O (%)	O-C=O (%)
				285.0 eV	286.5 eV	287.8 eV	289.2 eV
Untreated	95.0	5.0	0	93.3	5.7	0.1	0.9
Sample 1 2 x	90.6	9.4	0	88.1	6.9	1.8	3.2
Sample 1 5 x	84.2	15.8	0	81.5	10.4	4.0	4.1
Sample 1 20 x	82.8	15.4	1.8	84.6	7.9	3.3	4.3

Considering the results in Table 4.4, the shortest 2-time plasma treatment is too short but 5-time plasma treatment can be considered superior over longer 20-time plasma treatment. The amount of atomic oxygen on the surface is similar but in case of 5-time plasma treatment more oxygen is attached by single bonds. 5-time plasma treatment is therefore again confirmed as optimal treatment time, due to saturation of tube surface with oxygen functional groups. Longer treatment times have no significant influence on the concentration of oxygen groups and are therefore unnecessary.

4.1.3.4 Tensile Strength Measurements

Results obtained from tensile strength measurements display a different force needed to remove polymerized mixture from the tube (Sample 1), depending on the time of plasma treatment. From various treatment times/repetitions a peak of average force can be observed in Figure 4.8. The highest force is needed to remove a polymerized mixture from the 5-time plasma-treated tube, while longer treatments actually caused a decrease in adhesion force. The first measured value in the above-mentioned figure is for the untreated Sample 1, which exhibits the lowest average force needed for removal of polymerized mixture. The average force for untreated sample is around 30 N while the 5-time plasma-treated tube requires an average force of 90 N, which is three times higher.

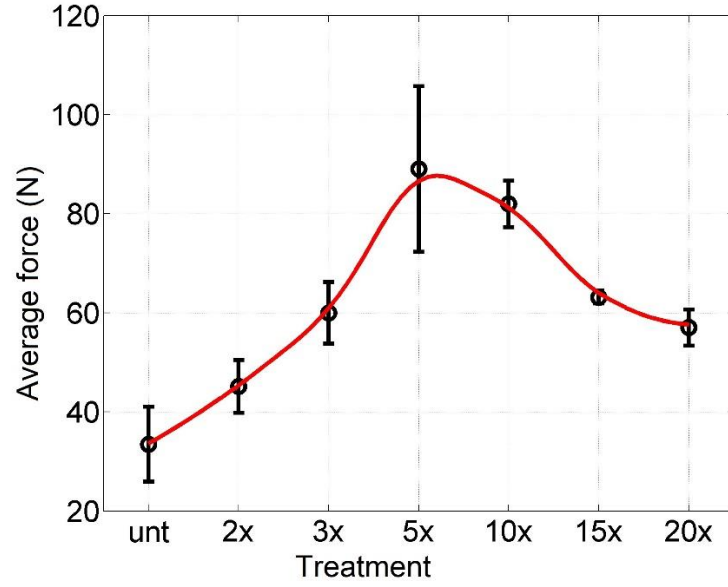


Figure 4.8: Average force needed to separate the polymerized mixture from the tube [94].

Representative force needed for polymerized mixture removal versus strain curve is presented in Figure 4.9. It can be observed that the highest force for removal is needed for 5-times plasma-treated tube and that the strain is also among the highest reached. In this case adhesion is improved for approximately 3 times and longer treatment times are again lowering the adhesion force. As observed from XPS analysis, surface seems to be saturated with oxygen functional groups after 5-time plasma treatment. Moreover, longer treatment times decrease the number of C-O as well as O-C-O and C=O functional groups, which may have an important role in adhesion between tube wall and polymerized mixture. AFM measurements also show that grain structures are growing up until 5-time plasma treatment and start to decrease in size after that.

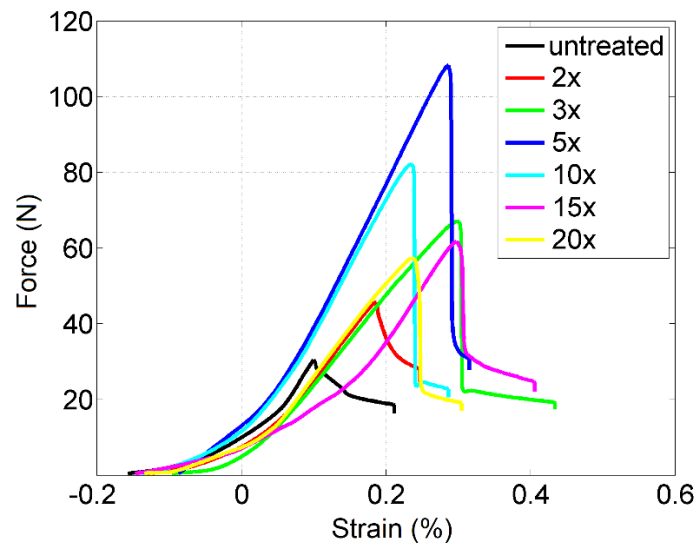


Figure 4.9: Example of representative force versus strain curves measured on tubes treated at different plasma jet repetitions [94].

Results are conclusive, showing that the 5-time plasma-treated tube has optimal characteristics which significantly increased the adhesion force between the tube and the polymerized mixture. XPS study revealed that novel oxygen functional groups are formed on the inner surfaces of the tube after plasma treatment. These results coincide well with the XPS results and confirm the formation and saturation of oxygen functional groups on the surface of tube. These functional groups could increase bonding between polymerized mixture and tube walls but as can be seen from Figure 4.9, the tensile strength measurements suggest that plasma treatment more than doubled the force needed to remove the polymerized mixture from the tube wall. This effect cannot be attributed only to chemical bonding but also to physical. With the nanostructured grains on the tube surfaces the area of contact between tube walls and polymerized mixture increases and due to orientation of grains perpendicular to removal force axis, the friction force increases substantially. It can be concluded that in case of a 5-time plasma-treated Sample 1, the combination of surface chemistry and roughness exhibited the highest removal force. We predict that the grain surface structures are favorable in case of microvesicle isolation, keeping the microvesicles away from flat surfaces where sheer stress occurs during contact, causing fragile structures to disintegrate.

4.1.4 Plasma Jet Treatment Improvements

4.1.4.1 Argon Atmosphere

The effects of argon atmosphere were also investigated. As suggested in the work of Reuter et al., [95], atmospheric plasma treatment can be highly sensitive to ambient species. Controlled atmosphere can therefore be applied to exclude the flux of ambient species and consequently control the effluent chemistry. We performed two experiments to confirm this theory. In the first case the tube was plasma treated for 15 seconds in air with argon only used as feed gas while in the second case, the tube was enclosed inside a container which was prefilled with argon for 15 seconds before the 15-second plasma treatment began. The volume of the container was approximately 0.1 L while the argon flow rate was set to 2 standard liters per minute (slm). This means that in 15 seconds 0.5 standard liter of argon should have entered the container and displaced most of the air out, as the container was not air tight. Hence the pressure inside the container was still atmospheric pressure but with argon as atmosphere gas instead of air.

The WCA of untreated PP foil was 94.1° . After plasma treatment without argon atmosphere and with argon atmosphere, the WCAs were $68.3^\circ \pm 4.8^\circ$ and $64.1^\circ \pm 2.6^\circ$, respectively. It can be observed that the resulting average water contact angle and standard deviation are lower in case of argon atmosphere. The conclusion is that argon atmosphere indeed improves uniformity of treatment which is of crucial importance for our future work.

4.1.4.2 Treatment Uniformity

In all of the WCA measurements, areas inside the tube further away from the plasma jet exhibited a little higher water contact angles. Considering that in the conical part of the tube WCA could not be measured due to smaller radius and there were already differences between the top and bottom measuring positions a new approach was needed. It was decided that the plasma jet has to move in and out of the tube to reach and evenly treat all inner surfaces. Best way to achieve such movement was to mount the plasma jet on a linear stage and control its movement with a software. The speed of linear stage was set to 12 mm/s and from this point on the amount of in-and-out movements was our treatment reference point.

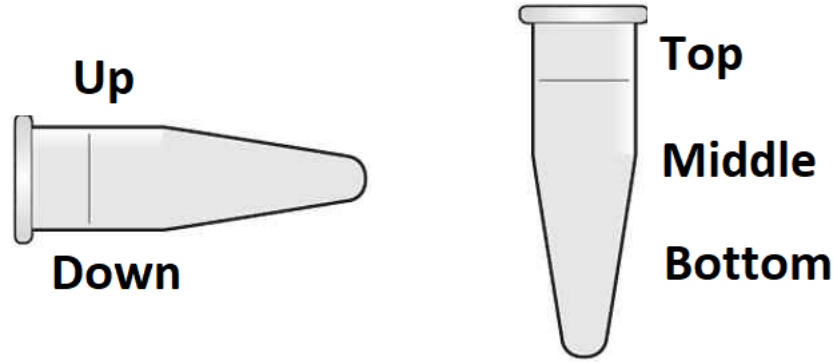


Figure 4.10: Measuring areas in horizontal (left) and vertical (right) position.

In Table 4.5 the results for 5 time treated tubes on both positions (Up and Down Figure 4.10) are presented. In case of Sample 2 and 3 the differences in WCAs are alarmingly high, while in case of Sample 1 a small increase with a little higher standard deviation can be observed. None of these are acceptable and that is why from this point on plasma treatment was performed in a vertical position. It is very important that plasma treatment results in evenly modified surfaces because this is the only way we can further analyze the effects of surface-to-sample interface.

Table 4.5: WCA on different positions of tube (Up-Down shown in Figure 4.10).

	WCA Up (°)	WCA Down (°)
Sample 1	66.9 ± 6.4	65.5 ± 2.2
Sample 2	59.7 ± 1.0	45.4 ± 2.0
Sample 3	80.2 ± 1.6	70.9 ± 2.8

In the vertical position, WCA measurements exhibited much more uniformity of plasma treatment. This is confirmed by the results obtained from automatically plasma-treated tubes presented in Figure 4.21. In case of these results, differences between the top and bottom position were within the measurement error and therefore negligible.

To further confirm the uniformity of vertical plasma treatment, XPS on top and bottom areas of the tube and AFM measurements on top, middle and bottom areas were performed for Sample 1. Both, XPS and AFM showed no significant difference amongst measured areas, therefore it can be concluded that morphology and chemistry inside the tube are uniform.

4.1.5 Optical Emission Spectroscopy

As can be seen from Figure 4.11, the optical emission spectrum main spectral features are OH emission band around 309 nm, nitrogen (N_2) second positive system and argon (Ar) atomic emission lines. For further analysis, we used OH emission line intensity, N_2 337 nm line and argon 763 nm line intensity.

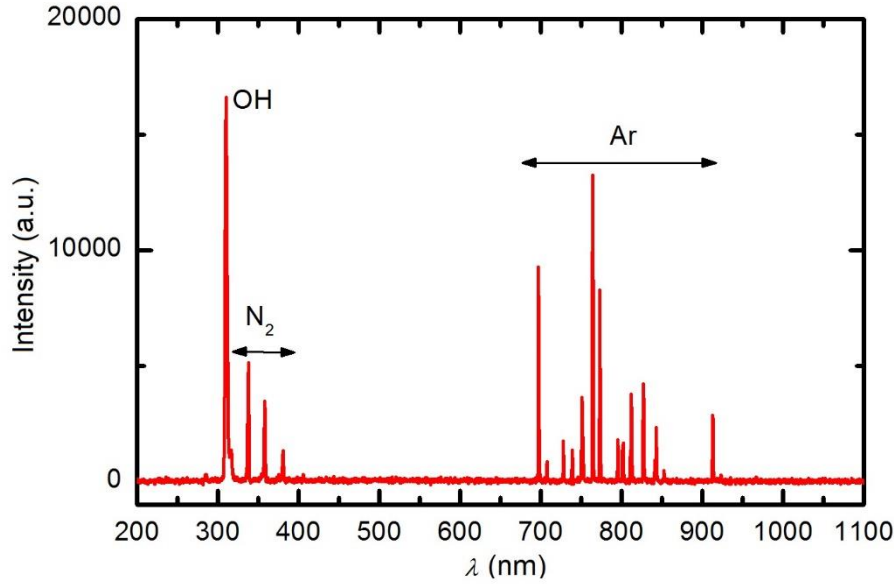


Figure 4.11: Optical emission spectrum of the atmospheric pressure plasma jet.

During the experimental work with the plasma jet, it became obvious that some warm-up time at the beginning is needed for the jet to start operating stably. To closely examine this phenomenon, two optical emission spectra were taken. Plasma jet was intentionally left unused for one month and then optical emission spectra was measured for 90 and 60 minutes on the first and second day, respectively. These spectral measurements are shown in Figure 4.12 and Figure 4.13. On both figures intensity of argon and OH changes over time. On the first day, plasma jet takes around 25 minutes (1500 s) to reach steady state operation while on the second day this is reached after approximately 15 minutes (960 s). Another improvement is that higher intensity of argon is observed on the second day. To better present the final result, the ratio between OH and argon intensities is presented in Figure 4.14. These are the dominant species presented in plasma jet and therefore their ratio plays an important role in providing a repeatable treatment. The higher amount of OH at the beginning is attributed to humidity of air. Whenever the plasma jet is not in operation, air penetrates the inside of the gas lines and small amounts of water condensate on surfaces. Herein described effect not only increases the amount of OH present in the plasma jet but also causes quenching of excited argon species [74].

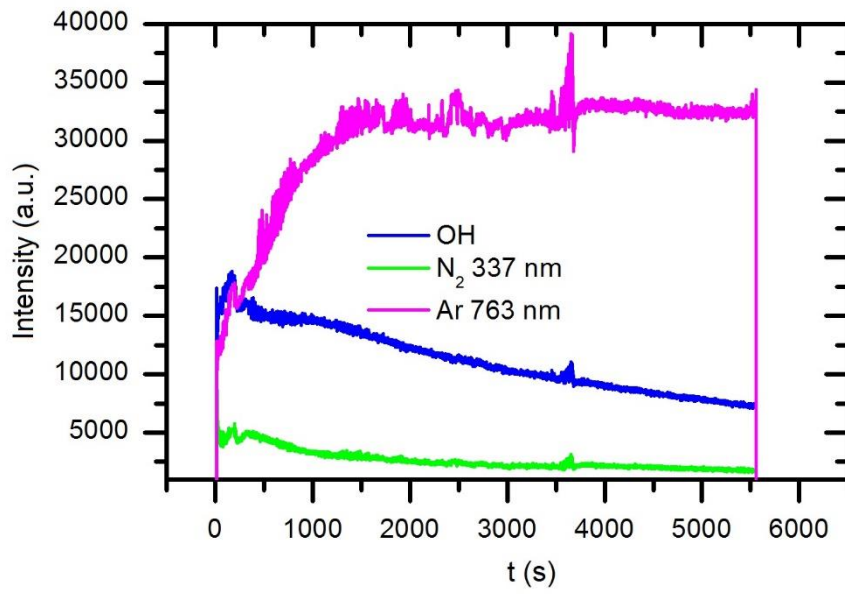


Figure 4.12: Optical emission spectrum of the atmospheric pressure plasma jet after it was not used for 30 days.

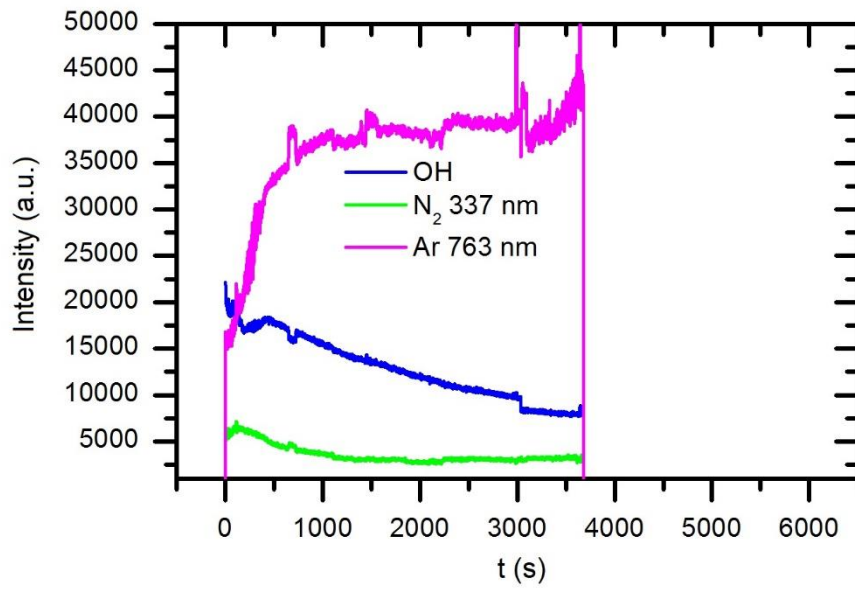


Figure 4.13: Optical emission spectrum of the atmospheric pressure plasma jet the next day after being used.

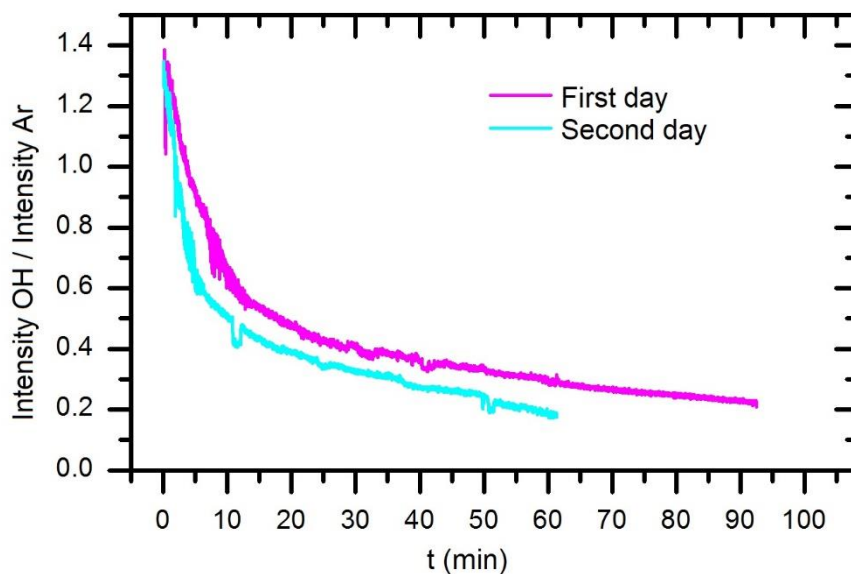


Figure 4.14: OH to argon ratio measured by optical emission spectroscopy.

Considering the plasma jet is not frequently used, measures must be taken to ensure equal treatment conditions. One of them is warm-up prior to treatment. Preventive measures can also be taken to make warm-up faster or even unnecessary. These measures are short gas lines, preferably metal not polymer, gas valves on both ends so after the treatment argon is kept inside the tube and air cannot enter, cap at the end of plasma jets' quartz tube, etc. Shorter gas lines mean less surface area for impurities to attach. Metal gas lines can be better sealed at the ends, preventing loss of argon and intrusion of atmospheric gases. Valves prevent atmospheric gases from entering the tubes when plasma jet is not working and there is no gas flow.

4.2 Design and Construction of Automatic Plasma Jet Device

4.2.1 Design and Optimization Steps

The treatment of internal surfaces of a long tube with gaseous plasma jet is a challenge by itself. Furthermore, curved surfaces present a problem for most analytic techniques. Hence most research groups decide to use foil made of what is declared to be the same polymer, due to more convenient surface analysis. In our case vast differences already appeared between tubes from different manufacturers, therefore foil was used for preliminary experiments only. It also has to be considered that the dynamic of CAP jet treatment cannot be simulated on a flat surface due to complex mechanisms of gaseous plasma-activated species being transported in a unique manner inside tubes. These species and their interaction with surface are of crucial importance when mechanisms of surface functionalization and etching are observed. The main requirement for successful tube modification is that all surfaces are evenly gaseous plasma treated. This was achieved by the settings listed below:

- Gaseous plasma treatment was conducted inside an enclosed container to ensure argon atmosphere where less quenching of excited plasma species occurs, plasma jet is wider and less focused, and streamers are more branched. Gas flow is initiated before the plasma jet is turned on. Argon is heavier than air and displaces most of the air from the bottom of the container. Figure 4.15 presents CAP treatment before the argon atmosphere was applied, it can be observed how the majority of streamers are in contact with the closest surface while plasma-activated species are at the bottom of the tube (pink glow). Another static CAP treatment is presented in Figure 4.16, where the tube is inside an argon prefilled container while being mounted coaxially to the plasma jet.

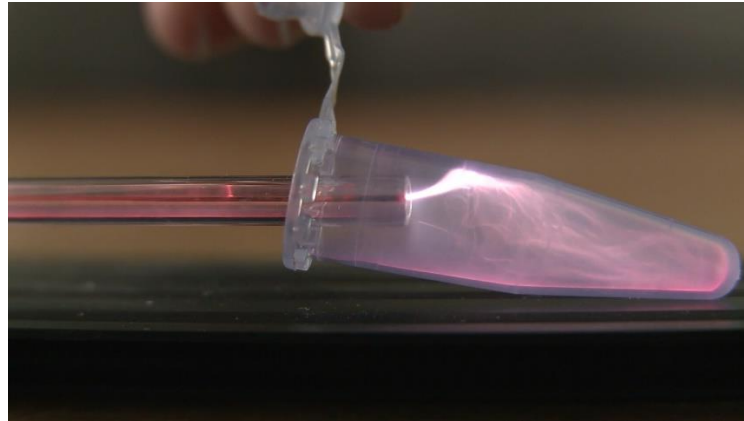


Figure 4.15: CAP treatment without argon atmosphere.

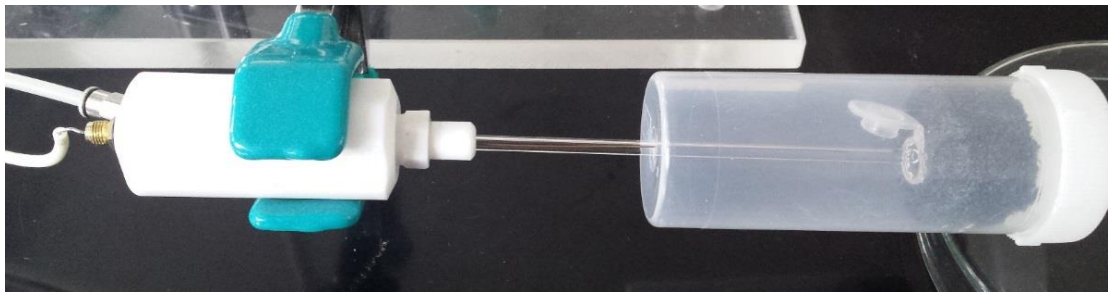


Figure 4.16: Static horizontal CAP treatment with argon atmosphere.

- Treatment is not stationary, gaseous plasma jet is moving in and out of the tube during treatment. It was found by XPS measurements that dynamic plasma treatment is much more effective in saturating the PP surface with oxygen functional groups. The movement is enabled by a linear stage to ensure equal speeds and acceleration/deceleration and consequently even and repeatable gaseous plasma treatment. This is also called dynamic plasma treatment and is shown in Figure 4.17. Other settings stayed unchanged.



Figure 4.17: Dynamic horizontal CAP treatment with argon atmosphere.

- Vertical position of gaseous plasma jet ensures that the ground is coaxially positioned with the axis of symmetry. WCA measurements supported by XPS and AFM analysis proved that up and down positions (Figure 4.10) are equally plasma treated only in the case of a vertically positioned plasma jet. In the case of a horizontal setup the lower part of the tube was more effectively gaseous plasma treated than the upper part due to the jet being attracted to the ground. In practice this resulted in streamers never touching the upper surface. The final solution can be seen in Figure 4.18, as this setup is designed for automatic plasma treatment of multiple tubes at once, the entire system is enclosed in an air tight housing and prefilled with argon.

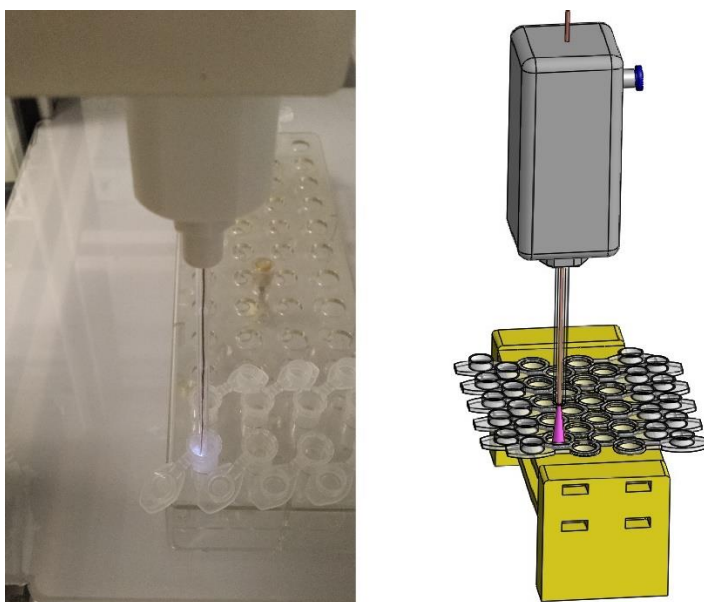


Figure 4.18: Dynamic vertical CAP treatment with argon atmosphere.

- Gas flow is optimized in a manner that backflow does not affect the main gaseous plasma jet and carries plasma-activated species close to the surface with as little turbulence as possible. This was done on the base of visual observations, measuring the length of plasma jet with different gas flows and on the base of measured power efficiency from Chapter 4.4.1., Table 4.2.

4.2.2 Final Construction of Device

The final version of the automatic plasma treatment device was constructed using one planar and one linear stage as the main components. X and Y axis movements were enabled by Festo EXCM (full name EXCM-30-400-360-KF-ST-B1-PF5-EN) portal with 400 mm and 360 mm movements in X and Y direction, respectively. Z axis was a separate unit, Festo EGSL (EGSL-BS-45-200-10P) with 200 mm stroke, powered by a Festo stepper motor (EMMS-ST-42-S-SEB-G2). The control unit used to operate both stages simultaneously was also from Festo (CMMO-ST-C5-1-DIOP). The plasma head was mounted directly onto the vertical stage (Z axis). The frame of the device was made from 40 x 40 mm Rexroth profiles with outer dimensions 830 x 700 x 480 mm, height, width and depth, respectively. The entire housing is enclosed by transparent Plexiglas panels to make the treatment under argon atmosphere possible and to keep the operator safe from ozone as a side effect of plasma jet. Large door is built-in so the sample manipulation is made as easy as possible. In case of additional space needed, side panels are removable. The treatment table is made out of Plexiglas which has minimalistic support profiles to minimize the effects of ground conductor effects on the plasma jet. The height of the treatment table is also adjustable. The design of the device is compact with all the components (except argon cylinder) incorporated inside the housing.

Software for controlling the device for automatic plasma treatment was written in C# language. This is an experimental version with a lot of available functions and error reporting. Connection to the device is done via Ethernet, where each device (EXCM for X and Y axis and CMMO for Z axis) has its own IP address. After successful connection the motors are powered up and homing is performed. The software itself can be divided into three parts and its schematics is presented in block diagram on Figure 4.19. The first part is pooling information from all three linear motors (X, Y, and Z axis). This includes:

- Actual position, velocity and error reporting for all axes.
- All sorts of status bits for all three axes: enabled, voltage applied, motion complete, warning, fault, etc.

The second part is controlling all axes by inputting the desired position in terms of X, Y, and Z (in millimeters) and choosing absolute or relative movement. An absolute motion always moves to a taught position without being affected by the previous motion, while a relative motion sets its reference to the current position of the result of executing the previous motion command and the previous motion command affects the motion. Prior to executing the movement command (“Start Positioning”), the user also has to set the velocity and acceleration for all axes.

The third part is automatic treatment, where the device automatically treats a set of tubes in a holder. At present, the software has to be re-programmed for a given set of tubes. We are currently improving automatic treatment so that the user only inputs the number of tubes in one row, the number of rows, the diameter and depth of a tube, the horizontal and vertical distance between two neighboring tubes, and plasma treatment time. By inputting these data, the computer software will be able to calculate all the necessary movements and automatically adjust all the parameters and perform the plasma treatment.

The entire program is shown in Appendix C.

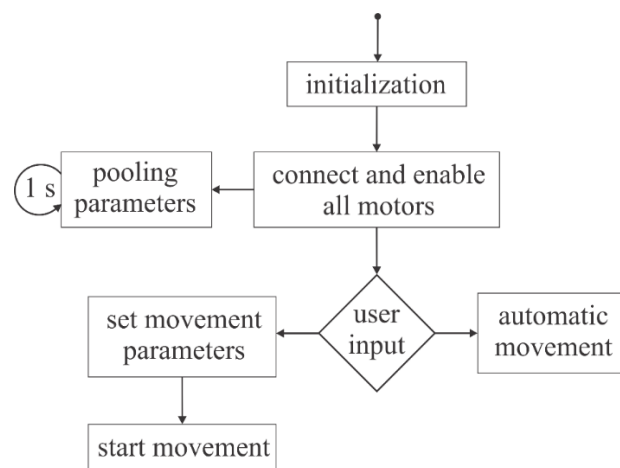


Figure 4.19: Basic block schematics of software operation.

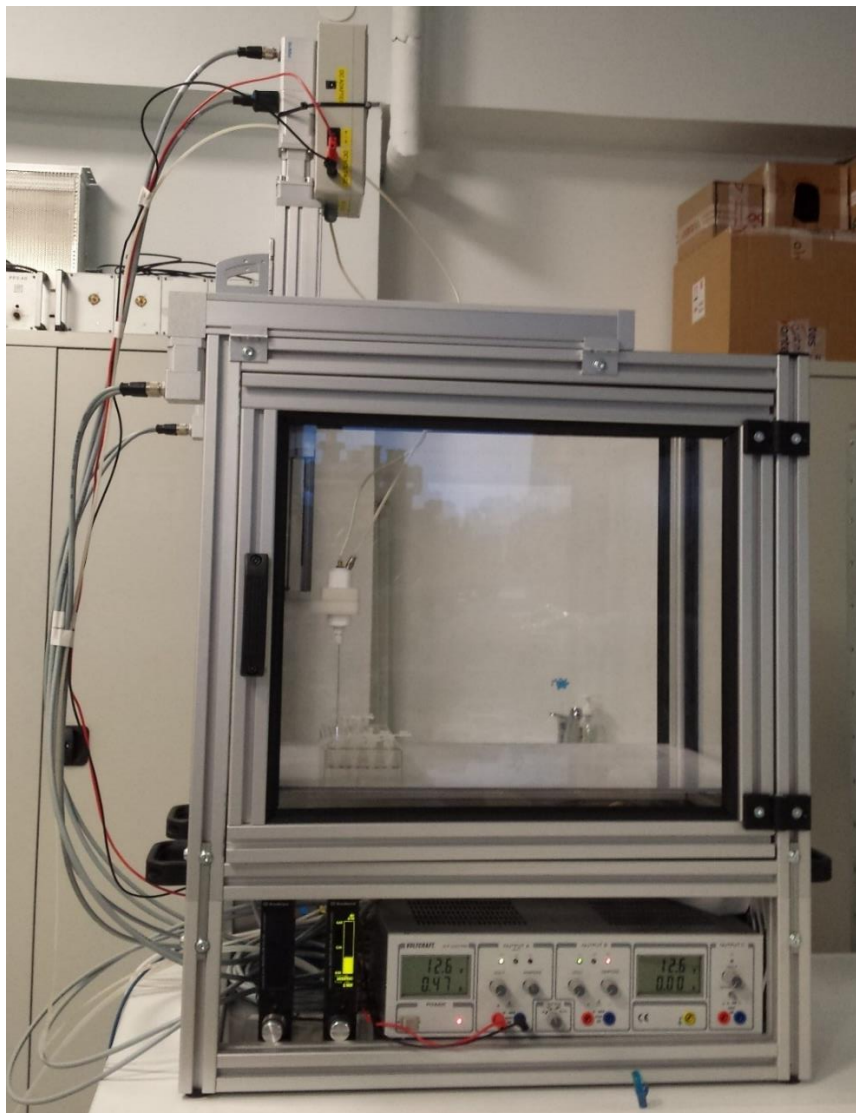


Figure 4.20: Automatic plasma treatment device, front side.

The front side of automatic plasma treatment device is visible in Figure 4.20. At the bottom there is electric power source, flow controller and stepper motor controllers and power supply. Above is the treatment chamber with plasma jet, sample holder, samples and doors for sample handling, surrounded by Plexiglas in order to visually control the treatment.

4.3 Modification of Polypropylene Tubes

Previously optimized plasma treatment and a newly developed automatic plasma treatment device were used to modify three types of tubes. In Table 4.6, an overview of analysis suggests what is presented in this chapter.

Table 4.6: Overview of analysis performed on tubes.

Material	Plasma treatment	Analyzing method
Sample 1, 2 and 3	5-time with argon atmosphere	WCA, ageing (18 days)
		Surface free energy
		AFM (morph. and topography)
		XPS (survey and high resolution s.), ageing (6 days)
		DSC

4.3.1 Water Contact Angles

During vertical plasma treatment experiments, tubes were always cut in four pieces and WCAs were measured on different places along all of them. As can be observed from Figure 4.21, untreated tubes have almost identical wettability around 90 degrees, while plasma-treated exhibit much more hydrophilic properties. Sample 2 changes its wettability in the most dramatic way and becomes almost super hydrophilic after a relatively short gaseous plasma treatment. This is definitely not what one might expect from PP at least not according to the literature. The lowest water contact angle achieved by atmospheric pressure argon plasma on PP was reported to be 30 degrees by Akishev et al. [46]. It has to be taken into consideration that WCAs measured on curved surfaces are of comparative nature and are not directly comparable to those found in articles.

Sample 1 displays better wettability improvement than Sample 3, but both are far less hydrophilic than Sample 2. As can be observed from standard deviations from 8 measurements on each sample, vertical plasma jet treatment homogenously changed tube characteristics. The differences between top and bottom positions are within measurement error (3 degrees) and are therefore neglected. The results in Figure 4.21 are for all measuring areas shown in Figure 4.10 combined.

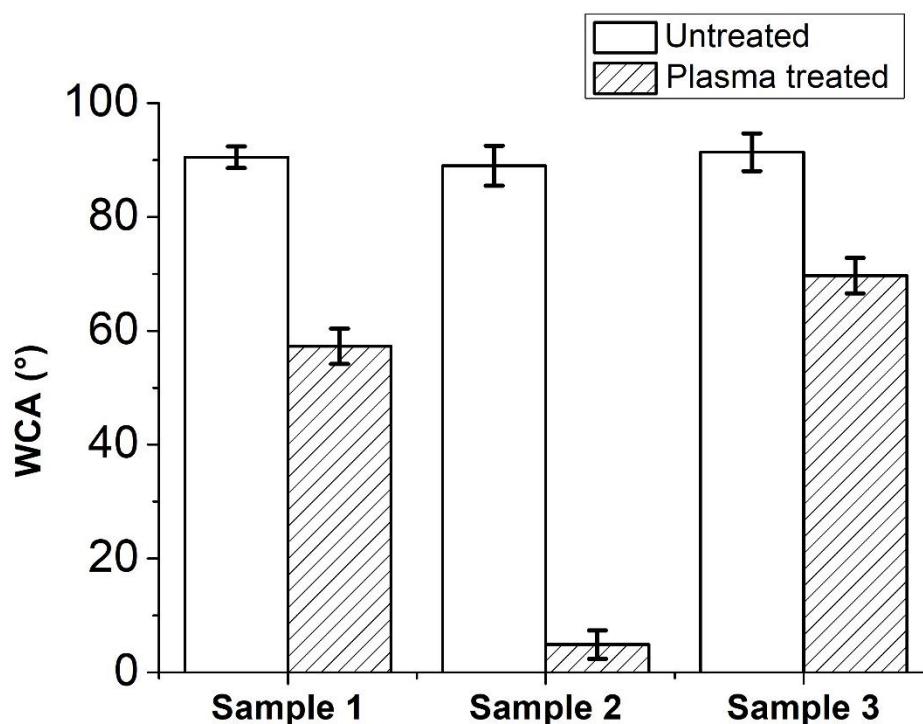


Figure 4.21: WCA of untreated and plasma-treated tubes.

4.3.2 Ageing of Water Contact Angles

Water contact angles were measured on plasma-treated samples over the period of 18 days after treatment. Each measurement in Figure 4.22 is an average of 8 measured WCAs from one specific sample. Some statistic error can be attributed to material differences inside each sample and to measurement conditions like room temperature and humidity that cannot be controlled over such a period of time. The first point of each line is the WCA of untreated sample. The second point or day zero represents a freshly treated sample, measured within one hour after plasma treatment. As can be seen from Figure 4.22, more hydrophilic samples tend to age faster and all samples have a tendency to have a WCA of around 70 degrees after 4 days of ageing. This value does not change considerably up to day 18 for any of the samples. After 18 days the WCAs are 73.9; 68.2 and 65.4 degrees for Sample 1, 2 and 3, respectively.

It can be concluded that plasma jet treatment lowers WCA of polypropylene tube for approximately 20 degrees and this change is permanent during a longer period of time. This improvement can be attributed to morphological and chemical changes, even though chemical changes are not completely permanent. The surface chemistry does not return to the initial state, but some double bonds are broken and some functional groups reorient. Most of the single-bonded oxygen (C-O) and carboxyl groups (COOH) are present even months after plasma treatment, but double-bonded oxygen (C=O) content tends to decrease as can be observed from our XPS measurements of aged samples in Table 4.10.

Furthermore, Sample 2 has an observably higher standard deviation than other two samples. This deviation is not observed on untreated and freshly treated sample therefore it can be attributed to chemical ageing effects. The process of defunctionalization and reorientation is fast for this sample and uneven surface results in uneven ageing effects.

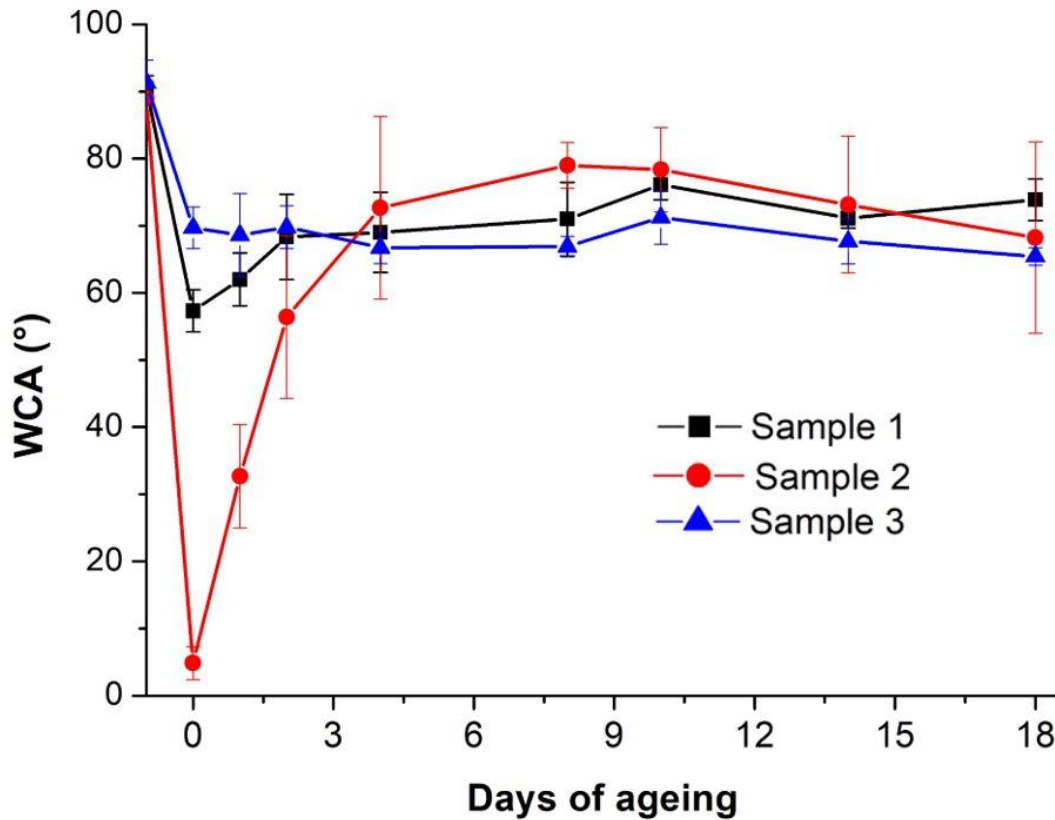


Figure 4.22: Ageing of WCA over an 18-day period for Samples 1-3.

4.3.3 Surface Free Energy

Total surface energy rises for all plasma-treated samples as expected (Table 4.7). The highest rise is observed for Sample 2 which is in accordance with WCA results. Literature usually reports the increase of the polar component of surface free energy (σ_{AB}) while the non-polar component (σ_{LW}) stays unchanged [40, 44]. This is roughly the case with Sample 1, where the non-polar component increases for less than 4 % while the polar component increases for more than 3-fold. These numbers are already higher for Sample 3, a 16 % increase of non-polar and over 5-fold increase of polar, but even higher for Sample 2. Sample 2 reaches the highest surface free energy value of 48.42 mJ/m², starting from the lowest surface free energy amongst all samples (33.44 mJ/m²). In case of Sample 2, both the non-polar and polar component increase dramatically (Figure 4.23). The increase of the non-polar component is 23 % while the polar component is increased for more than 6-fold.

All of the values are in good agreement with the literature describing PP modification by CAP [38, 90, 92, 96–98], where values around 30 and 50 mJ/m² are reported for untreated and plasma-treated PP, respectively.

Table 4.7: Surface free energy and its components for all samples.

Surface free energy (mJ/m ²)	Untreated			Plasma treated		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
σ total	38.4	33.4	37.6	42.1	48.4	45.7
σ LW	37.4	32.0	37.1	38.8	39.5	43.0
σ AB	1.1	1.4	0.5	3.3	8.9	2.7
σ +	0.1	0.1	0.1	0.2	1.1	0.2
σ -	2.7	3.8	0.4	15.7	18.5	7.6

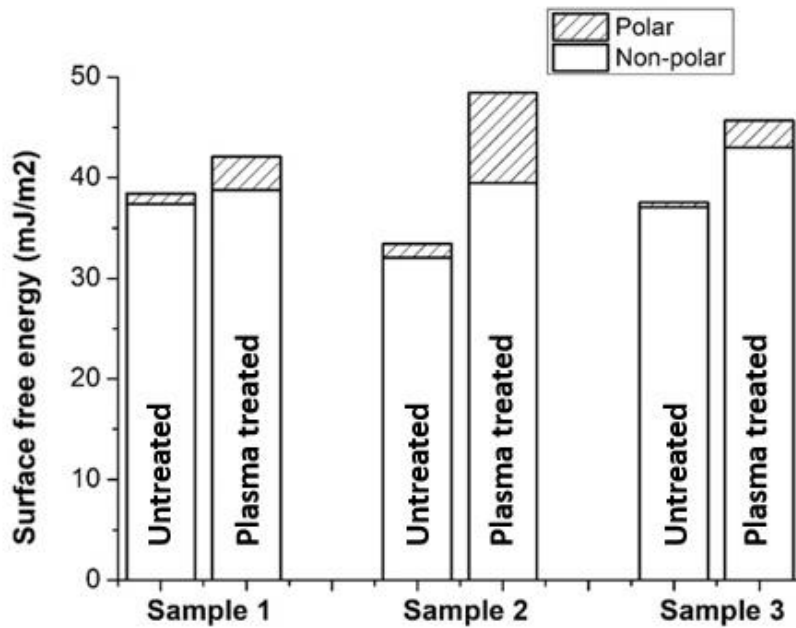


Figure 4.23: Surface free energy divided into a polar and non-polar component.

Results in Figure 4.23 are in good agreement with XPS results shown in Chapter 4.3.6. They confirm a direct link between the surface oxygen content and surface free energy.

4.3.4 Surface Morphology

In this chapter, AFM figures of untreated and treated samples are presented in 10 x 10 μm (Figure 4.24, Figure 4.25) and 2 x 2 μm areas (Figure 4.26, Figure 4.27). Both 2 and 3 dimensional figures are shown for the same area of each sample.

From 10 x 10 μm figures in 2D (Figure 4.24), it can be seen that every sample has its own unique morphological features. These were of course measured on multiple areas along the inner surface of tube and only the most representative are shown herein.

Sample 1 in an untreated state exhibits parallel lines/grooves, probably a result of the manufacturing process. When the tube was injection molded, the molds' surface presumably got imprinted onto the tubes' surface. Upon further examinations, there are large aggregates (5 μm and more) present on the surface, increasing roughness and making morphology uneven. At this point it is hard to predict their origin. After plasma treatment

they are not observed anymore, we predict these aggregates are loosely bound to surface and easily etched or removed in another manner.

Sample 2 has a different surface structure, exhibiting sheet-like forms, oriented in multiple directions. The edges of surface structures are sharp and clearly visible. There is no order and it can only be predicted that the formation of such structures is caused by a fast cooling down process after injection molding in combination with raw material blend.

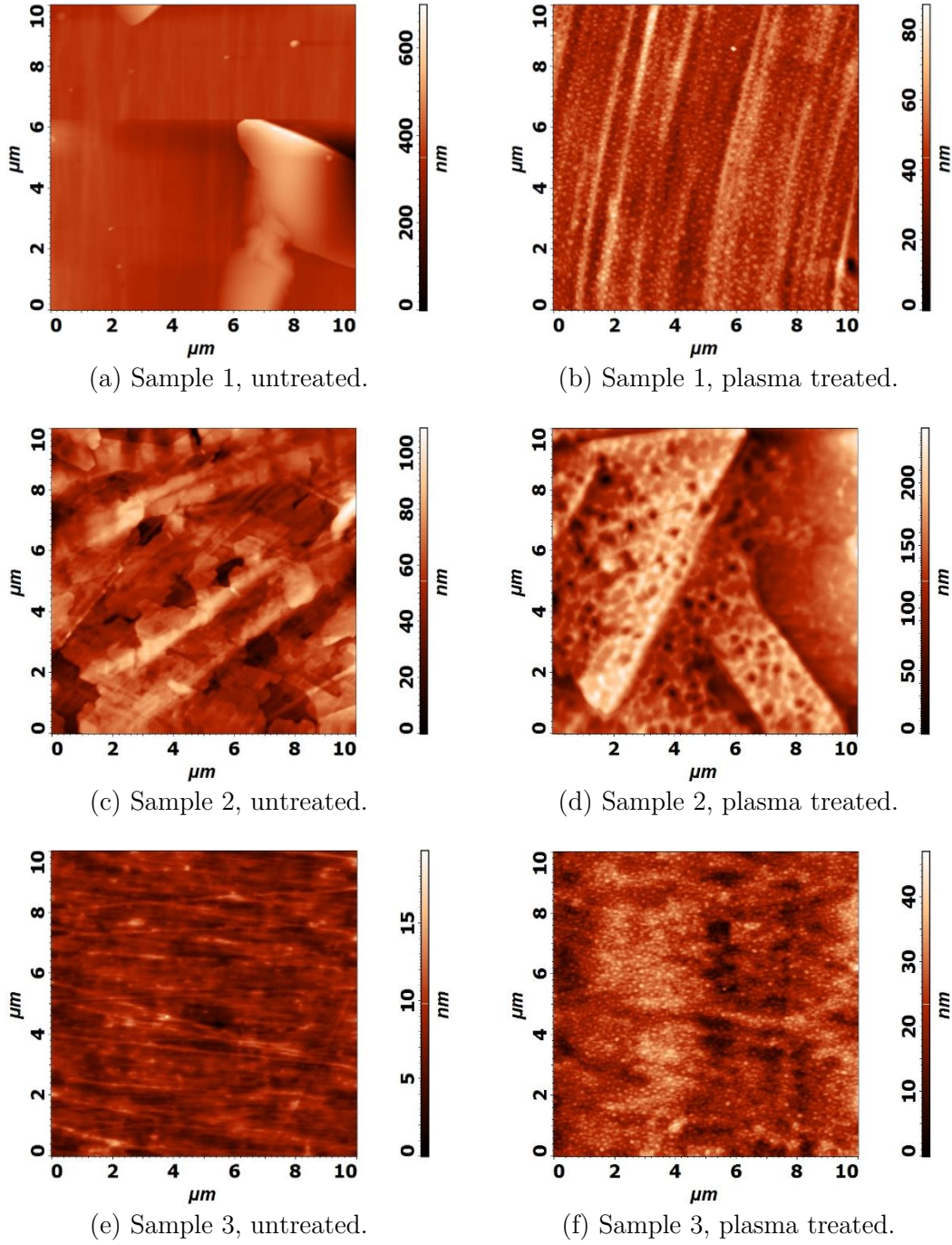
Sample 3 is the least rough of all the samples and has a really fine structure. There is a slightly visible orientation of surface texture. Surface is homogeneous even on larger areas than presented here. The manufacturing process must have been carefully optimized and the quality of injection mold's surface finish is superb, most probably polished to a mirror shine.

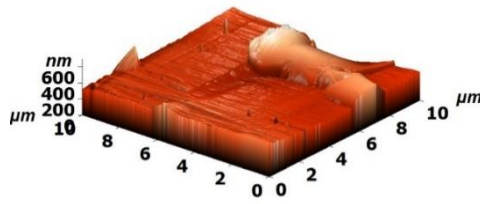
After plasma treatment all samples display changes in surface morphology. Grooves are still present and visible on Sample 1 and 3 and sheet-like structure can still be observed on Sample 2. The difference is that plasma etching has occurred and grain-like structure appears on Sample 1 and 3 while the surface of etched Sample 2 can be better described as crater-like structured. This phenomenon is mainly correlated with differences in etching of amorphous and crystalline parts of polymer, as it was already shown that amorphous parts are more easily etched compared to its crystalline counterparts [29, 30]. Same effect is also confirmed by the images from $2 \times 2 \mu\text{m}$ area (Figure 4.26).

In case of plasma-treated Sample 1 and 3 (Figure 4.24 (b) and (f)) the grain-like structures are approximately 250 and 120 nm in diameter and 10 and 8 nm in height, respectively. Grain-like structures are more commonly reported with grains growing with increased plasma treatment time [37, 42, 91] until a certain threshold after which the grains are etched away and the process of grain growth is periodically repeated [92]. The craters on Sample 2 are considerably larger compared to grains, the average crater is about 350 nm wide and 70 nm deep.

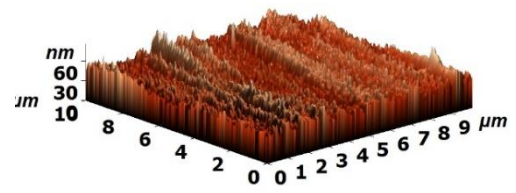
Three-dimensional AFM figures show a similar trend (Figure 4.25, Figure 4.27). The tube surface becomes highly nanostructured after plasma treatment. It should be mentioned that the height of nanostructures in Z axis is shown in nanometers while X and Y axis are in micrometers. This is to emphasize the results and improve the visual presentation. For Sample 1 and 3 the size and layout of grain structures are fairly uniform, while for Sample 2 craters form over previously uneven surface and take more random shapes. Samples 1 and 3 results are in good correlation with the results previously obtained on PP foil.

More on surface morphology can be found in Appendix D where AFM images of $5 \times 5 \mu\text{m}$ area are presented in 2D (Figure 5.30) and 3D (Figure 5.31).

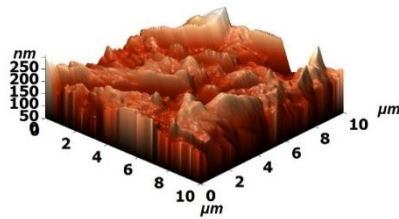
Figure 4.24: 2D AFM image on 10 x 10 μm area.



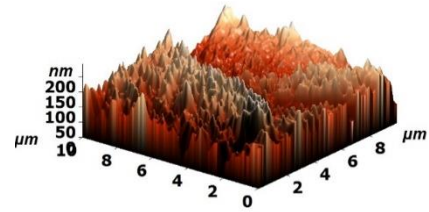
(a) Sample 1, untreated.



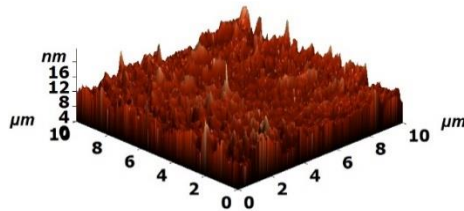
(b) Sample 1, plasma treated.



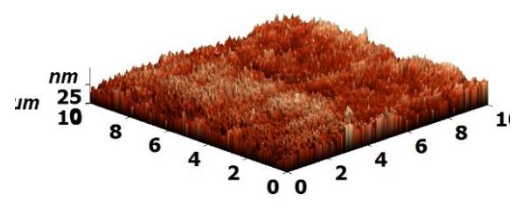
(c) Sample 2, untreated.



(d) Sample 2, plasma treated.



(e) Sample 3, untreated.



(f) Sample 3, plasma treated.

Figure 4.25: 3D AFM image on 10 x 10 μm area.

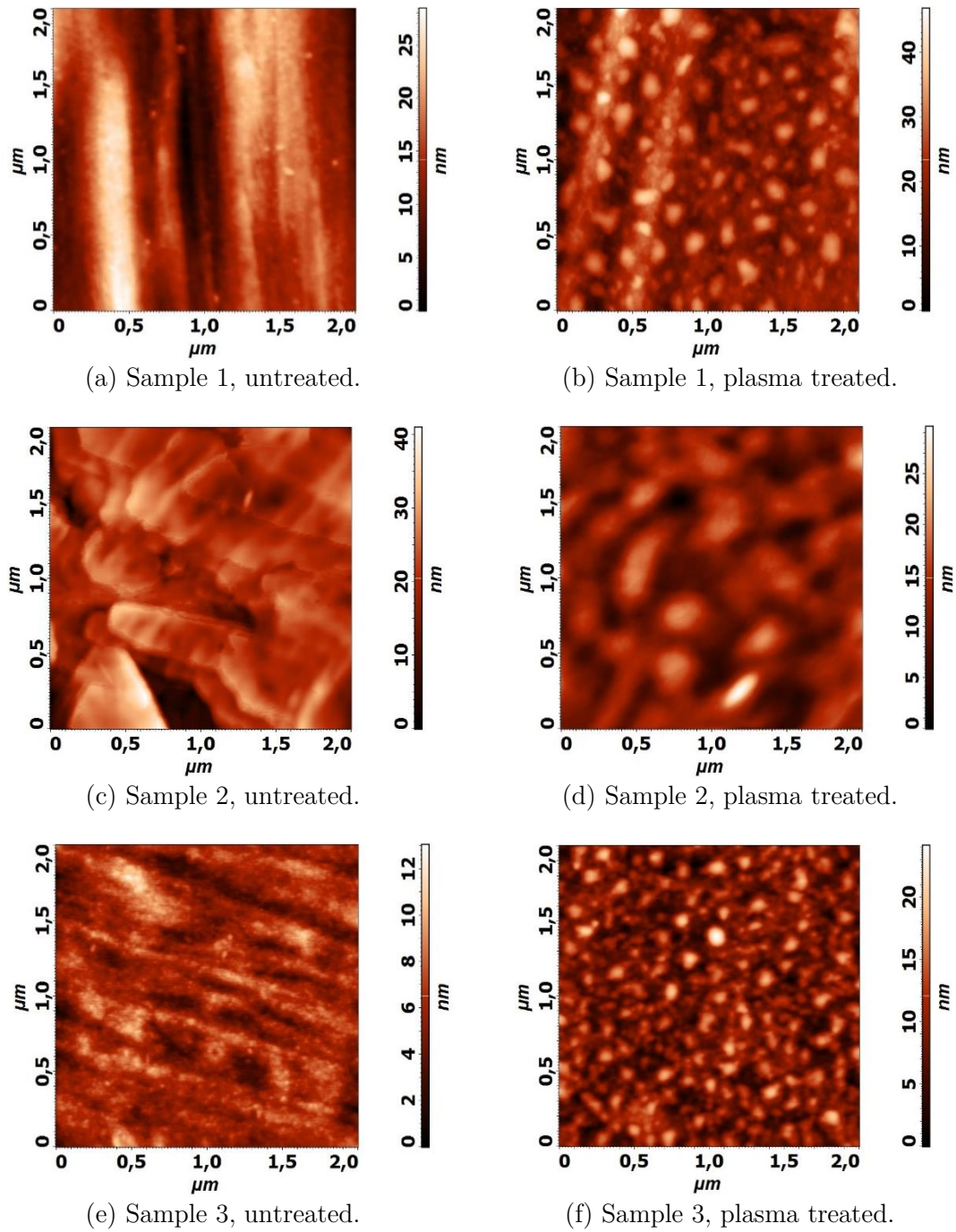
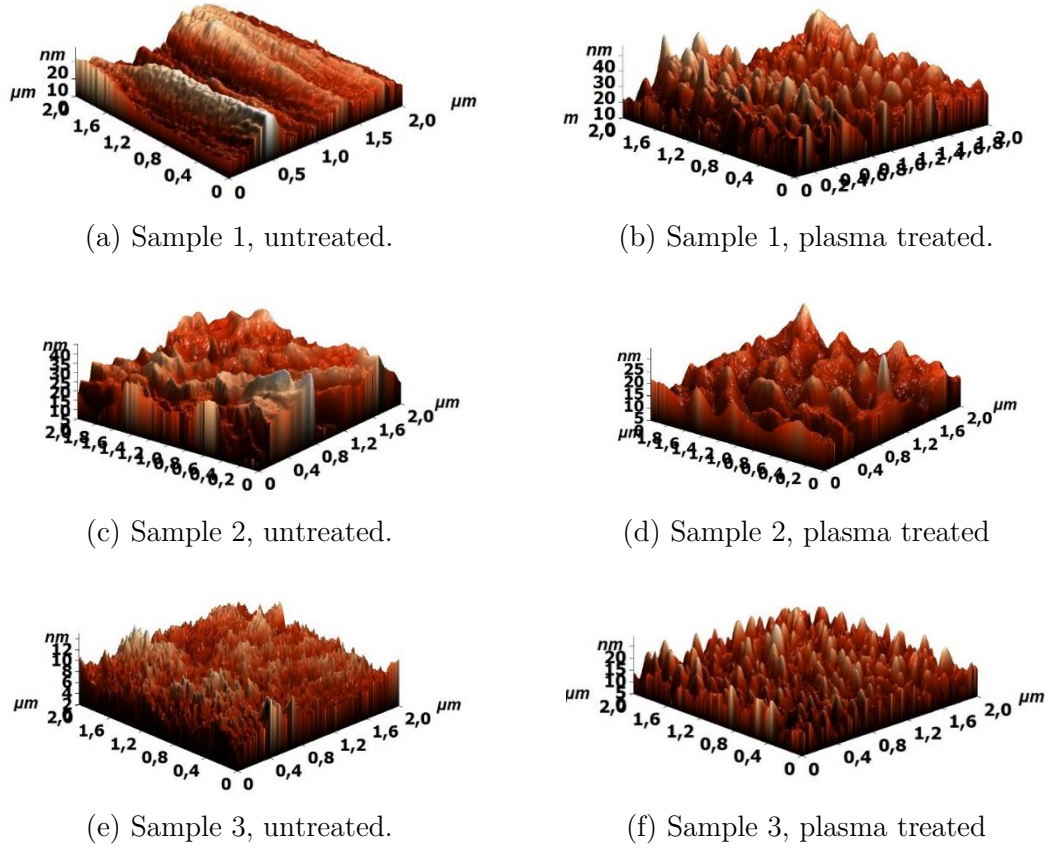


Figure 4.26: 2D AFM image on $2 \times 2 \mu\text{m}$ area.

Figure 4.27: 3D AFM image on 2 x 2 μm area.

4.3.5 Surface Topography

Expectations according to literature were for average roughness to increase due to plasma etching [39, 89, 91, 92, 96, 99], but some also report about roughness decrease [38]. As can be observed in Table 4.8, in case of tube samples, roughness increased for all of them after plasma treatment. These results were obtained on 1 x 1 μm areas, because it is easier to exclude macro roughness on smaller areas and show only the newly formed nanostructured one. Razes and other artefacts from the manufacturing process are present on larger areas and they affect the roughness result, making nanostructure roughness negligible. It has to be stated that it is normal to observe lower average roughness for smaller AFM measured areas. As this method is highly sensitive and none of the examined surfaces is perfectly flat, it is normal that on smaller areas, less grooves, craters or agglomerates are found and therefore roughness is lower.

Sample 2 has its unique surface nanostructure due to lower crystallinity (as seen in the Chapter 4.3.7.) but it does not stand out with its roughness results. According to measurements of the three samples presented here it is medium rough. As stated in the previous chapter, craters are observed visually but none of the existing surface roughness parameters could undoubtedly confirm that.

The measurements of root mean square surface roughness S_q (Figure 5.32) and highest peak to lowest valley distance S_z (Figure 5.33) correspond well with average surface roughness measurements and do not provide many additional insights (Appendix D). Calculated standard deviations are in some cases higher for highest peak to lowest valley distance.

Table 4.8: Average surface roughness (Sa) on 1 x 1 μm area.

1 x 1 μm area	Untreated - Sa (nm)	Treated - Sa (nm)
Sample 1	2.48 ± 0.51	3.90 ± 1.32
Sample 2	2.40 ± 0.34	2.64 ± 0.52
Sample 3	0.74 ± 0.21	2.58 ± 0.05

Sample 1 has the highest average surface roughness before and after plasma treatment. The average roughness of Sample 2 increases for 10 % while the average roughness of Sample 3 increases for more than 3-fold. These results can be correlated with crystallinity measurements on DSC in a way that higher crystallinity means more etching and a larger difference in average surface roughness between the untreated and plasma-treated sample.

4.3.6 Surface Chemistry

After acquisition and close examination of preliminary results from PP foil, it was decided that 5-time treatment of tubes is optimal and should be explored further. This was chosen not only on the base of XPS but also AFM and WCA analyses results. At this point tubes were plasma treated and analyzed by XPS. Results are shown in Figure 4.28 as O/C ratio and Table 4.9 where atomic concentration of elements calculated from XPS survey spectra is presented. The detection limit of XPS measurements is approximately 1 atomic percent.

Table 4.9: Atomic concentration for untreated and plasma-treated samples.

		C 1s (at %)	O 1s (at %)	Si 2p (at %)
Sample 1	Untreated	89.9	8.3	1.8
	Treated	84.8	15.2	0
	Treated 6 days aged	87.4	12.6	0
Sample 2	Untreated	90.2	9.8	0
	Treated	81.0	19.0	0
	Treated 6 days aged	83.9	16.1	0
Sample 3	Untreated	99.4	0.6	0
	Treated	85.1	14.9	0
	Treated 6 days aged	87.9	12.1	0

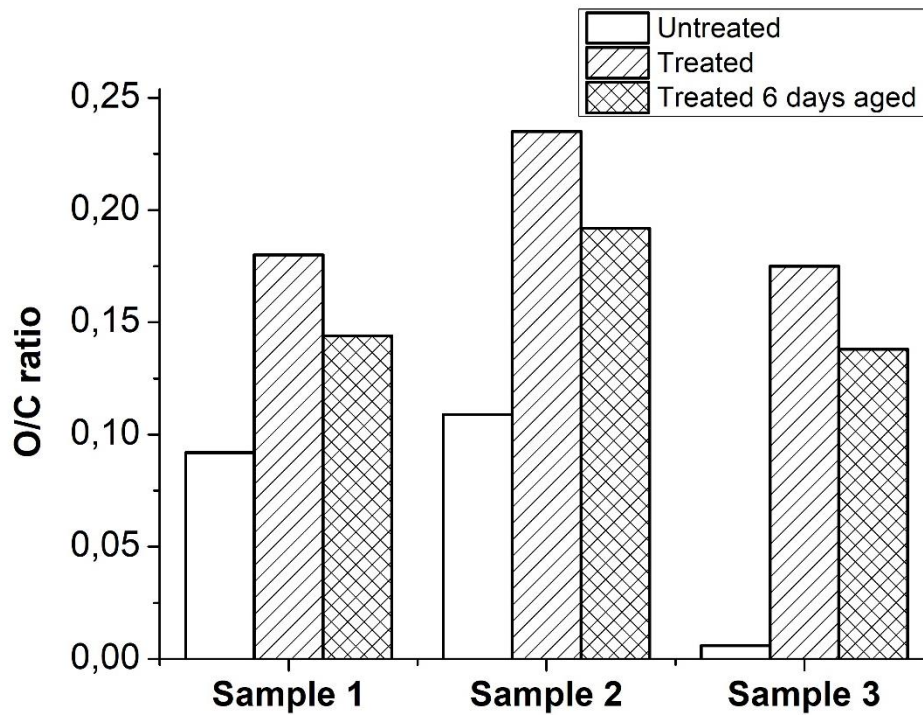


Figure 4.28: Oxygen/Carbon ratio for untreated and plasma-treated samples.

In Table 4.9 the chemical composition of the tube surface is given. As can be seen from Table 4.9, Sample 3 has almost no oxygen present on the surface prior to gaseous plasma treatment, which would be expected of polypropylene polymer, as no oxygen is present in its chain. However other PP samples have about 8 to 10 at % of oxygen on the surface. The moisture and oxygen from the atmosphere are removed by high vacuum inside the XPS, thus oxygen content on the surface is relevant and could be observed due to various fillers used in polymer manufacturing. For example, SiO_2 could be a possible filler used in Sample 1, as about 2 at % of Si are also observed on the polymer surface. Other results showing Si are on the limit of detection. Results of XPS analysis performed on different samples clearly indicate that PP samples differ in surface composition, which not only influences the microbiological interactions with microvesicles, but also on interaction of the surface with plasma species. After plasma treatment a higher oxygen content on the surface of all tubes was observed, however it seems that the highest concentration of oxygen on plasma-treated surfaces was about 15 to 20 at %. These results are in good agreement with most of the topic-related papers reporting concentrations around 9 to 15.1 at % of oxygen [92, 93, 97, 100], with two exceptions. Kwon et al. report the highest concentration at 42.9 at % of oxygen on the PP film after argon plasma treatment at atmospheric pressure [38]. This is probably the result of PP film preparation prior to plasma treatment and the use of much higher plasma treatment power and longer treatment time. The second is Kostov et al., where 27 at % of oxygen is reported before plasma-treated PP is rinsed with water and the concentration drops to 12.5 at % [39]. It is suggested that rinsing dissolves low molecular weight oxidized material (LMWOM) which is loosely bounded and is responsible for the hydrophobic recovery.

Nevertheless, small changes in initial chemical composition can highly influence surface functionalization and etching, as competition between functionalization and etching is taking part during plasma treatment. This process continues until the saturation limit is

reached. The oxygen functionalization is achieved by the surrounding air and moisture, which are excited and made reactive by excited argon species from the plasma jet.

Deconvolution of high resolution carbon spectra is presented in Table 4.10. Curves were adequately fitted and the percentage of each carbon bond was acquired. As expected, the C-O bonds increase the most after plasma treatment (increase of 7.8 to 9.1 at %). After 6 days of ageing under room conditions, the percentage of single bonded oxygen drops by 2.5 to 3.8 at %, which is a consequence of defunctionalization or reorientation of such bonds. More intriguing is how the C=O share drops after ageing in all cases while O=C-O remains almost constant.

Table 4.10: High resolution carbon spectra results.

		C-C (285.0 eV)	C-O (286.5 eV)	C=O (287.8 eV)	O=C-O (289.2 eV)
Sample 1	Untreated	96.3	2.4	0	1.2
	Treated	82.1	10.2	3.7	4.0
	Treated 6 days aged	86.8	7.7	1.5	4.0
Sample 2	Untreated	90.4	7.4	0	2.2
	Treated	75.0	16.5	3.2	5.3
	Treated 6 days aged	79.4	12.7	2.4	5.5
Sample 3	Untreated	100	0	0	0
	Treated	81.8	9.1	4.8	4.3
	Treated 6 days aged	88.8	5.5	2.3	3.4

4.3.7 Polymer Crystallinity

Crystallinity highly influences the process of plasma etching and consequential morphological surface changes. The DSC thermograms were obtained for all three samples in untreated and plasma-treated state to determine their crystallization and melting behavior. Plasma-treated samples were analyzed due to curiosity as DSC is a bulk and not surface analysis method and plasma is only expected to change the crystalline fraction of the topmost layer. There were no statistically relevant differences among untreated and plasma-treated samples, therefore only untreated are presented here. The results from Table 4.11 indicate that Sample 3 has the highest and Sample 2 has the lowest crystallinity. Samples 1 and 3 have similar crystallinities while Sample 2 stands out, it has considerably lower crystallinity, meaning there is more amorphous phase present.

Furthermore, height and width of melting and crystallization peaks from Figure 5.34. Figure 5.35 and Figure 5.36 in Appendix E can give us some insight on size uniformity of crystallites. Sample 1 and 3 have narrow melting peaks, meaning the crystallites are highly uniform and melt at the same time during heating. On the contrary, Sample 2 has a wide melting curve with two observable peaks. It can be concluded that there are two uniform groups of crystallites present and their sizes are not uniform. Due to different sizes and properties these crystallites melt at different temperatures, making the melting peak wider.

Table 4.11: DSC results and calculated crystallinities.

	T_m (°C)	T_c (°C)	Crystallinity (%)
Sample 1	161.3	119.1	53.6
Sample 2	149.3	120.6	41.8
Sample 3	166.0	133.4	58.0

It was shown on AFM images that Sample 2 stands out with its morphology. This can be correlated with its low crystallinity and furthermore with the two populations of crystallites suggested by DSC thermogram (Figure 5.35). The etching process is faster for the amorphous parts compared to crystalline parts [35], therefore it is possible that the craters are the largest on Sample 2 because it has the lowest crystallinity. Two populations of crystallites are on the other hand presumably different in size and well distributed across the surface and probably prevent the formation of a grain-like structure observed on Sample 1 and 3. Instead the two populations could slow down the etching process of the majority of the surface and only the fully amorphous parts are observably etched. This hypothesis can be nicely supported by the etching results reported by Wohlfart et al., [101]. It is suggested in their work that the so-called mesophases between purely crystalline and purely amorphous domains have intermediate etching rates, blurring the surface patterns. Sample 2 having the lowest melting temperature could also contribute to craters being as wide and deep as they are. The surface could be more intensively locally melted when impacted by highly energetic ions from plasma jet.

4.4 Biomedical Experiments

In this chapter, the interaction between microvesicles and proteins in contact with tube surfaces was closely studied. Flow cytometry of microvesicle isolates and UV-Vis protein adsorption measurements were performed on all samples while DLS and AFM were performed only on Sample 1 and 2. This is the result of the complexity level of such analyzes that were simply impossible to perform on all samples. 5-time plasma treatment inside argon atmosphere was again used for tube preparation as it was found to be optimal. The overview of biomedical experiments performed is presented in Table 4.12.

Table 4.12: Overview of analysis performed on tubes.

Material	Plasma treatment	Analyzing method
Sample 1, 2, 3 with MVs	5-time with argon atmosphere	Flow cytometry
Sample 1, 2 and MVs from Subject 1 and 2		DLS
		AFM
Sample 1, 2, 3 and proteins		UV-Vis adsorption

4.4.1 Experiments with Microvesicles

4.4.1.1 Isolation and Flow Cytometry

Preliminary experiments were performed within other studies where microvesicles were isolated. The first assumption was that all polypropylene tubes had the same characteristics and these characteristics can be changed by CAP treatment. It was soon obvious that tubes from different manufacturers had different characteristics before as well as after

plasma treatment. The preliminary results of microvesicle isolation performed in the scope of other larger microvesicle researches were not always favorable but the trend of plasma-improved tubes was visible (Table 5.2, Appendix F). Multiple tubes were compared but any statistical conclusions were impossible to make at this point. There were never enough repetitions measured at once because there were not enough blood donors. Over time, conditions, flow cytometer settings and personnel changed and cause the results to be incomparable. All the plasma-treated tubes involved in preliminary experiments were plasma treated in horizontal position (Figure 4.17) and therefore the repeatability might not have been perfect. Only later on we discovered that tubes have to be treated in vertical position to ensure even surface treatment. The last set of preliminary experiments involved only two subjects that were labelled Subject 1 and Subject 2 and were used also for further analysis first on the device for dynamic light scattering (DLS) and later on atomic force microscope (AFM). The results of these analyses are presented in the following two chapters. In the Table 4.13, microvesicle count of Subject 1 and 2 is presented for untreated Sample 2 and 1 and for plasma treated Sample 1. There is no trend and no conclusion can be made from these results. They are shown only as supplement to flow cytometer scatter diagrams, DLS and AFM results.

Table 4.13: MV count for Subject 1 and 2.

Donor	Untreated Sample 2	Untreated Sample 1	Treated Sample 1
Subject 1	1773/ μ L	1352/ μ L	1570/ μ L
Subject 2	2606/ μ L	6721/ μ L	4013/ μ L

In Figure 4.29 and Figure 4.30 results from flow cytometer are visually presented, showing forward and side scatter of microvesicle population for Subject 1 and 2. The population of measured particles is presented in scatter diagram with colors from red to blue where red is the highest concentration and blue the lowest. From left to right, top to bottom, graphs are showing results from untreated Sample 2, untreated Sample 1, plasma-treated Sample 1 and background, respectively. PBS might not have been as pure as in the following experiments as there are around 100 events per μ L detected in PBS itself. This means that the results from flow cytometry of Subject 1 and 2 deviate between 1.5 and 7.4 % only because of the PBS.

The first experiment was performed with 11 donors/volunteers. The aim was to make a statistically relevant study of all three types of tubes in untreated and plasma treated state at once. To get an information about predicted improvement of microvesicle isolation due to plasma treatment of tubes it was important to use the blood of one donor for one set of six different tubes (3 types, untreated and treated). Another important aspect was that all the samples were processed the same day under same conditions by the same person. The probability of events inside the measured populations were satisfying, between 82 and 97 %. This means that there was an almost uniform population and the majority of events were counted and present the final number of microvesicles in each isolate. In case of lower probabilities there might have been something wrong with isolation process and the isolate could potentially contain particles other than microvesicles. The highest amount of measured microvesicles per μ L was 1232 and the lowest 49. The difference in counted concentration is conditioned by each individual donor and any correlation between them is hard. Each donor is his or her own reference point. All of the results are presented in Table 5.3 in Appendix F.

The second experiment was conducted soon after the first to confirm the results of the first and to get a better idea about statistic repeatability. 9 donors/volunteers participated in this experiment. Results are presented in Table 5.4 and Table 5.5. The last three donors,

numbered donor 18, 19 and 20, had their isolates processed in the tubes of the same manufacturer to get an information on repeatability of one sample inside one type of tube, treated and untreated. The probability of events was again similar, between 81 and 98 %, with one exception of 63 %. This result was discarded. The highest amount of measured microvesicles per μL was 1428 and the lowest 122, which is similar to the first experiment. Only a short amount of time passed between both experiments, conditions were similar, same people prepared the samples and performed measurements and the flow cytometer was calibrated similarly. As can be seen from Table 5.5, the MV count results of each donor in one kind of the tube already deviates for more than 50 % on average. Similar deviations are confirmed by calculations from other measurements as well. The complexity of isolation including human factor (blood collection, pipetting precision and velocity, the sequence of measurements etc.) and flow cytometry error make the process that is already dealing with highly individual-dependent biomedical samples, even more difficult for interpretation.

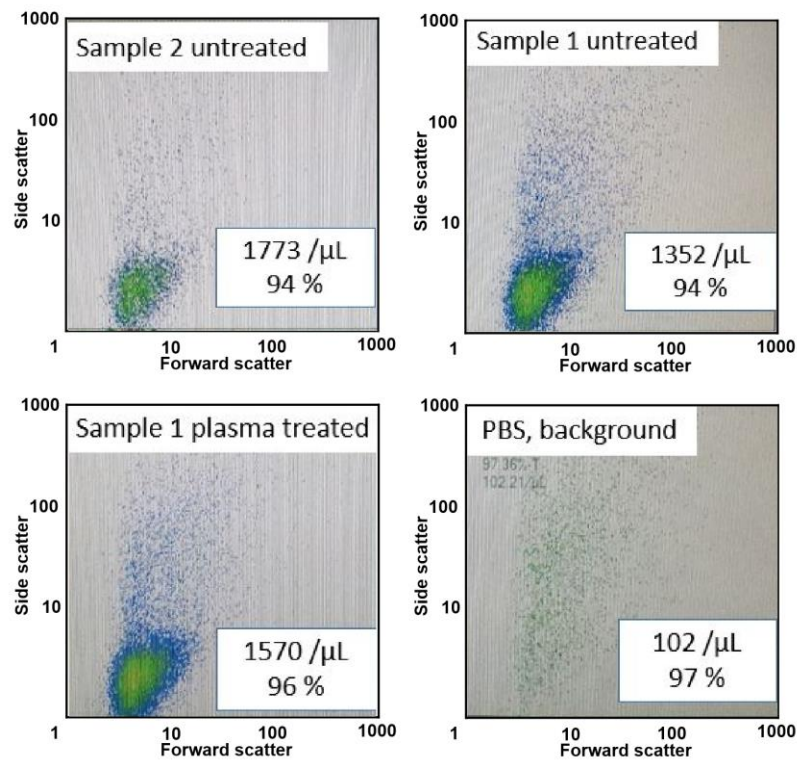


Figure 4.29: Flow cytometry results Subject 1.

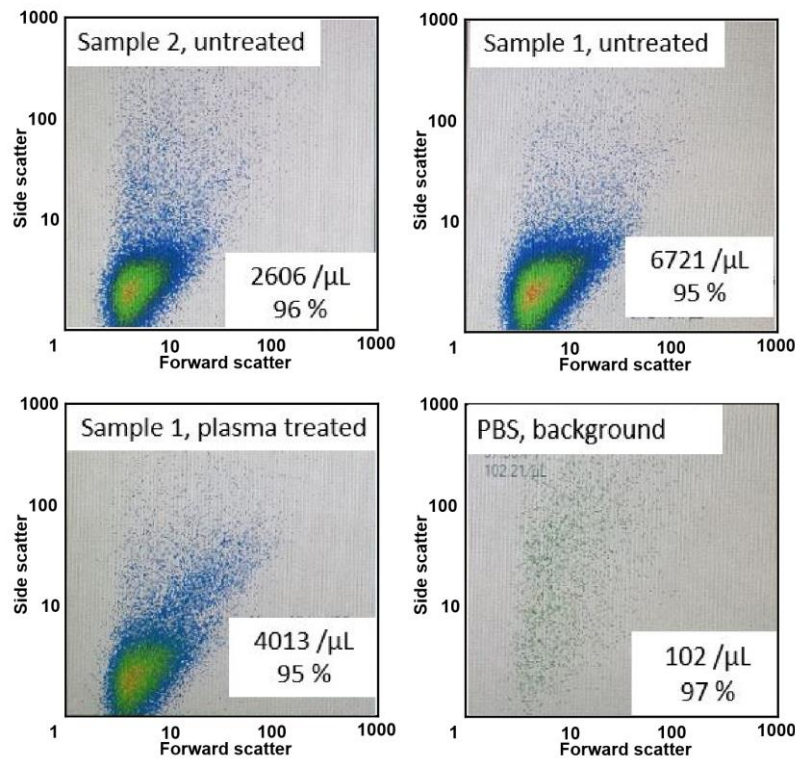


Figure 4.30: Flow cytometry results Subject 2.

Ratio between plasma-treated and untreated tube results was calculated to get a better idea of how much the plasma treatment of tubes improved microvesicle isolation yields. The sample from each donor could only be compared to itself but the ratio of improvement can be compared among all the samples and the average can be calculated. The final average of results can be seen in Table 4.14.

Table 4.14: Average MV count and average calculated improvement.

	MV untreated (/ μL)	MV treated (/ μL)	Improvement (%)
Sample 1	388	394	24
Sample 2	365	455	23
Sample 3	431	487	15

The discrepancies (especially in case of Sample 1) between results in Table 4.14 originate from the different number of subjects included in the study. In the calculation of improvement all 20 donors were included while in case of average MV count donors 18, 19 and 20 had to be excluded. These donors had their blood isolated only in one kind of tubes and therefore could not be used for the comparison of different tubes.

It can be concluded from this study that even though Sample 1 has the best calculated average improvement, both Sample 2 and 3 have a significantly higher average MV count for plasma-treated tubes. Both Sample 2 and 3 also have their calculated improvements in good correlation with the average MV count while Sample 1 does not.

4.4.1.2 Dynamic Light Scattering

The DLS analysis showed two relevant populations of particles present in the isolate. Both populations correspond to EVs, the smaller one to exosomes and the larger one to MVs, but apoptotic bodies may also be present. Due to sample complexity the results of DLS analysis are not unambiguous. The refractive index also known as the particle shape coefficient (ρ) gives an information about the shape and mass distribution of analyzed particles. In the case of microvesicles, coefficient value around 1 is expected. It must be kept in mind that in our case, it is only an estimate due to the particles being on the lower margin of detection limit. Other potential deviations can be the result of unfiltered buffer during isolation, relatively low concentration of parts in the final solution or insufficient filtration.

The results from Subject 1 and 2 show a trend for ratio between light scatter intensity and laser intensity (I_0/I_{tot}). The ratio is the highest for untreated Sample 2 and the lowest for plasma-treated Sample 1. Along with the scatter intensity ratio, the amount of large particles also drops from untreated Sample 2 to plasma-treated Sample 1, as can be seen from scatter intensity contribution in Table 4.15 and Table 4.16. This effectively suggests that either the size or amount of large particles is the highest for untreated Sample 2 and the lowest for plasma-treated Sample 1. It is also possible that large particles, in this case microvesicles, agglomerated in untreated samples and therefore exhibit a larger size and a lower count. Smaller particles could also be microvesicle fragments.

The shape coefficient factor of larger particles corresponds to the spherical shape (around value 1) as presented in Table 3.5. The deviations are within normal limits. In case of smaller particles, the shape coefficient is around 1.4 to 1.8, which suggests a random ball shape from the shape coefficients in Table 3.5. The shape coefficient (ρ) is too high due to overrated Rg value, which is the result of the particles being on the margin of measurement detection.

Table 4.15: DLS results Subject 1.

	$I_0/I_{tot}, 90^\circ$	Rh 0° (nm)	Rg (nm)	ρ
Untreated Sample 2	1.8	14	20	1.41
		172	129	0.75
Untreated Sample 1	1.2	17	24	1.45
		175	146	0.83
Treated Sample 1	0.8	14	21	1.44
		168	133	0.79

Table 4.16: DLS results Subject 2.

	$I_0/I_{tot}, 90^\circ$	Rh 0° (nm)	Rg (nm)	ρ
Untreated Sample 2	1.2	15	37	1.79
		188	189	1.00
Untreated Sample 1	1.0	20	36	1.62
		198	185	0.93
Treated Sample 1	0.8	20	35	1.68
		160	130	0.83

The main goal of this analysis was to get a confirmation that MVs represent the majority of particles in our isolates. According to the size and shape coefficient results from Table 4.15 and Table 4.16, it can be concluded that our isolates contain MVs as predicted.

4.4.1.3 Atomic Force Microscopy of Microvesicles

As microvesicles were not abundant in the isolates they proved to be difficult to locate by AFM Solver Pro which only has a maximum scan area of $45 \times 45 \mu\text{m}$. Once microvesicle or a group of them was located, images on $1 \times 1 \mu\text{m}$ area were made for higher magnification and better representation of microvesicles. Apart from height images presented here in 2 and 3D, phase images were also taken. On these images we can observe different hardness or chemical composition of measured surface and microvesicles are supposed to stand out.

Isolates from two different subjects were analyzed (Subject 1 and 2), both also measured on flow cytometer and DLS. Only the images of Subject 2 are presented here because the isolate of Subject 2 contained more MVs.

AFM images of Subject 2 isolate from Sample 2 untreated tube presented in Figure 4.31 show agglomerates roughly 100-200 nm in diameter, composed of smaller spheres with a diameter under 50 nm. Spheres are best noticeable on a 3D AFM height image (Figure 4.31 (c)). This corresponds well with the DLS measurements where agglomeration is suggested as one of the possible scenarios for larger detected size and lower count of MVs. This potential agglomeration is of course not desired in the process of microvesicle isolation for further detection or treatments in medicine.

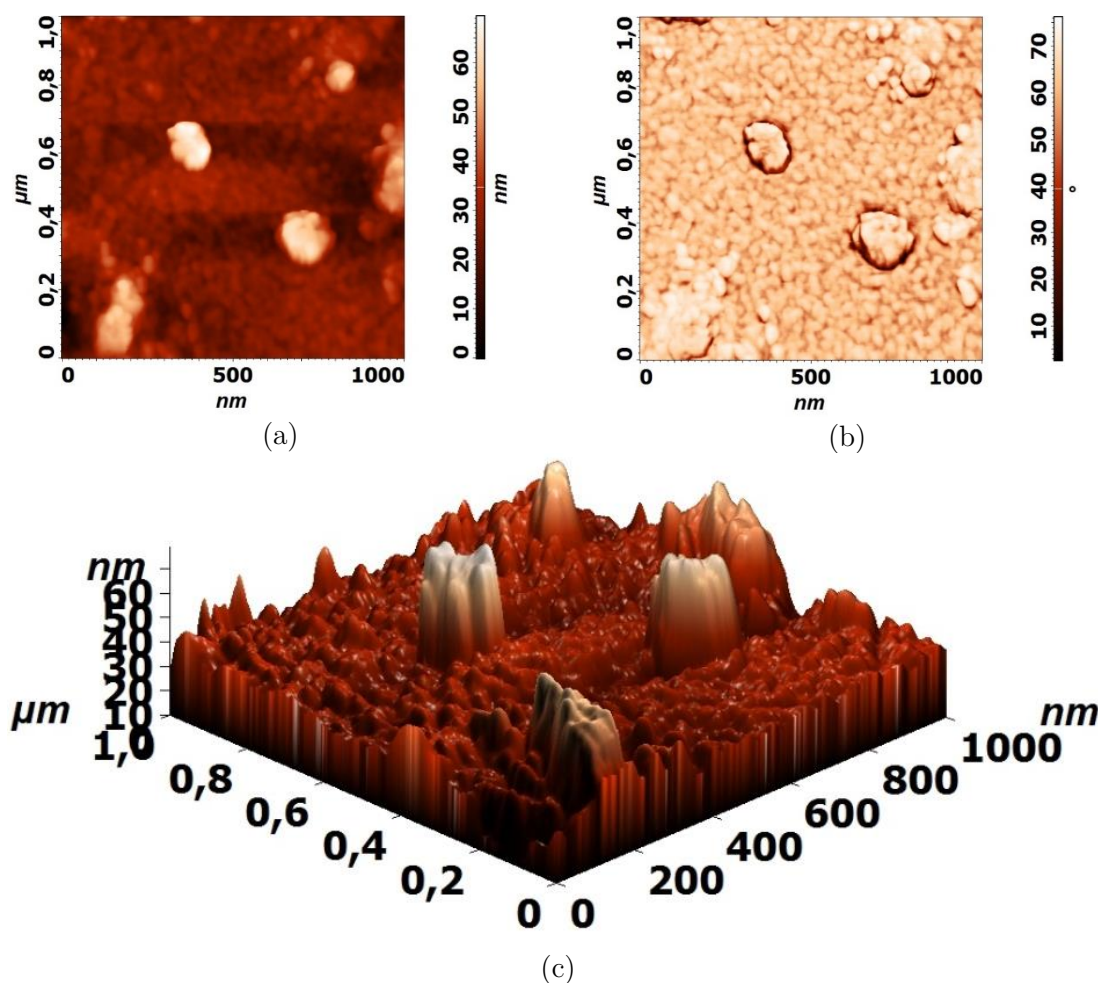


Figure 4.31: AFM of Subject 2, isolated in Sample 2 untreated tube. (a) is height image, (b) is phase image and (c) is 3D height image for better visualization.

In Figure 4.32, MV from Subject 2 isolated in Sample 1 tube in untreated state are presented. One larger MV (around 120 nm in diameter) and one smaller MV or exosome (50 nm in diameter) are presented. It is interesting to observe the almost perfectly round shape and similarities between both. The phase image (Figure 4.32 (b)) suggests there is some sort of membrane around the central part and this membrane also has a circular shape. The phase image detects this unlike the height image as it is sensitive to different attractive forces between surface and AFM needle.

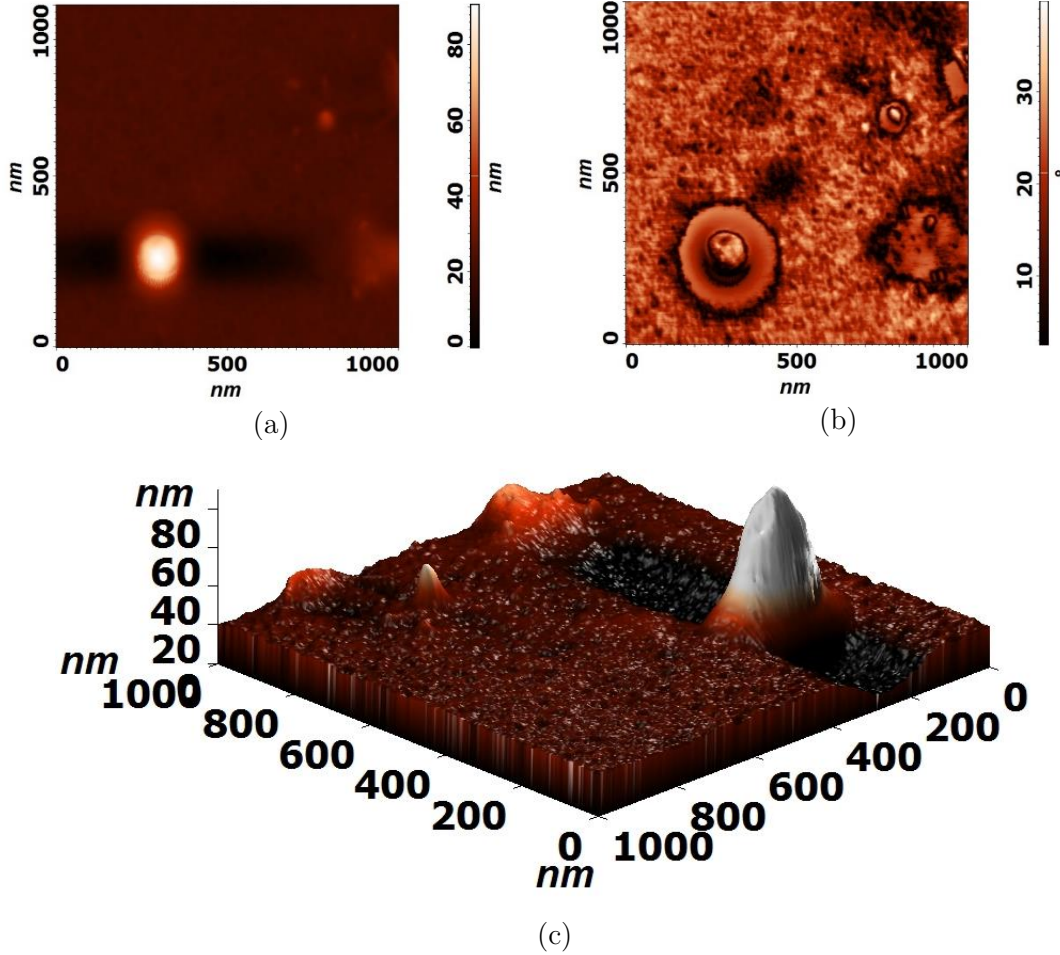


Figure 4.32: AFM of Subject 2, isolated in Sample 1 untreated tube. (a) is height image, (b) is phase image and (c) is 3D height image for better visualization.

In Figure 4.33, there is a large amount of small-sized particles present (approximately 50 nm in diameter), isolated from Subject 2 in plasma-treated Sample 1 tube. This result does not correspond well with flow cytometry measurements in Table 5.2, which suggest the lowest concentration of MVs in plasma-treated Sample 1. Once again it has to be stated that AFM is not a quantitative technique and that measuring spots are randomly chosen on a relatively large sample. Thus AFM cannot necessarily give the right quantitative information but can only be used for qualitative measurements. With this in mind, we can suggest that plasma treatment might improve MV isolation quality. This is evident if we compare Figure 4.31 where we have agglomerates and Figure 4.33 where MV are not agglomerated.

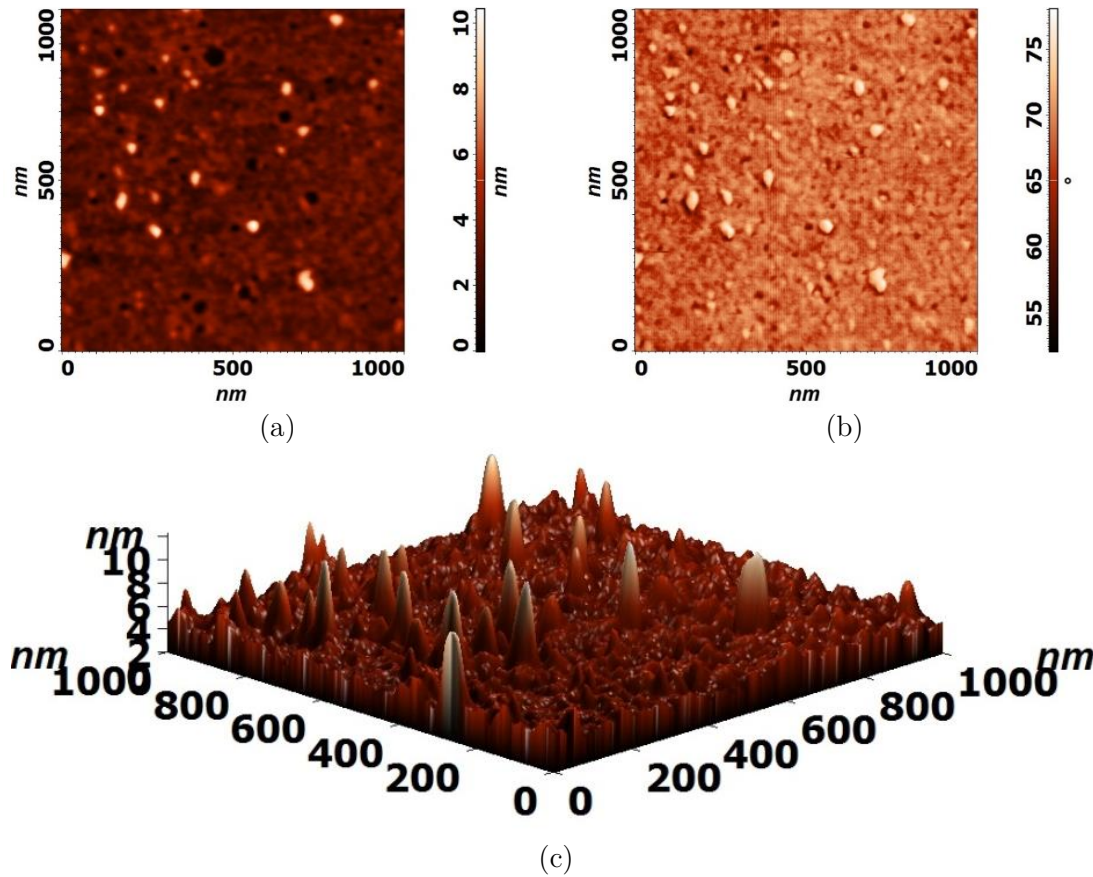


Figure 4.33: AFM of Subject 2, isolated in Sample 1 plasma-treated tube. (a) is height image, (b) is phase image and (c) is 3D height image for better visualization.

4.4.2 Protein Adsorption

The results of protein adsorption experiment show that the amount of proteins adsorbed on the tubes' inner surfaces increases after plasma treatment for all samples. The values of adsorbance in Figure 4.34 suggest that Sample 3 has the highest and Sample 1 has the lowest adsorbance of proteins. Values are rather similar among samples but are undoubtedly statistically relevant as the measurements were repeated twice in quadruplets and results were consistent.

It was of great importance to us that the analysis error was low and results were repeatable after the highly inconsistent results with MVs. In our opinion, this experiment confirmed that plasma treatment was indeed even and repeatable and that deviations of MV results were the consequence of isolation process error.

As mentioned in the methods section, proteins are also present in the blood and to some extent also in isolates with MVs. Our theory is that increased adsorption of proteins to the tubes' inner surface acts like a coating, preventing MVs from coming into contact with these surfaces. Such "coating" is naturally present in the MV isolation process but enhanced by plasma treatment. It should prevent MVs from experiencing shear stress and consequently damage, deform, agglomerate or irreversibly stick onto surfaces. In such case, increase of adsorbed proteins is preferable and useful in the process of MV isolation.

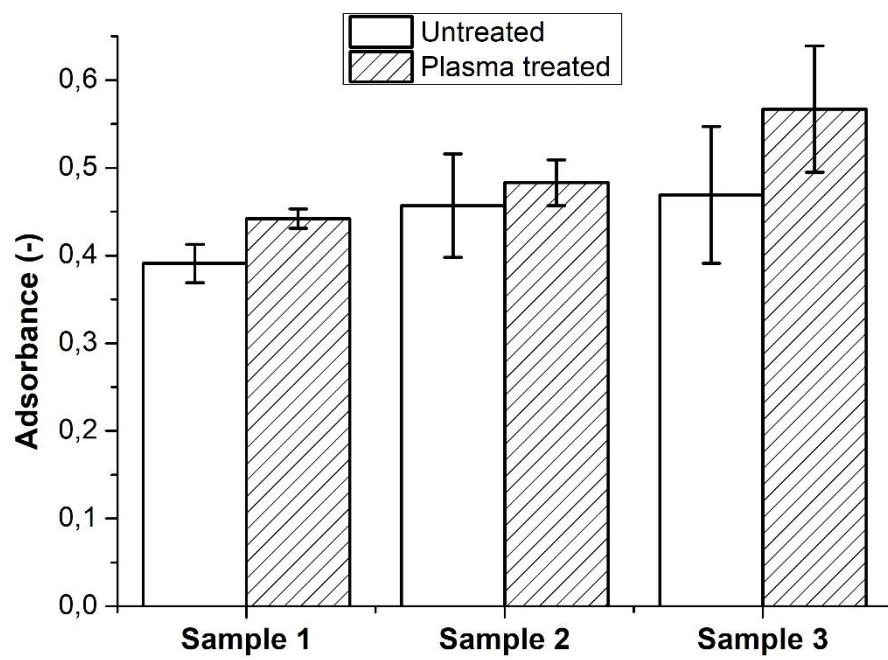


Figure 4.34: Protein adsorption results.

Chapter 5

Conclusions

Herein, properties of cold atmospheric pressure argon plasma-treated polypropylene tubes and the response of biomedical samples (MVs and proteins) to these tubes were presented. Each segment was studied in depth and finally a study of combined segments was performed. Plasma jet parameters were characterized and plasma treatment of polypropylene tubes optimized to a point where an automatic device for plasma treatment was constructed. All available analyzing techniques were employed to gather information on tubes' inner surfaces for tubes made by three different manufacturers. Large variations occurred among them as can be observed in Table 5.1, numbers 1, 2 and 3 stand for Samples 1, 2 and 3.

Table 5.1: Main findings combined.

		WCA (°)	Surface free energy (mJ/m ²)	Sa (nm)	Morph.	O 1s (at.%)	Cryst. (%)	Improvement in MV isolation (%)
1	Untr.	90.5	38.4	2.5	/	8.5	53.6	24
	Trea.	57.3	42.1	3.9	grains	15.2		
2	Untr.	88.9	33.4	2.4	/	11.0	41.8	23
	Trea.	5.0	48.4	2.6	craters	19.0		
3	Untr.	91.3	37.6	0.7	/	0.6	58.0	15
	Trea.	69.7	45.7	2.6	small grains	14.9		

All of the hypotheses were either confirmed or at least partially confirmed:

- Cold atmospheric pressure argon plasma can be used to modify the tubes' inner surface. Wettability increased, surface free energy and atomic oxygen concentration also increased, so does the average surface roughness on 1 x 1 μm area. The MV isolation improves for all three plasma-treated tube samples while the protein adsorption increases due to plasma treatment. This increase of adsorbed proteins can be favorable in MV isolation.
- Tubes' inner surface can be evenly plasma treated by appropriately optimized plasma jet parameters. Optimization steps led us to dynamic plasma treatment with the jet moving in and out of the tube in vertical position for 5-times. Treatment was confirmed to be even and repeatable by WCA, AFM and XPS measurements along the tubes' inner surface.

- An automatic device for cold atmospheric pressure argon plasma treatment was designed, optimized and programmed to treat batches of long narrow tubes under argon atmosphere in a fast, reliable and repeatable manner.
- Plasma treatment of polypropylene tubes results in higher yields of isolated MVs, calculated from the results of 20 donors. Considering the large process error involved in MV isolation, this is a positive result and a confirmation of our hypothesis.

Some other intriguing and relevant conclusions that can be drawn from this work are also:

- Surface free energy of polypropylene is closely correlated with its atomic oxygen content on the surface measured by X-ray photoelectron spectroscopy. The rise of polar component of surface free energy after plasma treatment can be directly contributed to the rise of oxygen functional groups.
- Morphological changes are in good agreement with differential scanning calorimetry results, suggesting that higher crystallinity reflects in finer surface nanostructure (smaller grains). Furthermore, the morphology of Sample 2 can be interpreted by two populations of crystallites found on DSC thermogram, causing the formation of crater-like structures.
- X-ray photoelectron spectroscopy results propose that no matter what the initial atomic oxygen content on the surface is, saturation is reached between 15 and 19 atomic percents of oxygen after plasma treatment. With ageing, this percent drops for approximately 3 atomic percents in 6 days on the account of single (C-O) and double (C=O) bonds reorientation, for all samples.
- Dynamic light scattering confirmed that particles isolated were indeed microvesicles and that the sizes are within expectations. Atomic force microscopy contributed an important insight into microvesicle isolation quality while flow cytometry only gives information about their quantity. The figures in Chapter 4.4.1.3 show how in case of untreated tube, microvesicles can agglomerate or damage, while this does not occur for the plasma-treated tube. Therefore, it can be concluded that cold atmospheric pressure argon plasma not only improves microvesicle isolation yields, but also improves the quality of isolation and furthermore decreases the chance of false results.

Appendix A

Electrical Characteristics of Plasma Jet

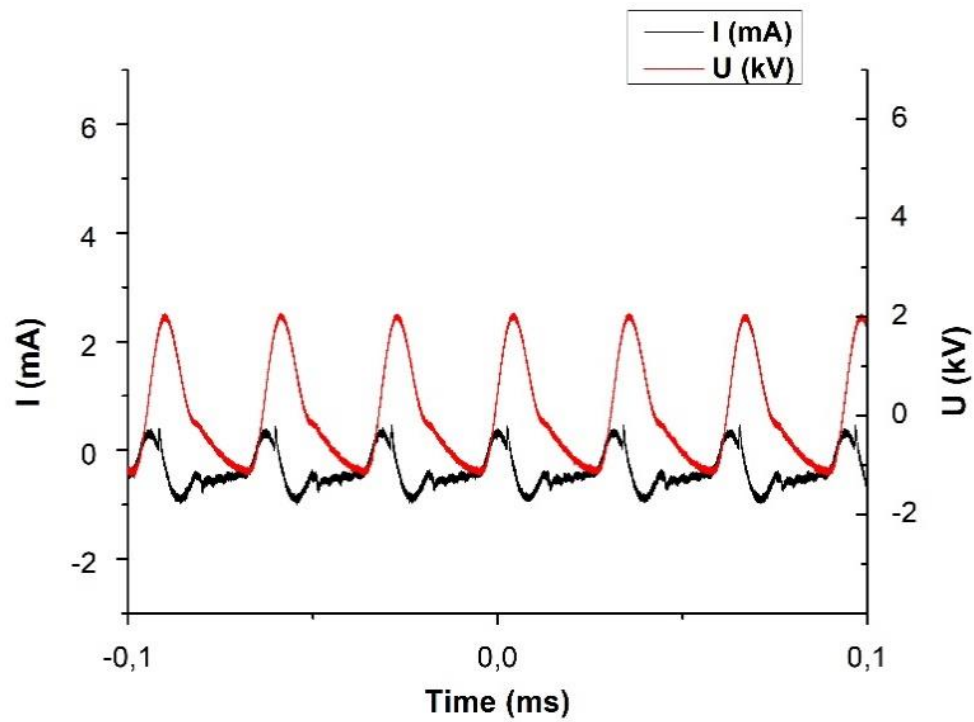


Figure 5.1: $I_{in}=0.21$ A, $U_{in}=4.1$ V, output signal.

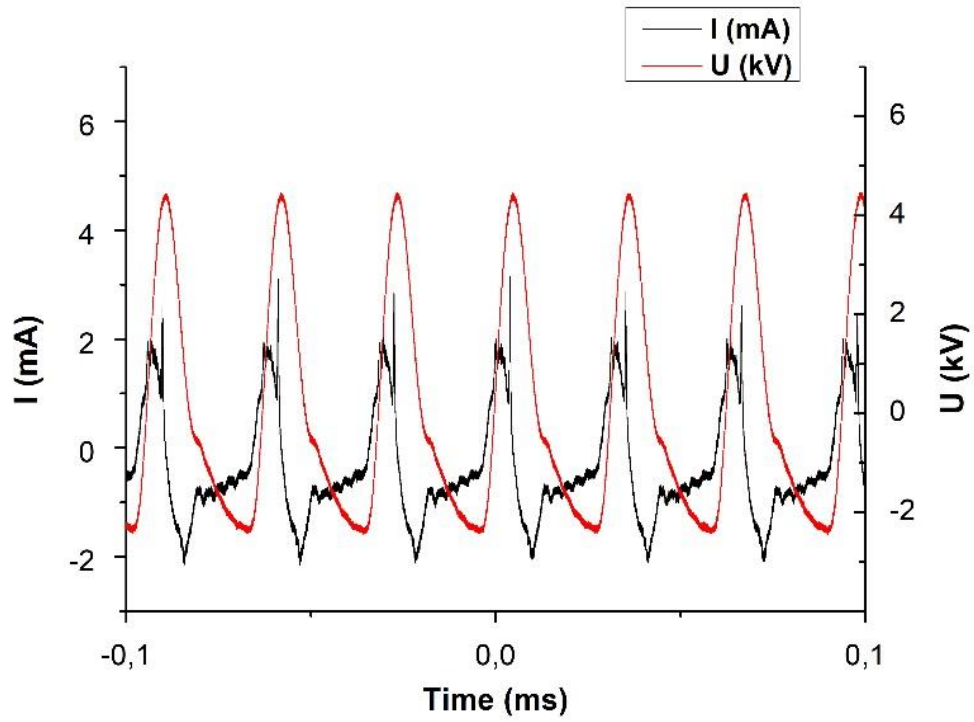


Figure 5.2: $I_{in}=0.45$ A, $U_{in}=9.0$ V, output signal.

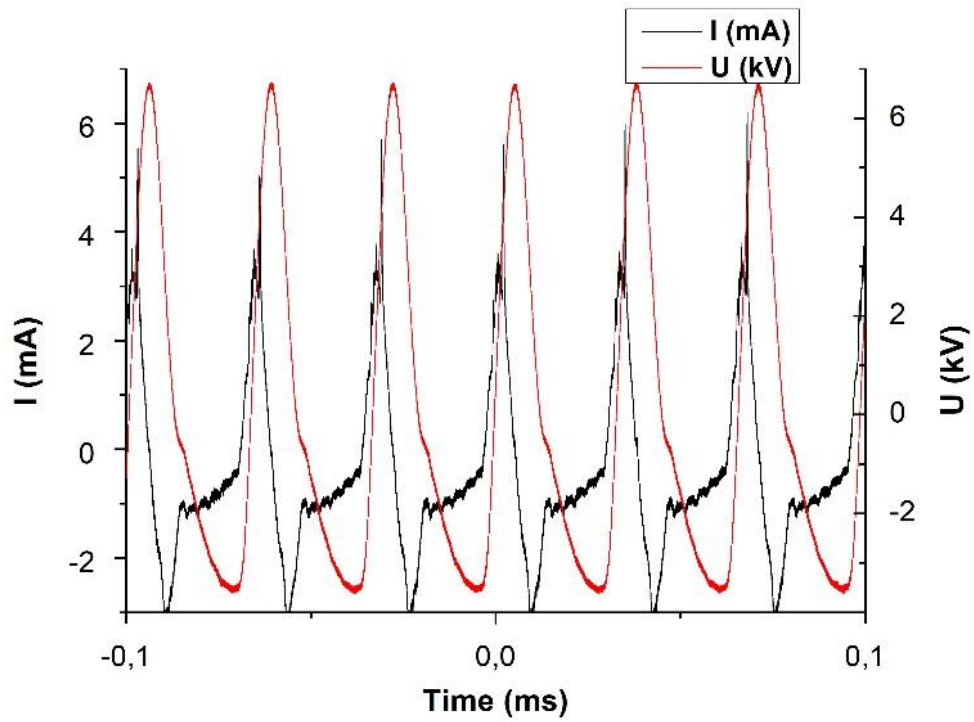


Figure 5.3: $I_{in}=0.70$ A, $U_{in}=15$ V, output signal.

Appendix B

X-Ray Photoelectron Spectroscopy

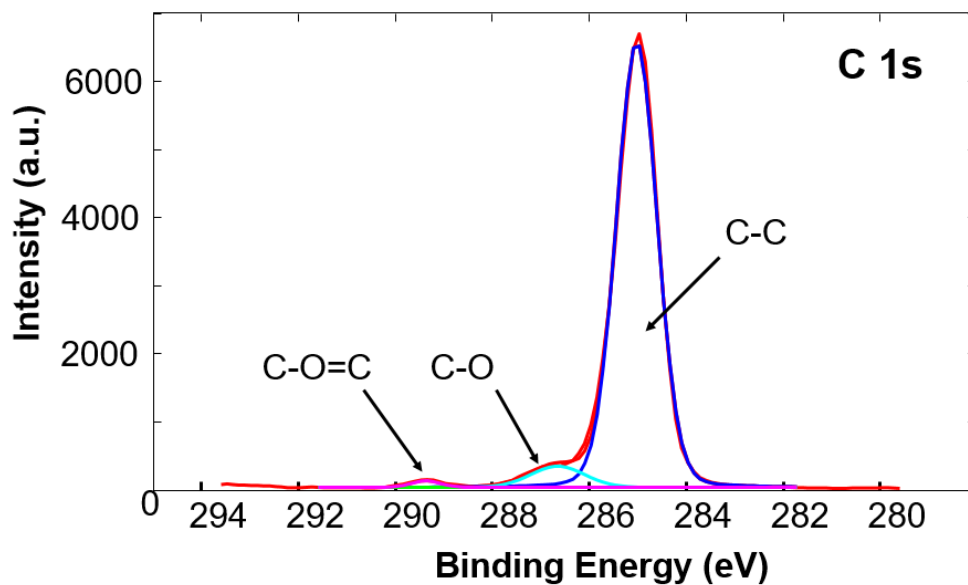


Figure 5.4: High resolution spectra of the untreated foil.

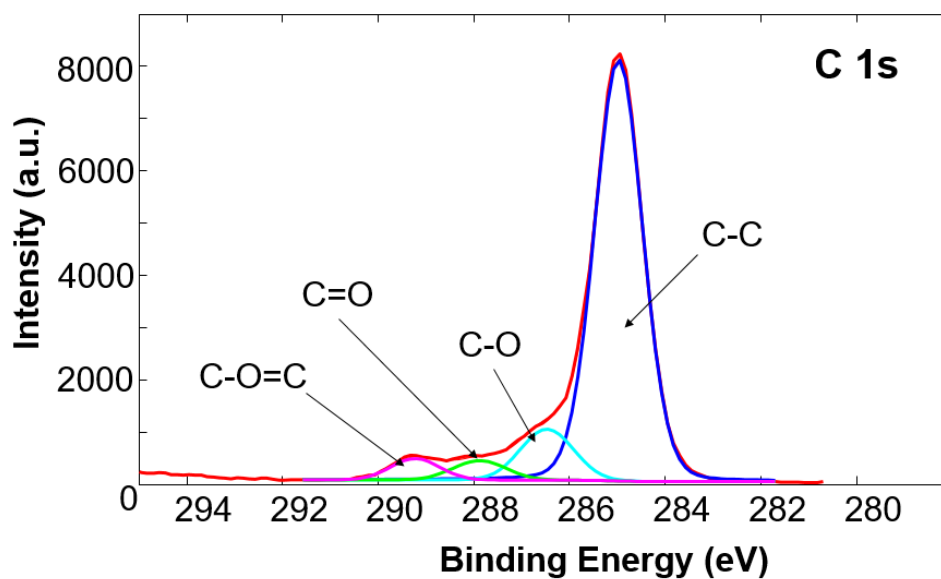


Figure 5.5: High resolution spectra of 5-time plasma-treated foil.

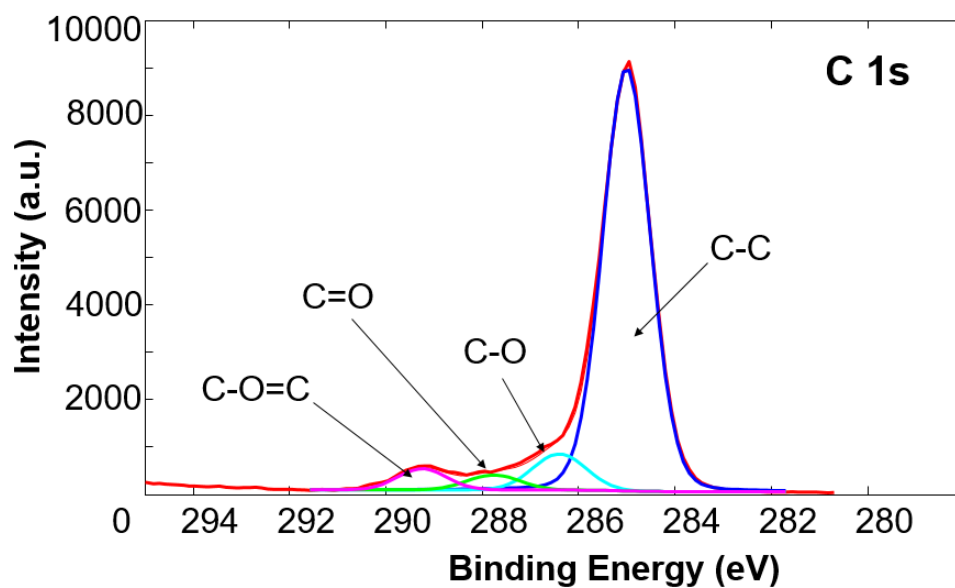


Figure 5.6: High resolution spectra of 20-time plasma-treated foil.

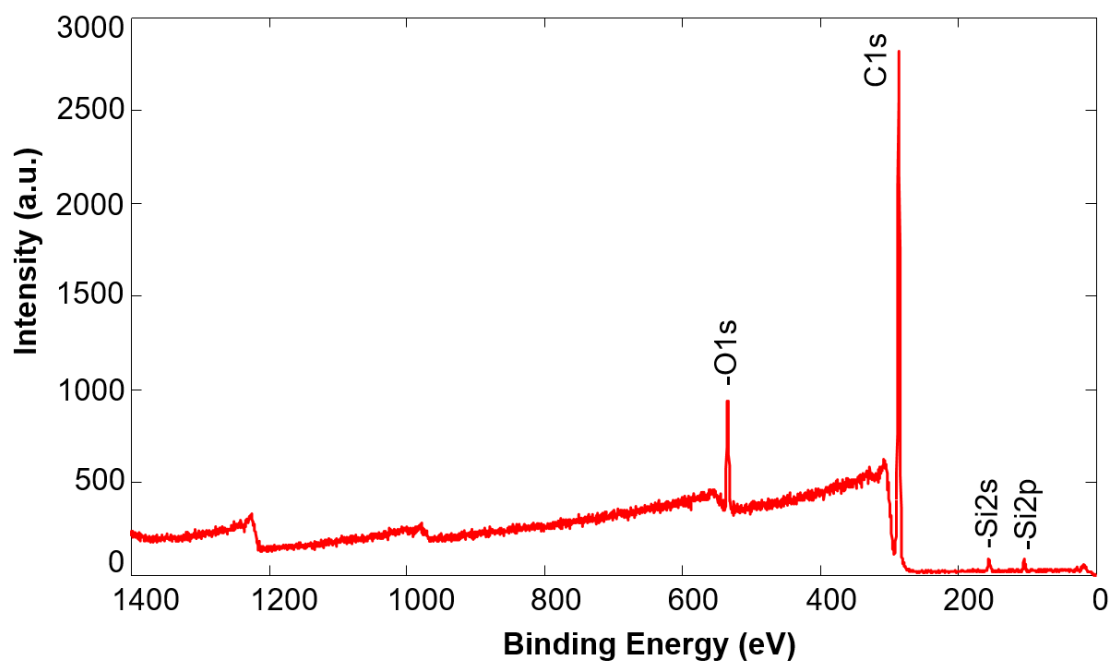


Figure 5.7: XPS of untreated Sample 1.

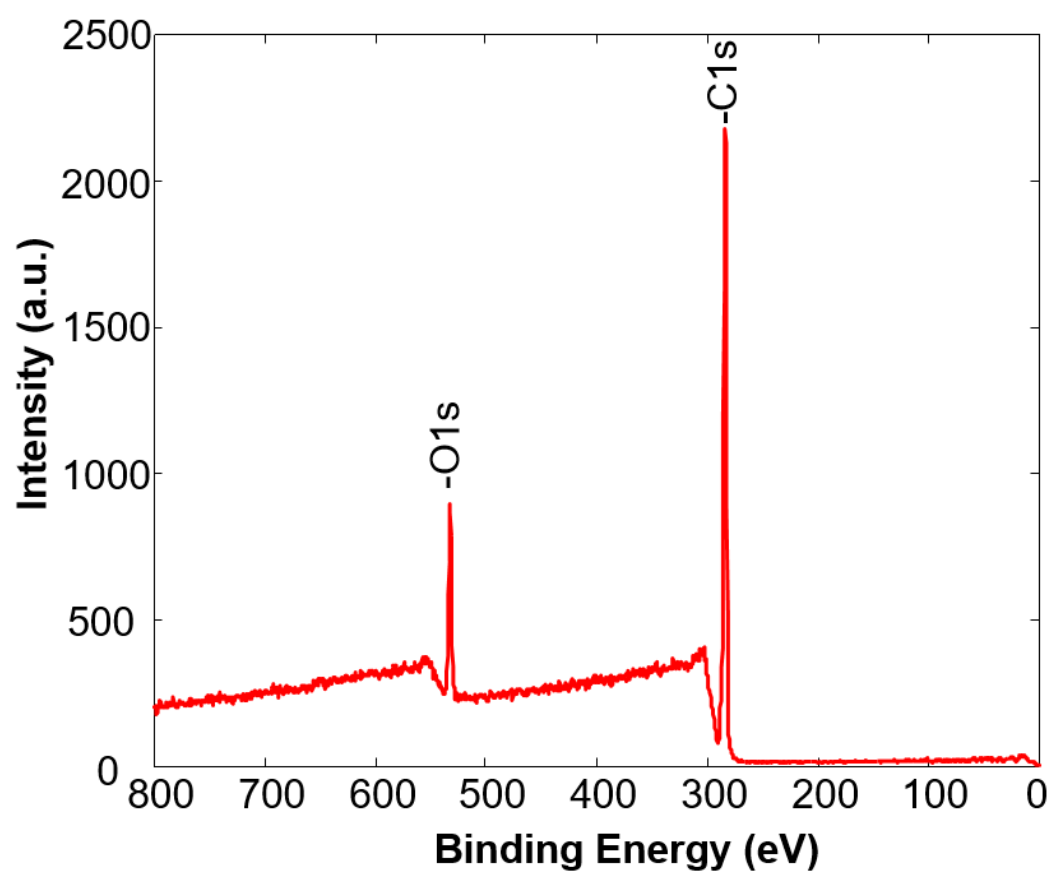


Figure 5.8: XPS of plasma-treated Sample 1.

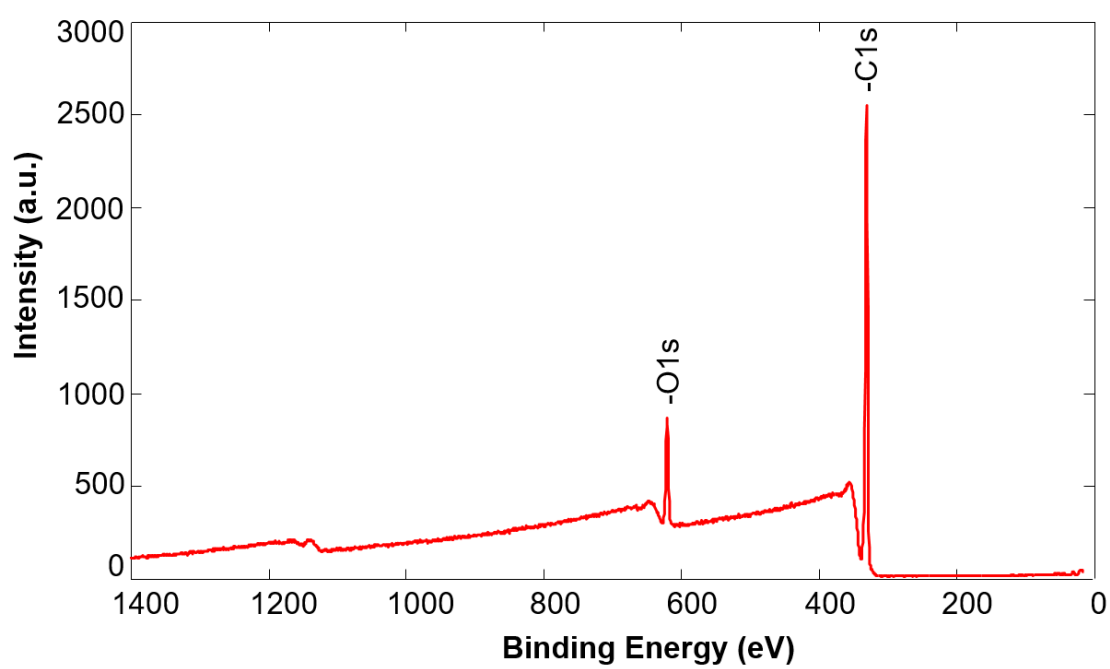


Figure 5.9: XPS of untreated Sample 2.

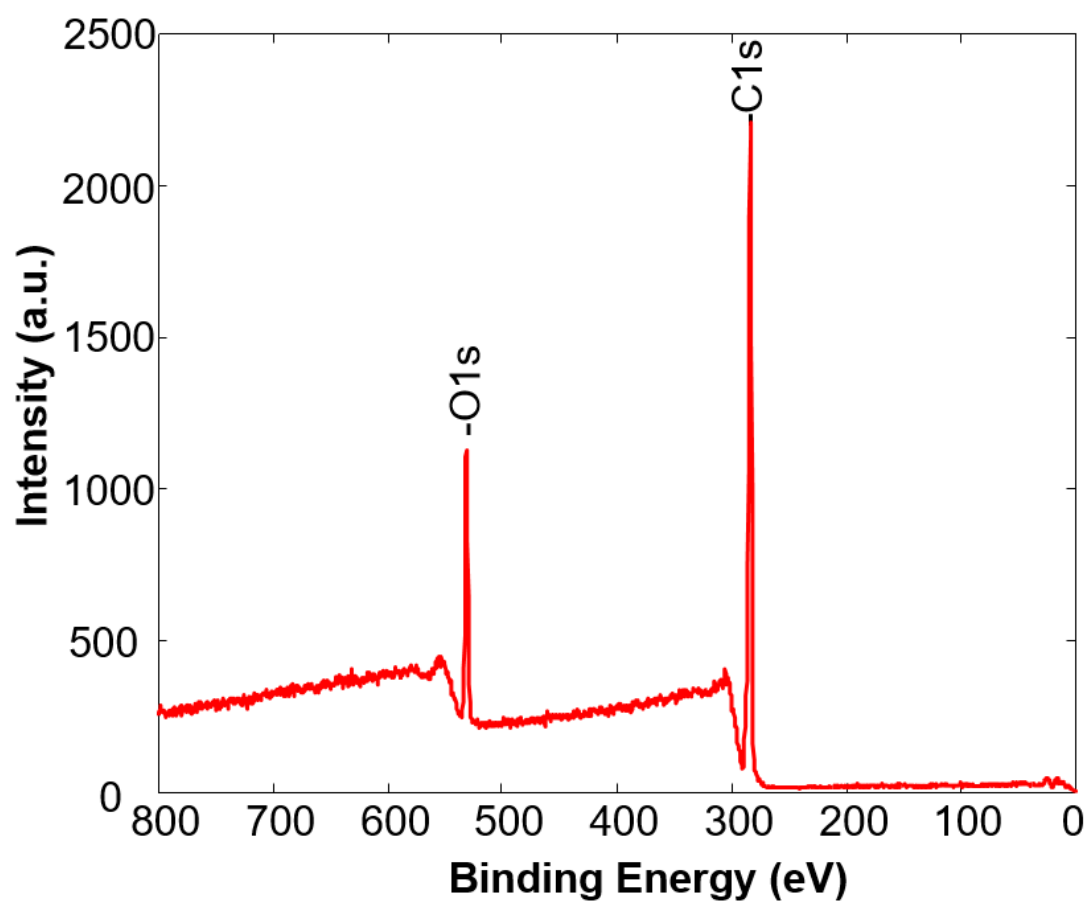


Figure 5.10: XPS of plasma-treated Sample 2.

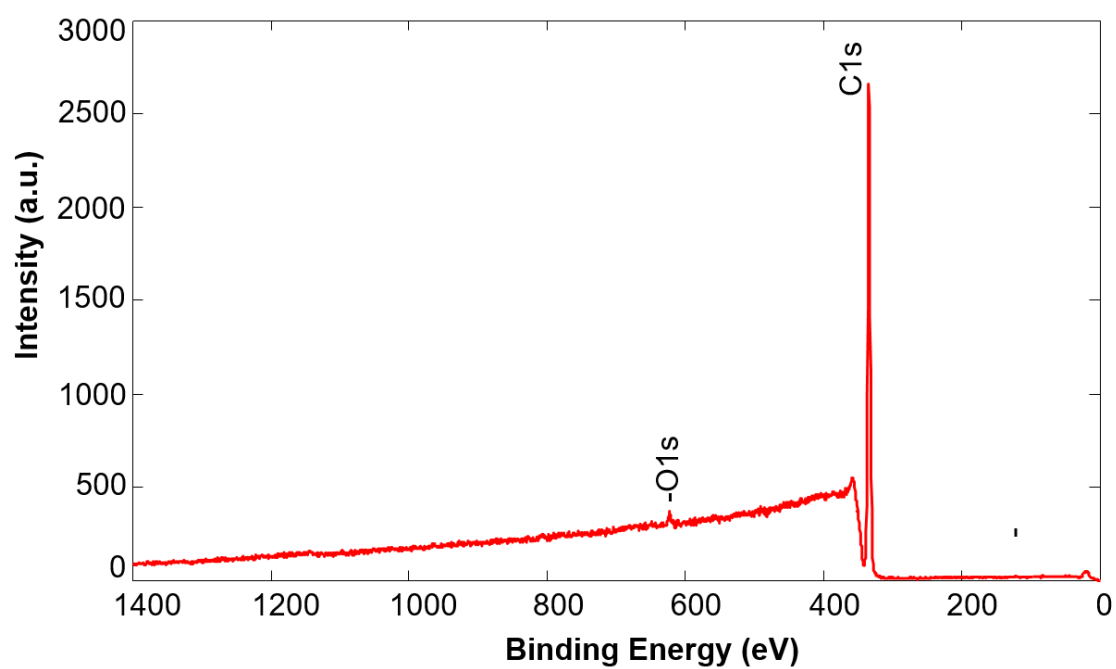


Figure 5.11: XPS of untreated Sample 3.

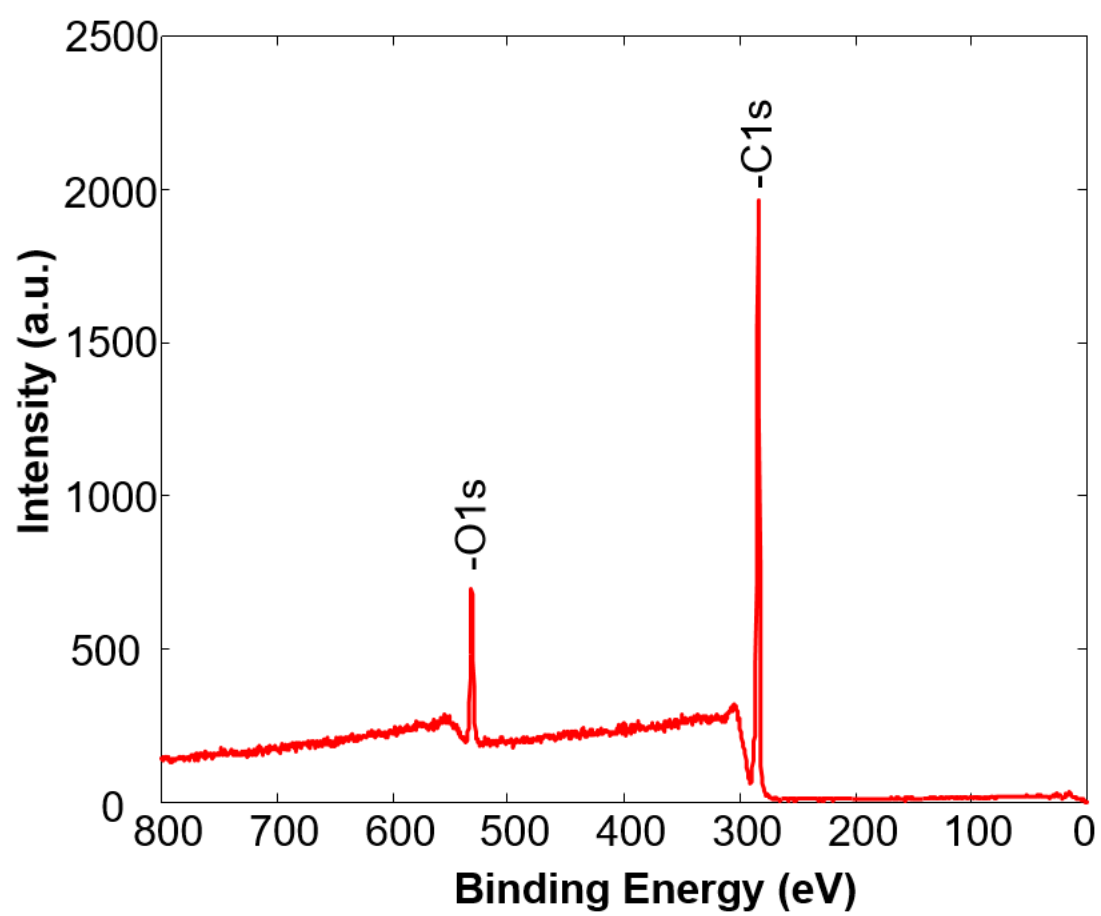


Figure 5.12: XPS of plasma-treated Sample 3.

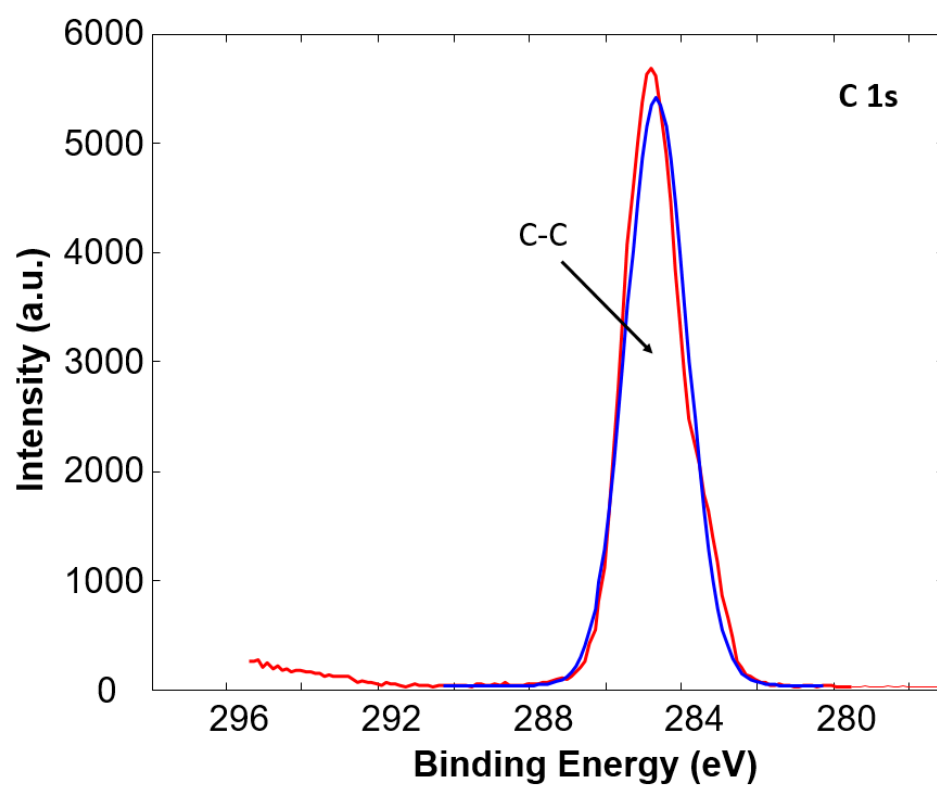


Figure 5.13: High resolution carbon spectra of untreated Sample 3.

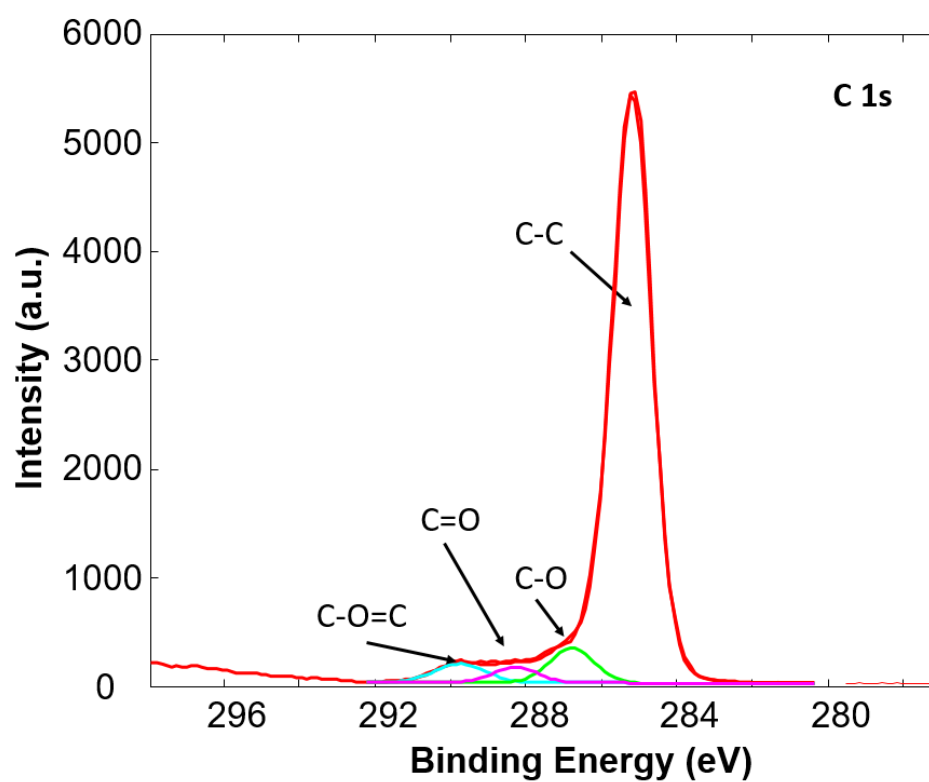


Figure 5.14: High resolution carbon spectra of 5-time plasma-treated Sample 3.

Appendix C

Device Control Program

```

..mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 1
1 using System;
2 using System.Linq;
3 using System.Windows;
4 using System.Windows.Input;
5 using System.Threading;
6 using System.Windows.Media;
7
8 using Festo.CMMO_ST;
9 using Festo.CVE;
10 using Festo.EXCM;
11 using System.Net;
12 using System.ComponentModel;
13
14 namespace Matic_mizica
15 {
16     /// <summary>
17     /// Interaction logic for MainWindow.xaml
18     /// </summary>
19     public partial class MainWindow : Window
20     {
21
22         public CMMO_ST myCMMO_ST = new CMMO_ST();
23         public EXCM myEXCM = new EXCM();
24
25         private System.Timers.Timer _timerUpdateUI;
26
27         public bool absoluteRelative = false;
28
29         double xEXCM = 20;
30         double yEXCM = 20;
31         double zDeltaCMMO = 20;
32         double vEXCM, vCMMO = 0;
33         double aEXCM, aCMMO = 0;
34
35         private System.Threading.AutoResetEvent DoWork;
36         private BackgroundWorker BGWorker;
37
38
39         public MainWindow()
40         {
41             InitializeComponent();
42
43             _timerUpdateUI = new System.Timers.Timer(1000);
44             _timerUpdateUI.AutoReset = true;
45             _timerUpdateUI.Elapsed += myEXCM_UpdateUI;
46
47             vEXCM = Convert.ToDouble(txtEXCMControlVV.Text);
48             vCMMO = Convert.ToDouble(txtCMMOControlVV.Text);
49
50             aEXCM = Convert.ToDouble(txtEXCMControlAV.Text);
51             aCMMO = Convert.ToDouble(txtCMMOControlAV.Text);
52
53             BGWorker = new BackgroundWorker();
54             BGWorker.DoWork += BGWorkerDoWork;
55             BGWorker.RunWorkerCompleted += new RunWorkerCompletedEventHandler ?

```

Figure 5.15: Code for automatic plasma treatment device control, page 1.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 2
    (BGWorkerDoWork);
57    BGWorker.WorkerSupportsCancellation = true;
58
59    //myEXCM.ConnectionStateChanged += myEXCM_ConnectionStateChanged;
60    //myEXCM.UpdateUI += myEXCM_UpdateUI;
61    //myEXCM.OnError += myEXCM_OnError;
62    //myCMMO_ST.ConnectionStateChanged += myCMMO_ST_ConnectionStateChanged;
63    //myCMMO_ST.UpdateUI += myCMMO_ST_UpdateUI;
64    //myCMMO_ST.OnError += myCMMO_ST_OnError;
65
66    }
67
68    private void btnConnect_Click(object sender, RoutedEventArgs e)
69    {
70        if (myEXCM.isConnected && myCMMO_ST.isConnected)
71        {
72            try
73            {
74                myEXCM.Disable();
75                myEXCM.ResetError();
76                myEXCM.Disconnect();
77            }
78            catch (Exception eDisconnectEXCM)
79            {
80                MessageBox.Show(this, "Couldn't disconnect EXCM\r\n" +
81                    eDisconnectEXCM.Message,
82                    "Warning", MessageBoxButton.OK,
83                    MessageBoxImage.Information);
84            }
85            try
86            {
87                myCMMO_ST.Disable();
88                myCMMO_ST.ResetError();
89                myCMMO_ST.Disconnect();
90            }
91            catch (Exception eDisconnectCMMO)
92            {
93                MessageBox.Show(this, "Couldn't disconnect CMMO\r\n" +
94                    eDisconnectCMMO.Message,
95                    "Warning", MessageBoxButton.OK,
96                    MessageBoxImage.Information);
97            }
98            txtConnect.Text = "Disconnected\nPress to connect";
99            txtBoxEXCMipAdd.IsReadOnly = false;
100            txtBoxCMMOipAdd.IsReadOnly = false;
101            btnConnect.Background = Brushes.LightGray;
102        }
103        else
104        {
105            try
106            {
107                myEXCM.ip = IPAddress.Parse(txtBoxEXCMipAdd.Text);
108                myEXCM.Connect();

```

Figure 5.16: Code for automatic plasma treatment device control, page 2.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 3
107 myEXCM.higherOrderControl =
Festo.Device.HigherOrderControl.CVE;
108 myEXCM.ResetError();
109 myCMMO_ST.ip = IPAddress.Parse(txtBoxCMMOipAdd.Text);
110
111 txtBoxEXCMipAdd.IsReadOnly = true;
112 txtConnect.Text = "Connected\nPress to disconnect";
113 btnConnect.Background = Brushes.LightGreen;
114
115 _timerUpdateUI.Start();
116 }
117 catch (Exception eConnectEXCM)
118 {
119     MessageBox.Show(this, "Couldn't connect EXCM\r\n" +
eConnectEXCM.Message,
120         "Warning", MessageBoxButton.OK,
MessageBoxImage.Information);
121 }
122 try
123 {
124     myCMMO_ST.Connect();
125     myCMMO_ST.higherOrderControl =
Festo.Device.HigherOrderControl.CVE;
126     myCMMO_ST.ResetError();
127
128     txtBoxCMMOipAdd.IsReadOnly = true;
129     txtConnect.Text = "Connected\nPress to disconnect";
130     btnConnect.Background = Brushes.LightGreen;
131
132     _timerUpdateUI.Start();
133 }
134 catch (Exception eConnectCMMO)
135 {
136     MessageBox.Show(this, "Couldn't connect CMMO\r\n" +
eConnectCMMO.Message,
137         "Warning", MessageBoxButton.OK,
MessageBoxImage.Information);
138 }
139 }
140 }
141
142 private void checkBoxEnableEXCM_Click(object sender, RoutedEventArgs
e)
143 {
144     if (checkBoxEnableEXCM.IsChecked == true)
145     {
146         try
147         {
148             myEXCM.Enable();
149         }
150         catch (Exception eEnableEXCM)
151         {
152             MessageBox.Show(this, "Couldn't enable EXCM\r\n" +
eEnableEXCM.Message,
153                 "Warning", MessageBoxButton.OK,
MessageBoxImage.Information);

```

Figure 5.17: Code for automatic plasma treatment device control, page 3.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 4
154         }
155         homingEXCM();
156     }
157     else
158     {
159         try
160         {
161             myEXCM.Disable();
162         }
163         catch (Exception eDisableEXCM)
164         {
165             MessageBox.Show(this, "Couldn't disable EXCM\r\n" +
166                             eDisableEXCM.Message,
167                             "Warning", MessageBoxButton.OK,
168                             MessageBoxImage.Information);
169         }
170         homingEXCM();
171     }
172     private void checkBoxEnableCMMO_Click(object sender, RoutedEventArgs
173     e)
174     {
175         if (checkBoxEnableCMMO.IsChecked == true)
176         {
177             try
178             {
179                 myCMMO_ST.Enable();
180             }
181             catch (Exception eEnableCMMO)
182             {
183                 MessageBox.Show(this, "Couldn't enable CMMO\r\n" +
184                                     eEnableCMMO.Message,
185                                     "Warning", MessageBoxButton.OK,
186                                     MessageBoxImage.Information);
187             }
188             homingCMMO();
189         }
190         else
191         {
192             try
193             {
194                 myCMMO_ST.Disable();
195             }
196             catch (Exception eDisableCMMO)
197             {
198                 MessageBox.Show(this, "Couldn't disable CMMO\r\n" +
199                                     eDisableCMMO.Message,
200                                     "Warning", MessageBoxButton.OK,
201                                     MessageBoxImage.Information);
202             }
203             homingCMMO();
204         }
205     }
206     public SolidColorBrush checkStatusBit(bool statusBit)

```

Figure 5.18: Code for automatic plasma treatment device control, page 4.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 5
203     {
204         if (statusBit)
205         {
206             return Brushes.LightGreen;
207         }
208         else
209         {
210             return Brushes.LightGray;
211         }
212     }
213     public SolidColorBrush checkStatusBitWarning(bool statusBit)
214     {
215         if (statusBit)
216         {
217             return Brushes.LightYellow;
218         }
219         else
220         {
221             return Brushes.LightGray;
222         }
223     }
224     public SolidColorBrush checkStatusBitError(bool statusBit)
225     {
226         if (statusBit)
227         {
228             return Brushes.Red;
229         }
230         else
231         {
232             return Brushes.LightGray;
233         }
234     }
235
236     private void myEXCM_UpdateUI(object sender, EventArgs e)
237     {
238         try
239         {
240             Dispatcher.BeginInvoke((Action)(
241             () =>
242             {
243                 txtEXCMStatusX.Text = String.Format("{0:0.00;-0.00;0.00}",
244                 myEXCM.actualPositionX);
245                 txtEXCMStatusY.Text = String.Format("{0:0.00;-0.00;0.00}",
246                 myEXCM.actualPositionY);
247                 txtCMMOStatusZ.Text = String.Format("{0:0.00;-0.00;0.00}",
248                 myCMMO_ST.actualPosition);
249
250                 txtEXCMStatusVV.Text = String.Format
251                 ("{0:0.00;-0.00;0.00}", myEXCM.velocity);
252                 txtCMMOStatusVV.Text = String.Format
253                 ("{0:0.00;-0.00;0.00}", myCMMO_ST.velocity);
254
255                 txtEXCMStatusER.Text = String.Format("0x{0:X} {1}", (int)
256                 myEXCM.currentError, myEXCM.currentError.ToString());
257                 txtCMMOStatusER.Text = String.Format("0x{0:X} {1}", (int)
258                 myCMMO_ST.currentError, myCMMO_ST.currentError.ToString())

```

Figure 5.19: Code for automatic plasma treatment device control, page 5.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 6

    ());

252
253         borderEXCMStatusB0.Background = checkStatusBit
254         (myEXCM.statusWord.Bit00_Enabled);
255         borderEXCMStatusB1.Background = checkStatusBit
256         (myEXCM.statusWord.Bit01_OperationEnabled);
257         borderEXCMStatusB2.Background = checkStatusBitWarning
258         (myEXCM.statusWord.Bit02_Warning);
259         borderEXCMStatusB3.Background = checkStatusBitError
260         (myEXCM.statusWord.Bit03_Error);
261         borderEXCMStatusB4.Background = checkStatusBit
262         (myEXCM.statusWord.Bit04_LoadVoltageIsApplied);
263         borderEXCMStatusB6.Background = checkStatusBit
264         (myEXCM.statusWord.Bit06_OperationMode1);
265         borderEXCMStatusB7.Background = checkStatusBit
266         (myEXCM.statusWord.Bit07_OperationMode2);
267         borderEXCMStatusB9.Background = checkStatusBit
268         (myEXCM.statusWord.Bit09_AcknowledgeStart);
269         borderEXCMStatusB10.Background = checkStatusBit
270         (myEXCM.statusWord.Bit10_MotionComplete);
271         borderEXCMStatusB11.Background = checkStatusBit
272         (myEXCM.statusWord.Bit11_AcknowledgeTeach);
273         borderEXCMStatusB15.Background = checkStatusBit
274         (myEXCM.statusWord.Bit15_AxisIsReferenced);
275
276         borderCMMOStatusB0.Background = checkStatusBit
277         (myCMMO_ST.statusWord.Bit00_ReadyToSwitchOn);
278         borderCMMOStatusB1.Background = checkStatusBit
279         (myCMMO_ST.statusWord.Bit01_SwitchedOn);
280         borderCMMOStatusB3.Background = checkStatusBitError
281         (myCMMO_ST.statusWord.Bit03_Error);
282         borderCMMOStatusB7.Background = checkStatusBitWarning
283         (myCMMO_ST.statusWord.Bit07_Warning);
284         borderCMMOStatusB8.Background = checkStatusBit
285         (myCMMO_ST.statusWord.Bit08_Move);
286         borderCMMOStatusB10.Background = checkStatusBit
287         (myCMMO_ST.statusWord.Bit10_TargetReachedMotionComplete);
288         borderCMMOStatusB12.Background = checkStatusBit
289         (myCMMO_ST.statusWord.Bit12_SetpointAcknowledge);
290         borderCMMOStatusB15.Background = checkStatusBit
291         (myCMMO_ST.statusWord.Bit15_Referenced);
292
293     }
294     ));
295 }
296
297 catch (Exception eStatusBit)
298 {
299     MessageBox.Show(this, "Couldn't check status bit of EXCM and
300     CMMO\r\n" + eStatusBit.Message,
301     "Warning", MessageBoxButton.OK,
302     MessageBoxImage.Information);
303 }
304
305 }
306
307 public void allowNumbersAndControl(TextCompositionEventArgs e)
308 {

```

Figure 5.20: Code for automatic plasma treatment device control, page 6.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 7
286     char c = e.Text.ToCharArray().First();
287     e.Handled = !(char.IsNumber(c) || char.IsControl(c) ||
                char.IsPunctuation(c));
288 }
289
290 private void btnEXCMControlB3_Click(object sender, RoutedEventArgs e)
291 {
292     try
293     {
294         myEXCM.ResetError();
295     }
296     catch (Exception eResetErrorEXCM)
297     {
298         MessageBox.Show(this, "Couldn't reset EXCM error\r\n" +
                eResetErrorEXCM.Message,
299             "Warning", MessageBoxButton.OK,
300             MessageBoxImage.Information);
301     }
302     try
303     {
304         myCMMO_ST.ResetError();
305     }
306     catch (Exception eResetErrorCMMO)
307     {
308         MessageBox.Show(this, "Couldn't reset CMMO error\r\n" +
                eResetErrorCMMO.Message,
309             "Warning", MessageBoxButton.OK,
310             MessageBoxImage.Information);
311     }
312 }
313
314 private void btnEXCMControlB9_Click(object sender, RoutedEventArgs e)
315 {
316     double xEXCM, yEXCM, zCMMO = 0;
317     double vEXCM, vCMMO = 0;
318     double aEXCM, aCMMO = 0;
319
320     xEXCM = Convert.ToDouble(txtEXCMControlXV.Text);
321     yEXCM = Convert.ToDouble(txtEXCMControlYV.Text);
322     zCMMO = Convert.ToDouble(txtCMMOControlZV.Text);
323
324     vEXCM = Convert.ToDouble(txtEXCMControlVV.Text);
325     vCMMO = Convert.ToDouble(txtCMMOControlVV.Text);
326
327     aEXCM = Convert.ToDouble(txtEXCMControlAV.Text);
328     aCMMO = Convert.ToDouble(txtCMMOControlAV.Text);
329
330     try
331     {
332         if (myEXCM.statusWord.Bit10_MotionComplete)
333         {
334             myEXCM.MoveToPosition(xEXCM, yEXCM, absoluteRelative,
335                 vEXCM, aEXCM);

```

Figure 5.21: Code for automatic plasma treatment device control, page 7.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 8
336         MessageBox.Show(this, "Wait until XY movement ends\r\n",
337             "Warning", MessageBoxButton.OK,
338             MessageBoxImage.Information);
339     }
340     catch (Exception eMoveEXCM)
341     {
342         MessageBox.Show(this, "Couldn't move EXCM to position\r\n" +
343             eMoveEXCM.Message,
344             "Warning", MessageBoxButton.OK,
345             MessageBoxImage.Information);
346     }
347     try
348     {
349         if (myCMMO_ST.statusWord.Bit10_TargetReachedMotionComplete)
350         {
351             myCMMO_ST.MoveToPosition(zCMMO, absoluteRelative, vCMMO,
352                 aCMMO);
353         }
354         else
355         {
356             MessageBox.Show(this, "Wait until Z movement ends\r\n",
357                 "Warning", MessageBoxButton.OK,
358                 MessageBoxImage.Information);
359         }
360     }
361     catch (Exception eMoveCMMO)
362     {
363         MessageBox.Show(this, "Couldn't move CMMO to position\r\n" +
364             eMoveCMMO.Message,
365             "Warning", MessageBoxButton.OK,
366             MessageBoxImage.Information);
367     }
368 }
369
370 private void btnEXCMControlB10_Click(object sender, RoutedEventArgs e)
371 {
372     homingEXCM();
373     homingCMMO();
374 }
375
376 public void homingEXCM()
377 {
378     //if (!myEXCM.statusWord.Bit15_AxisIsReferenced &&
379     //    myEXCM.statusWord.Bit00_Enabled)
380     //{
381     try
382     {
383         if (myEXCM.statusWord.Bit10_MotionComplete)
384         {
385             myEXCM.Home();
386         }
387         else
388         {
389             MessageBox.Show(this, "Wait until XY movement ends\r\n",
390                 "Warning", MessageBoxButton.OK,
391                 MessageBoxImage.Information);
392         }
393     }
394     catch (Exception eHomingEXCM)
395     {
396         MessageBox.Show(this, "Couldn't home EXCM\r\n" +
397             eHomingEXCM.Message,
398             "Warning", MessageBoxButton.OK,
399             MessageBoxImage.Information);
400     }
401 }

```

Figure 5.22: Code for automatic plasma treatment device control, page 8.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 9
    MessageBoxImage.Information));
384     }
385 }
386 catch (Exception eHomingEXCM)
387 {
388     MessageBox.Show(this, "Couldn't perform homing EXCM\r\n" +
        eHomingEXCM.Message,
389         "Warning", MessageBoxButton.OK,
        MessageBoxImage.Information);
390 }
391 //}
392 //else
393 //{
394 //    return;
395 //}
396 }
397
398 public void homingCMMO()
399 {
400     //if (!myCMMO_ST.statusWord.Bit15_Referenced && myCMMO_ST.state ==
        State.OperationEnabled)
401     //{
402     try
403     {
404         if (myCMMO_ST.statusWord.Bit10_TargetReachedMotionComplete)
405         {
406             myCMMO_ST.Home();
407         }
408         else
409         {
410             MessageBox.Show(this, "Wait until Z movement ends\r\n",
411                 "Warning", MessageBoxButton.OK,
412                 MessageBoxImage.Information);
413         }
414     }
415     catch (Exception eHomingCMMO)
416     {
417         MessageBox.Show(this, "Couldn't perform homing CMMO\r\n" +
418             eHomingCMMO.Message,
419             "Warning", MessageBoxButton.OK,
420             MessageBoxImage.Information);
421     }
422     //}
423     //else
424     //{
425     //    return;
426     //}
427 }
428
429 private void deviceStopSequence()
430 {
431     BGWorker.DoWork -= BGWorkerDoWork;
432     if (BGWorker.IsBusy)
433     {
434         BGWorker.CancelAsync();
435     }
436 }

```

Figure 5.23: Code for automatic plasma treatment device control, page 9.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 10
433     }
434
435     homingEXCM();
436     homingCMMO();
437
438     myEXCM.Disable();
439     myEXCM.ResetError();
440     myEXCM.Disconnect();
441
442     myCMMO_ST.Disable();
443     myCMMO_ST.ResetError();
444     myCMMO_ST.Disconnect();
445
446 }
447
448 private void txtEXCMControlXV_PreviewTextInput(object sender, 7
    TextCompositionEventArgs e)
449 {
450     allowNumbersAndControl(e);
451     //if (Convert.ToDouble(txtEXCMControlXV.Text) > 400)
452     //{
453         // txtEXCMControlXV.Text = "400.00";
454     //}
455 }
456
457 private void txtEXCMControlYV_PreviewTextInput(object sender, 7
    TextCompositionEventArgs e)
458 {
459     allowNumbersAndControl(e);
460     //if (Convert.ToDouble(txtEXCMControlYV.Text) > 360)
461     //{
462         // txtEXCMControlYV.Text = "360.00";
463     //}
464 }
465
466 private void txtCMMOControlZV_PreviewTextInput(object sender, 7
    TextCompositionEventArgs e)
467 {
468     allowNumbersAndControl(e);
469     //if (Convert.ToDouble(txtCMMOControlZV.Text) > 200)
470     //{
471         // txtEXCMControlXV.Text = "200.00";
472     //}
473 }
474
475 private void txtEXCMControlVV_PreviewTextInput(object sender, 7
    TextCompositionEventArgs e)
476 {
477     allowNumbersAndControl(e);
478 }
479
480 private void txtCMMOControlVV_PreviewTextInput(object sender, 7
    TextCompositionEventArgs e)
481 {
482     allowNumbersAndControl(e);
483 }

```

Figure 5.24: Code for automatic plasma treatment device control, page 10.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 11
484
485     private void txtEXCMControlAV_PreviewTextInput(object sender,
486         TextCompositionEventArgs e)
487     {
488         allowNumbersAndControl(e);
489     }
490     private void txtCMOControlAV_PreviewTextInput(object sender,
491         TextCompositionEventArgs e)
492     {
493         allowNumbersAndControl(e);
494     }
495     private void radioAbsolute_Click(object sender, RoutedEventArgs e)
496     {
497         absoluteRelative = false;
498     }
499     private void radioRelative_Click(object sender, RoutedEventArgs e)
500     {
501         absoluteRelative = true;
502     }
503
504     private void AppWindow_Closing(object sender, CancelEventArgs e)
505     {
506         MessageBoxResult result = MessageBox.Show(this, "Do you really
507             want to close the application?",
508             "Close", MessageBoxButton.YesNo,
509             MessageBoxImage.Warning);
510         if (result == MessageBoxResult.No)
511         {
512             e.Cancel = true;
513         }
514         else
515         {
516             e.Cancel = false;
517             deviceStopSequence();
518             App.Current.Shutdown();
519         }
520     }
521     private void FileExit_Click(object sender, RoutedEventArgs e)
522     {
523         this.Close();
524     }
525
526     public void EXCMMove(double x, double y, bool type, double v, double
527         a)
528     {
529         while (true)
530         {
531             try
532             {
533                 if (myEXCM.statusWord.Bit10_MotionComplete)
534                     {

```

Figure 5.25: Code for automatic plasma treatment device control, page 11.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 12
535         myEXCM.MoveToPosition(x, y, type, v, a);
536         myEXCM.MoveToPositionEvent.Wait();
537         break;
538     }
539     else
540     {
541         MessageBox.Show(this, "Wait until X, Y movement ends\r\n",
542             "Warning", MessageBoxButton.OK,
543             MessageBoxImage.Information);
544     }
545     catch (Exception eMoveEXCM)
546     {
547         MessageBox.Show(this, "Couldn't move EXCM to position\r\n" +
548             eMoveEXCM.Message,
549             "Warning", MessageBoxButton.OK,
550             MessageBoxImage.Information);
551     }
552 }
553 public void CMMOMove(double z, bool type, double v, double a)
554 {
555     while (true)
556     {
557         try
558         {
559             if
560             (myCMMO_ST.statusWord.Bit10_TargetReachedMotionComplete)
561             {
562                 myCMMO_ST.MoveToPosition(z, type, v, a);
563                 myCMMO_ST.MoveToPositionEvent.Wait();
564                 break;
565             }
566             else
567             {
568                 MessageBox.Show(this, "Wait until Z movement ends\r\n",
569                     "Warning", MessageBoxButton.OK,
570                     MessageBoxImage.Information);
571             }
572         }
573         catch (Exception eMoveCMMO)
574         {
575             MessageBox.Show(this, "Couldn't move CMMO to position\r\n" +
576                 eMoveCMMO.Message,
577                 "Warning", MessageBoxButton.OK,
578                 MessageBoxImage.Information);
579         }
580     }
581 }
582 public void CMMODownUp5x20(double v, double a)
583 {
584     CMMOMove(20, true, v, a);

```

Figure 5.26: Code for automatic plasma treatment device control, page 12.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 13
582         CMMOMove(-20, true, v, a);
583         CMMOMove(20, true, v, a);
584         CMMOMove(-20, true, v, a);
585         CMMOMove(20, true, v, a);
586         CMMOMove(-20, true, v, a);
587         CMMOMove(20, true, v, a);
588         CMMOMove(-20, true, v, a);
589         CMMOMove(20, true, v, a);
590         CMMOMove(-20, true, v, a);
591     }
592
593     private void btnHolderTestSet_Click(object sender, RoutedEventArgs e)
594     {
595         double z0CMMO = 150;
596         double vCMMO = 0;
597         double aCMMO = 0;
598
599         vCMMO = Convert.ToDouble(txtCMMOControlVV.Text);
600         aCMMO = Convert.ToDouble(txtCMMOControlAV.Text);
601
602         CMMOMove(z0CMMO, false, vCMMO, aCMMO);
603     }
604
605     private void btnHolderTest_Click(object sender, RoutedEventArgs e)
606     {
607         BGWorker.RunWorkerAsync();
608
609         btnEXCMControlB9.IsEnabled = false;
610         btnEXCMControlB10.IsEnabled = false;
611
612         txtTest2.Text = "Starting movement...";
613     }
614
615     void BGWorkerDoWork(object sender, DoWorkEventArgs e)
616     {
617         // Move plasma to eppendorf tube
618         CMMOMove(100, false, 100, 50);
619
620         CMMODownUp5x20(100, 50);
621         EXCMMove(0, 10, false, 100, 50);
622         CMMODownUp5x20(100, 50);
623         EXCMMove(0, 20, false, 100, 50);
624         CMMODownUp5x20(100, 50);
625         EXCMMove(0, 30, false, 100, 50);
626         CMMODownUp5x20(100, 50);
627         EXCMMove(0, 40, false, 100, 50);
628         CMMODownUp5x20(100, 50);
629         EXCMMove(0, 50, false, 100, 50);
630         CMMODownUp5x20(100, 50);
631         EXCMMove(10, 10, false, 100, 50);
632         CMMODownUp5x20(100, 50);
633         EXCMMove(10, 20, false, 100, 50);
634         CMMODownUp5x20(100, 50);
635         EXCMMove(10, 30, false, 100, 50);
636         CMMODownUp5x20(100, 50);
637         EXCMMove(10, 40, false, 100, 50);

```

Figure 5.27: Code for automatic plasma treatment device control, page 13.

```

...mizica new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 14
638      CMMODownUp5x20(100, 50);
639      EXCMMove(10, 50, false, 100, 50);
640      CMMODownUp5x20(100, 50);
641      EXCMMove(20, 10, false, 100, 50);
642      CMMODownUp5x20(100, 50);
643      EXCMMove(20, 20, false, 100, 50);
644      CMMODownUp5x20(100, 50);
645      EXCMMove(20, 30, false, 100, 50);
646      CMMODownUp5x20(100, 50);
647      EXCMMove(20, 40, false, 100, 50);
648      CMMODownUp5x20(100, 50);
649      EXCMMove(20, 50, false, 100, 50);
650      CMMODownUp5x20(100, 50);
651      EXCMMove(30, 10, false, 100, 50);
652      CMMODownUp5x20(100, 50);
653      EXCMMove(30, 20, false, 100, 50);
654      CMMODownUp5x20(100, 50);
655      EXCMMove(30, 30, false, 100, 50);
656      CMMODownUp5x20(100, 50);
657      EXCMMove(30, 40, false, 100, 50);
658      CMMODownUp5x20(100, 50);
659      EXCMMove(30, 50, false, 100, 50);
660      CMMODownUp5x20(100, 50);
661      EXCMMove(40, 10, false, 100, 50);
662      CMMODownUp5x20(100, 50);
663      EXCMMove(40, 20, false, 100, 50);
664      CMMODownUp5x20(100, 50);
665      EXCMMove(40, 30, false, 100, 50);
666      CMMODownUp5x20(100, 50);
667      EXCMMove(40, 40, false, 100, 50);
668      CMMODownUp5x20(100, 50);
669      EXCMMove(40, 50, false, 100, 50);
670      CMMODownUp5x20(100, 50);
671      EXCMMove(50, 10, false, 100, 50);
672      CMMODownUp5x20(100, 50);
673      EXCMMove(50, 20, false, 100, 50);
674      CMMODownUp5x20(100, 50);
675      EXCMMove(50, 30, false, 100, 50);
676      CMMODownUp5x20(100, 50);
677      EXCMMove(50, 40, false, 100, 50);
678      CMMODownUp5x20(100, 50);
679      EXCMMove(50, 50, false, 100, 50);
680      CMMODownUp5x20(100, 50);
681
682      BGWorker.CancelAsync();
683  }
684
685  void BGWorkerDoWork(object sender, RunWorkerCompletedEventArgs e)
686  {
687      // The background process is complete. We need to inspect
688      // our response to see if an error occurred, a cancel was
689      // requested or if we completed successfully.
690      if (e.Cancelled)
691      {
692          txtTest2.Text = "Task Cancelled.";
693      }

```

Figure 5.28: Code for automatic plasma treatment device control, page 14.

```

...mizica_new\Matic_mizica\Matic_mizica\MainWindow.xaml.cs 15
694
695     // Check to see if an error occurred in the background process.
696
697     else if (e.Error != null)
698     {
699         txtTest2.Text = "Error while performing background
700             operation.";
701     }
702     else
703     {
704         // Everything completed normally.
705         txtTest2.Text = "Task Completed...";
706     }
707
708     //Change the status of the buttons on the UI accordingly
709     btnEXCMControlB9.IsEnabled = true;
710     btnEXCMControlB10.IsEnabled = true;
711 }
712 }
713 }
714

```

Figure 5.29: Code for automatic plasma treatment device control, final page.

Appendix D

Atomic Force Microscopy

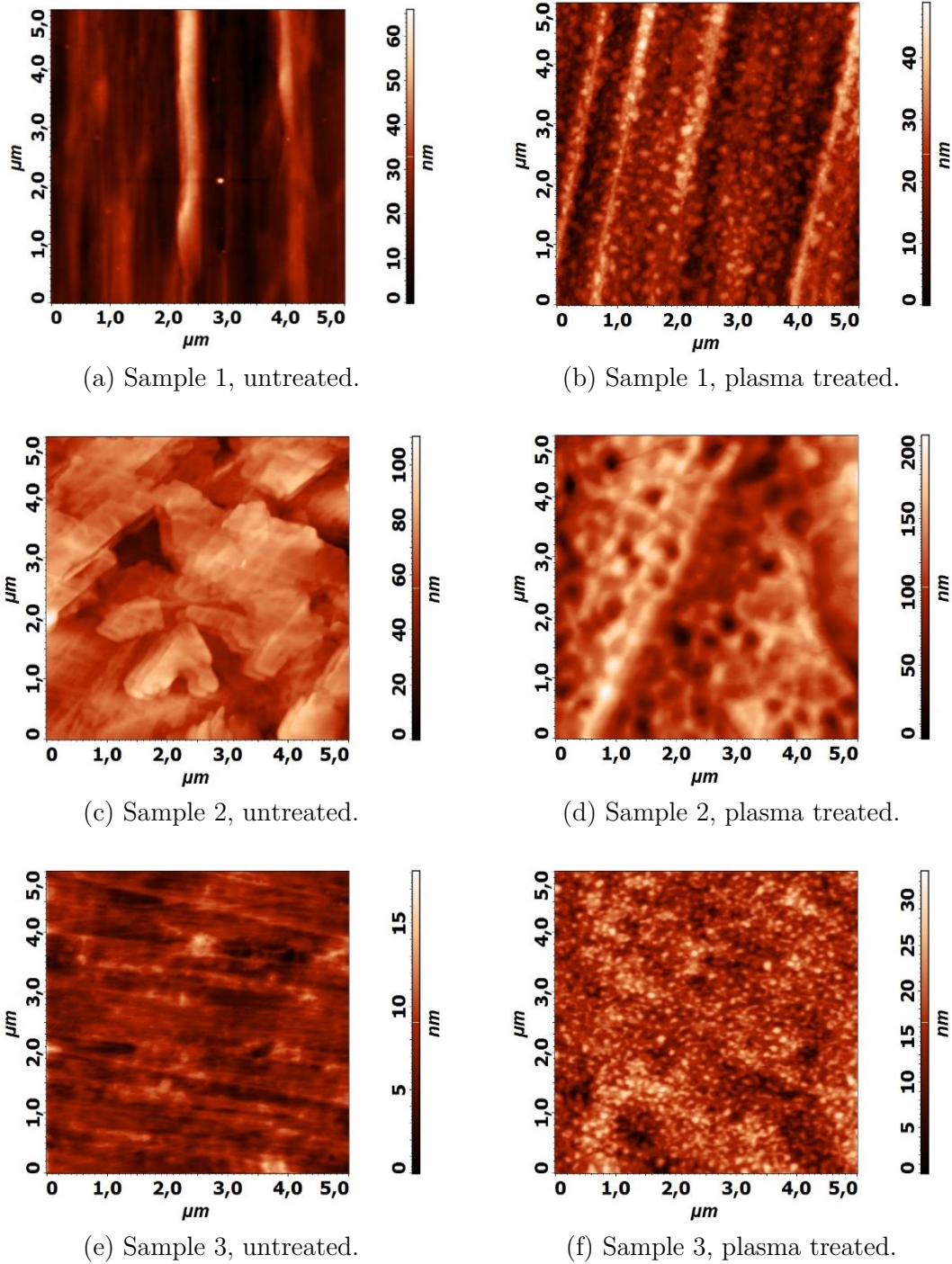
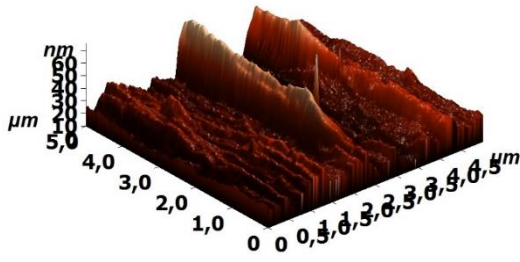
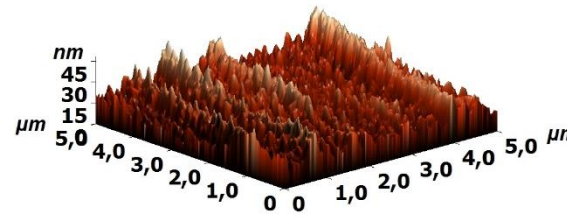


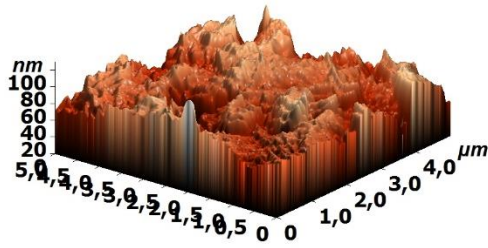
Figure 5.30: 2D AFM image on 5 x 5 μm area.



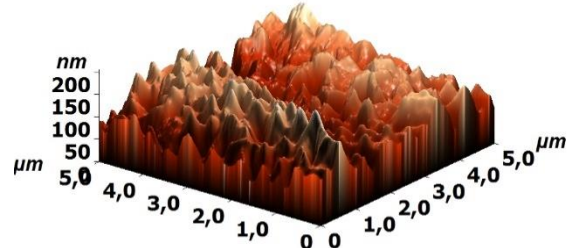
(a) Sample 1, untreated.



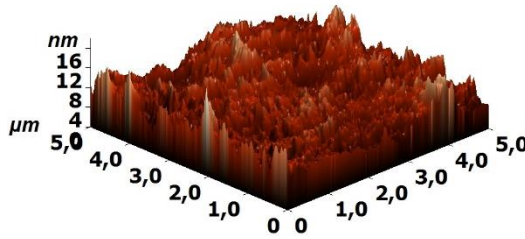
(b) Sample 1, plasma treated.



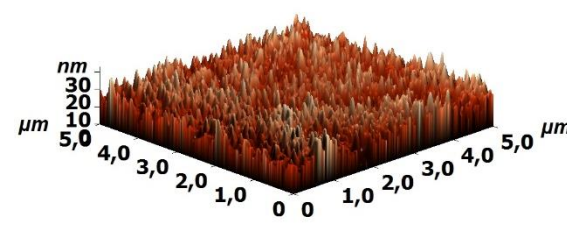
(c) Sample 2, untreated.



(d) Sample 2, plasma treated.



(e) Sample 3, untreated.



(f) Sample 3, plasma treated.

Figure 5.31: 3D AFM image on 5 x 5 μm area.

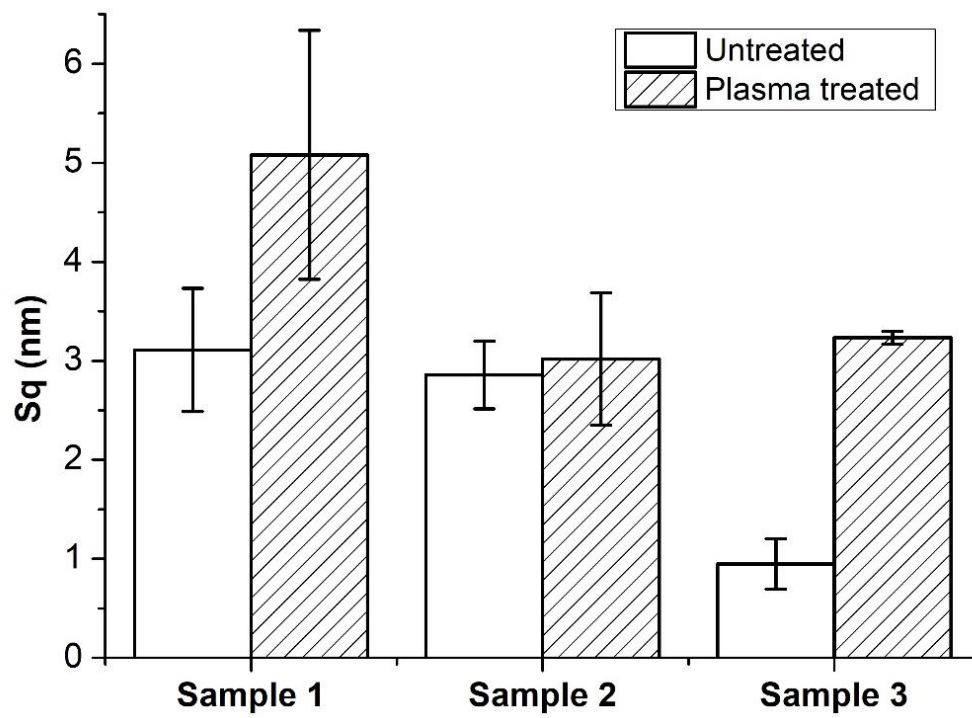


Figure 5.32: Root mean square surface roughness on $1 \times 1 \mu\text{m}$ area.

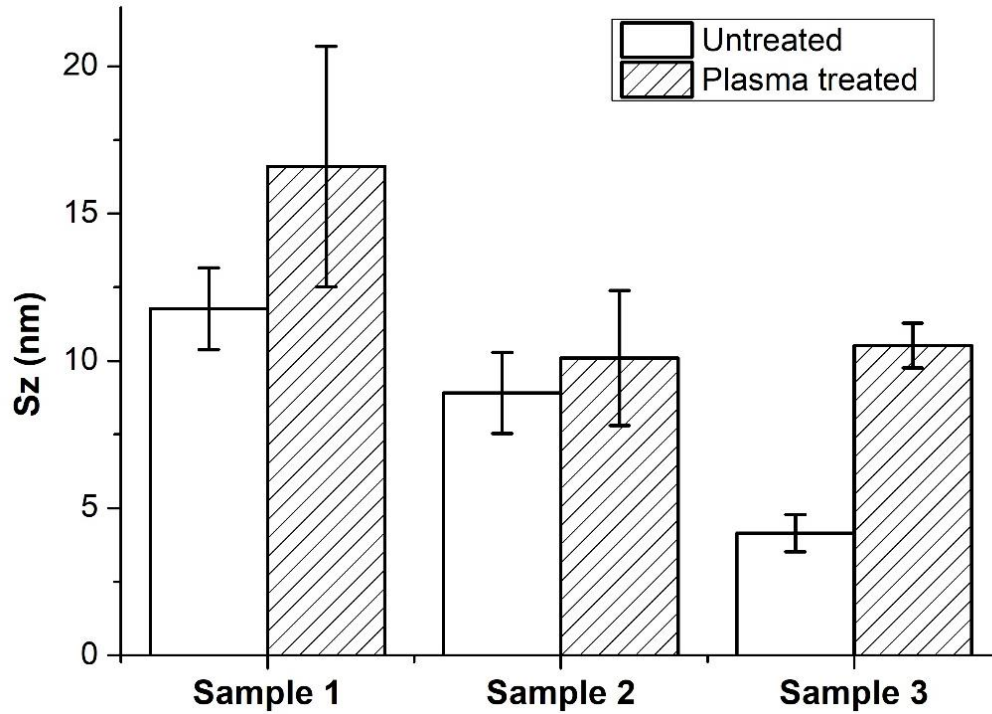


Figure 5.33: Distance from the highest peak to the lowest valley on $1 \times 1 \mu\text{m}$ area.

Appendix E

Differential Scanning Calorimetry

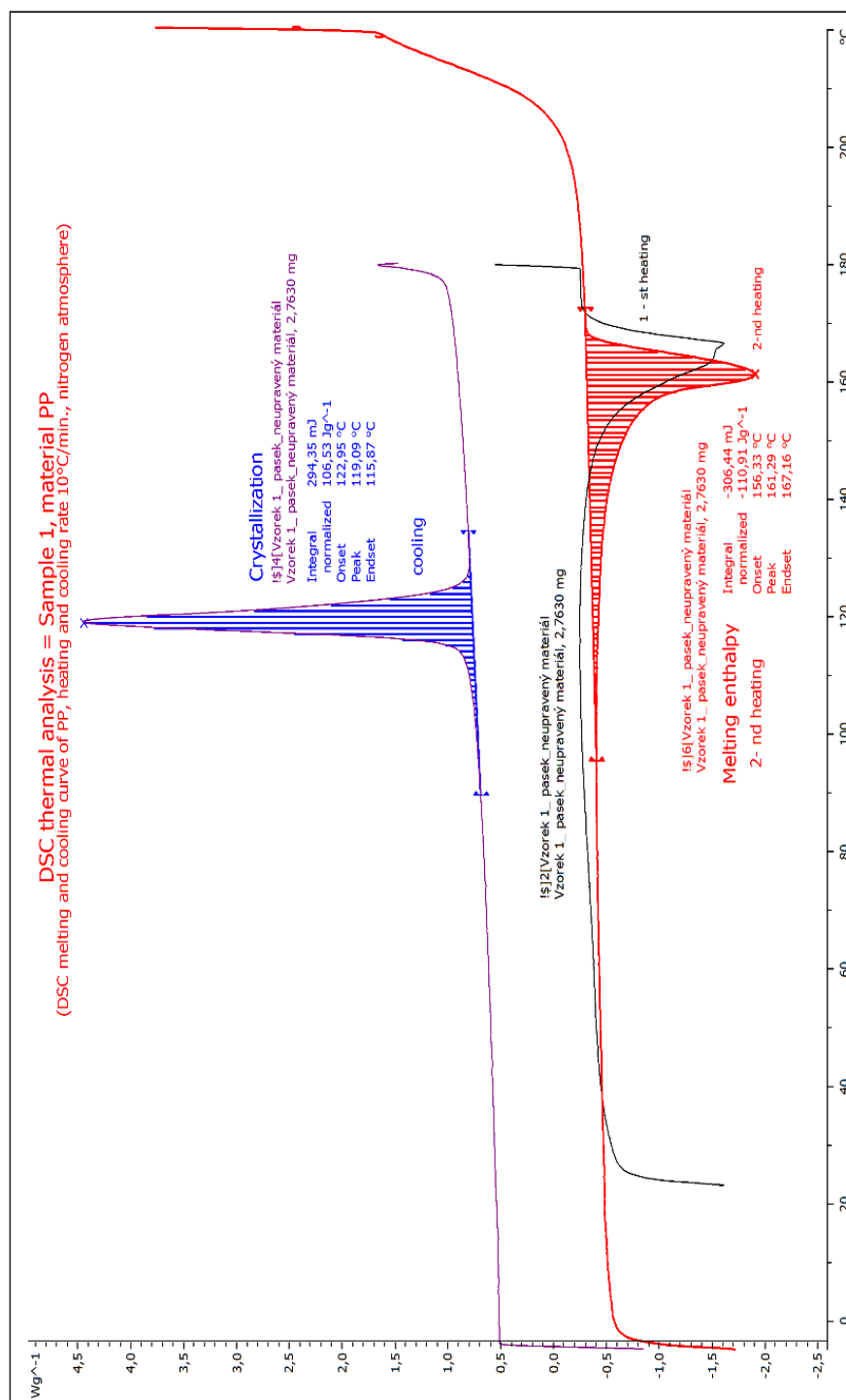


Figure 5.34: DSC analysis of Sample 1.

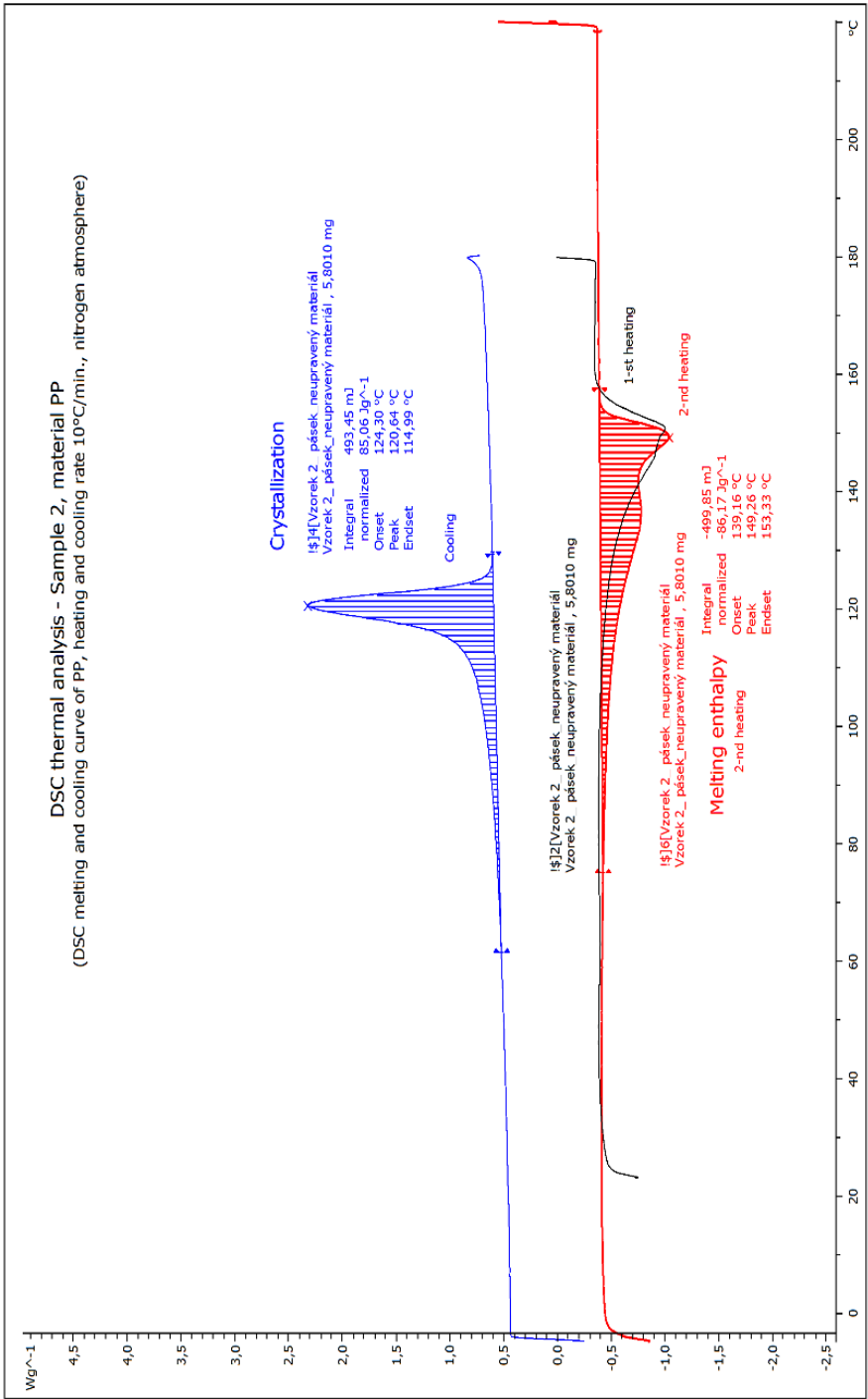


Figure 5.35: DSC analysis of Sample 2.

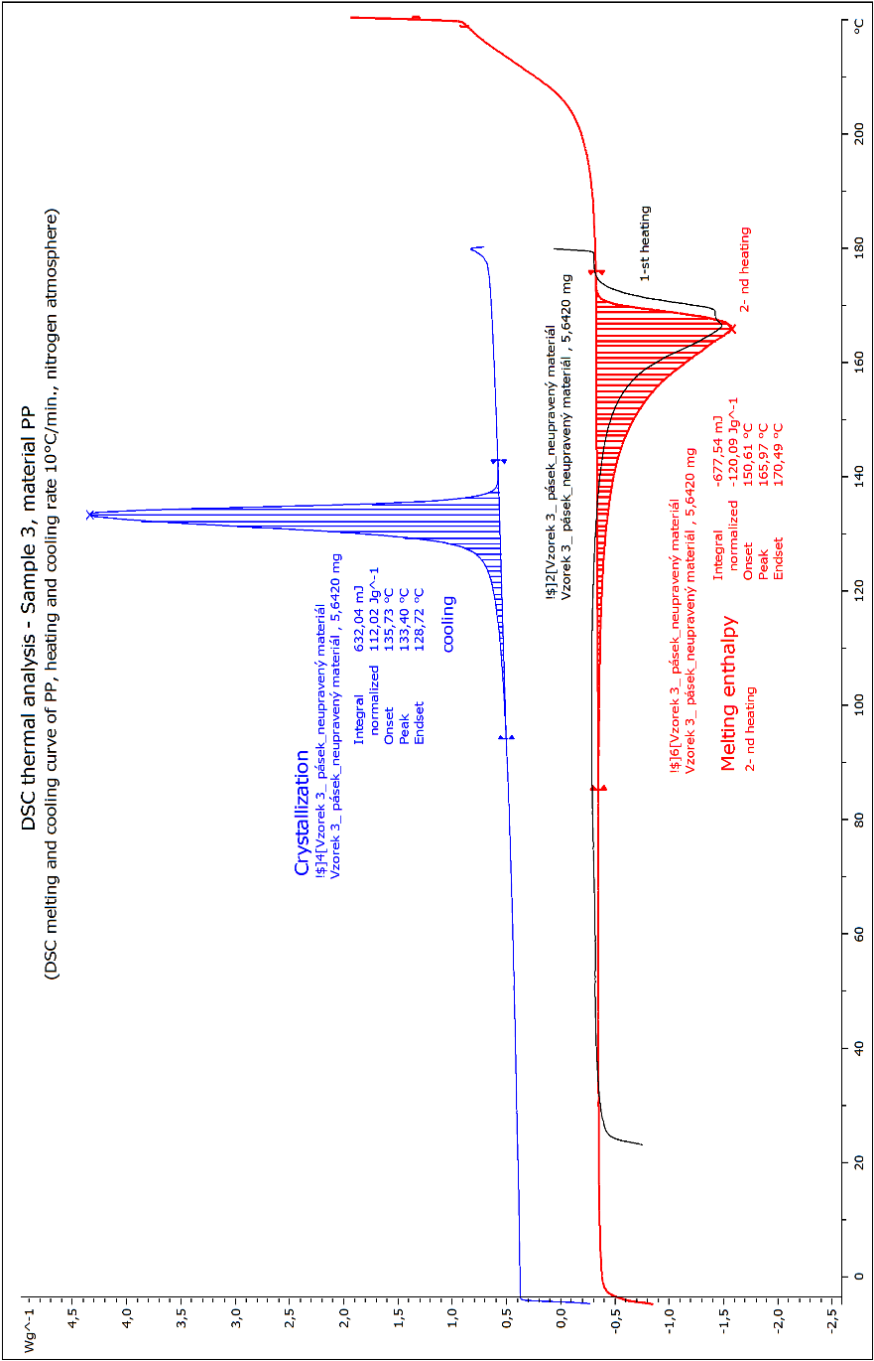


Figure 5.36: DSC analysis of Sample 3.

Appendix F

Flow Cytometry

Table 5.2: Preliminar flow cytometry results.

Donor Number	Untreated Sample 2	Plasma treated Sample 2	Untreated Sample 3	Untreated Sample 1	Plasma treated Sample 1
31	996	1578	1917	/	/
39	2526	4597	3287	/	/
37	4564	2036	3569	/	/
42	3000	2758	3143	/	/
27	2983	2078	4829	/	/
38	2651	1771	4485	/	/
49	799	1718	1458	/	/
25	1402	1615	1514	/	/
28	2458	3195	3935	/	/
23	1574	4389	2499	/	/
28	853	708	933	/	/
66	1709	/	1268	/	/
51	4784	2174	6149	/	8129
45	2056	1393	2867	/	2850
49	2907	1786	4014	/	4222
54	1307	2264	1865	/	2247
63	1157	/	1367	1125	1047
64	1673	/	1598	1702	2264
65	1020	/	2927	3032	3468
Subject 1	1773	/	/	1352	1570
Subject 2	4013	/	/	6721	2606

Table 5.3: Flow cytometry of the first experiment.

	Sample 1		Sample 2		Sample 3	
	Untreated	Plasma t.	Untreated	Plasma t.	Untreated	Plasma t.
Donors	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)
1	466 (95)	379 (92)	359 (92)	522 (94)	429 (93)	368 (90)
2	116 (93)	78 (92)	113 (94)	156 (92)	49 (90)	115 (92)
3	205 (90)	162 (87)	256 (90)	323 (89)	174 (86)	112 (87)
4	139 (96)	137 (93)	138 (95)	103 (95)	122 (94)	106 (92)
5	249 (92)	198 (91)	226 (90)	592 (93)	285 (92)	175 (90)
6	356 (94)	502 (95)	583 (96)	494 (94)	434 (94)	883 (93)
7	509 (88)	511 (89)	409 (92)	1232 (90)	343 (91)	463 (92)
8	341 (94)	206 (91)	164 (90)	275 (92)	184 (88)	203 (90)
9	416 (91)	426 (92)	390 (92)	606 (86)	473 (91)	351 (82)
10	153 (95)	259 (92)	357 (97)	228 (94)	203 (95)	111 (91)
11	183 (93)	285 (91)	229 (92)	443 (91)	160 (91)	85 (88)

Table 5.4: Flow cytometry of the second experiment.

	Sample 1		Sample 2		Sample 3	
	Untreated	Plasma t.	Untreated	Plasma t.	Untreated	Plasma t.
Donors	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)
12	158 (90)	323 (93)	183 (92)	166 (94)	282 (93)	200 (93)
13	377 (89)	224 (86)	364 (90)	131 (81)	551 (90)	1054 (84)
14	720 (95)	876 (92)	493 (95)	747 (92)	944 (93)	1282 (95)
15	729 (91)	977 (93)	989 (91)	838 (93)	1428 (91)	1215 (94)
16	551 (94)	707 (93)	348 (92)	416 (92)	470 (91)	755 (95)
17	929 (93)	446 (89)	609 (94)	462 (90)	800 (89)	808 (90)

Table 5.5: Flow cytometry of the second experiment, repeatability test.

	Sample 1		Sample 2		Sample 3	
	Untreated	Plasma t.	Untreated	Plasma t.	Untreated	Plasma t.
Donors	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)	/ μ L (%)
18	211 (84)	324 (80)	/	/	/	/
	211 (82)	443 (87)	/	/	/	/
	137 (85)	380 (63)	/	/	/	/
19	/	/	302 (97)	175 (96)	/	/
	/	/	491 (98)	153 (96)	/	/
	/	/	244 (98)	225 (94)	/	/
20	/	/	/	/	186 (94)	228 (93)
	/	/	/	/	153 (90)	160 (87)
	/	/	/	/	122 (91)	199 (93)

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Publications Related to the Thesis

Journal Articles

- D. Carralero, P. Manz, G. Birkenmeier, M. Brix, M. Groth, H. W. Müller, U. Stroth, Nicola Vianello, E. Wolfrum, I. Lengar, L. Snoj, A. Drenik, N. Osterman, M. Panjan, G. Primc, M. Resnik, R. Zaplotnik, et al., ASDEX Upgrade team, JET Contributors, and EUROfusion MST1 Team, "Experimental validation of a filament transport model in turbulent magnetized plasmas", *Phys. rev. lett.*, iss. 21, vol. 115, 215002, 2015, doi: 10.1103/PhysRevLett.115.215002.
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Biography

The author of this thesis, Matic Resnik, was born on September 21, 1989 in Ljubljana, Slovenia. His parents are Mojca and Drago Resnik. He has a younger brother Vid.

After graduating from Gymnasium Bežigrad in 2008, he was admitted to the Faculty of Mechanical Engineering at the University of Ljubljana. During his study he joined LAT (Laboratory for Alternative Technologies) led by Prof. Dr. Mihael Junkar and worked on several industrial projects for Gorenje, BSH Group and research-oriented simulation of passive microchannel mixers. He obtained his Bachelor's degree in Mechanical Engineering in 2011.

The same year he enrolled in the Master's study at the Faculty of Mechanical Engineering at the University of Ljubljana and continued his study under the program "Production Engineering". He finished his Master thesis entitled "Heat exchanger tube cutting in nuclear power plants with electrothermal process" under the supervision of A/Prof. Dr. Joško Valentinčič on August 29, 2014. The author was acknowledged for his work with the Prešeren Prize by the Faculty of Mechanical Engineering.

Afterwards he applied for the position of a young researcher at the Department of Surface engineering and Optoelectronics, Jožef Stefan Institute, Ljubljana. The author was granted the position and started his research career on November 1, 2014.

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