The influence of surface properties of materials on biofilm formation

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Doctoral Thesis Summary

Tomas Bata University in Zlín

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Faculty of Technology

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The influence of surface properties of materials on biofilm formation

Vliv povrchových vlastností materiálů na tvorbu biofilmu

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ABSTRACT

Conducting polymers (CPs), have become the subject of intensive research thanks to their unique properties, such as conductivity, simple and low-cost synthesis or easy coating of various surfaces by CP. Between the unique properties of CP can be assigned the easy modification either chemically (e.g. by using various doping acids), by plasma treatment or incorporation of antimicrobial agents onto their surface. The ability of easy surface modification is crucial for their application as biointerface materials. Due to these, CPs can be also used for the improvement of the surface properties of other materials. Improved surface properties may subsequently influence the reaction and attachment of various proteins, eukaryotic cells, tissues and especially microorganisms.

The presence of microbial biofilm and overall the adhesion of microorganisms onto the material surfaces cause severe problems in many fields of industry or medicine. The surface properties of materials are a key factor influencing the reciprocal interaction between surface and microorganisms, e.g. in the context of microorganism attachment.

Thus, in the continuity of this issue, the main aim of the doctoral study was to provide novel information about the possible anti-biofilm properties of pristine CPs or their modification.

ABSTRAKT

Elektricky vodivé polymery se staly předmětem výzkumu z důvodu potenciálního využití v mnoha odvětvích průmyslu či biomedicíny. Zájem o vodivé polymery vychází z jejich unikátních vlastností jako je elektrická vodivost, snadná a nízkonákladová syntéza či jednoduchá tvorba tenkých filmů na různých površích.

Vodivé polymery se vyznačují jednoduchou modifikovatelností jak chemicky, pomocí dopujících kyselin, tak pomocí plazmového ošetření či inkorporací antimikrobiálních látek. Vodivé polymery tak mohou být využity k ovlivnění chování rozličných biologických systémů, jako jsou proteiny, jednotlivé buňky (prokaryotické i eukaryotické) či tkáně. V řadě aplikací jsou pak biofilm tvořící mikroorganismy významnější než častěji studované planktonní kmeny. To je dáno tím, že přítomnost biofilmu způsobuje vážné problémy v mnoha oblastech průmyslu a medicíny. Odstranění již vytvořeného mikrobiálního biofilmu se stává rovněž náročnou záležitostí, jelikož v tomto společenství se buňky dokážou lépe chránit vnějším vlivům a extracelulární matrix zabraňuje penetraci biocidních látek. Povrchové vlastnosti materiálů proto hrají klíčovou roli při interakci materiálu s mikroorganismy, protože mohou ovlivnit již počáteční adhezi mikroorganismů.

V návaznosti na problematiku vzniku a rozvoje biofilmu je hlavním cílem této dizertační práce poskytnout základní informace a poznatky o schopnosti omezit tvorbu bofilmu pomocí vodivých polymerů a jejich modifikací stejně jako pomocí dalších materiálů.

1 INTRODUCTION

One group of synthetic polymers which can contribute to antimicrobial materials is a group of conducting polymers (CP). CPs are widely studied for their outstanding properties. They can easily form thin films on other materials and cover their entire area. Moreover, CPs have low-cost production and rapid polymerization process (Anselme, Ploux and Ponche, 2010). However, it still persists the desire to further optimize their surfaces and forms with targeting to specific applications.

The important fact for utilizing CPs in any application is to determine their properties. Concerning the antibacterial, or especially the antibiofilm properties, the surface parameters among which belong to chemical, electrical, thermal or atomic properties are crucial (Rodríguez-Hernández, 2016). It is moreover well-known that the first substances, which are in contact with the colonized surface, may not be microorganisms themselves, but trace organics. However, trace organics form a thin layer neutralizing excessive surface charge and surface free energy. This founding might contribute to knowledge about bacterial attachment onto the surface and prevent the initial bacterial approach. Furthermore, most microorganisms adhere more rapidly to hydrophobic, nonpolar surfaces (De-la-Pinta *et al.*, 2019).

To solve the problem of biofilm formation the scientists endeavor to develop new effective antibiofilm surfaces focused on prevention microbial attachment from the initial contact. Therefore, the subject of this doctoral thesis is to describe preferably the effect of CPs, and further of titanium-based materials, on microbial biofilm formation.

1.1 Conducting polymers

CPs, as a novel generation of organic materials, were firstly produced in the 1970s (Shirakawa *et al.*, 1977; Guimard, Gomez and Schmidt, 2007). Interest in CPs arose mainly due to their electrical and optical properties, which are often similar to metals and inorganic semiconductors. Their attractive characteristics associated with properties similar to those of conventional polymers (flexibility in processing, easy of synthesis) are also in the center of interest (Guimard, Gomez and Schmidt, 2007; Le, Kim and Yoon, 2017). One of the major discoveries related to CPs is attributed to the team of H. Shirakawa, who has synthesized the simplest CP, polyacetylene. The discovery of Shirikawa's team and the development of electronically CPs were awarded by the Chemistry Nobel Prize in 2000 (Rasmussen, 2011).

However, it would be also appropriate to mention the greatest advantage of CPs, which is versatility. Thanks to versatility, the CPs may be used in a diverse array of fields, ranging from sensors devices to tissue engineering. The unique property of CPs that links all their applications together is conductivity (Kaur *et al.*, 2015; Liu *et al.*, 2018; Ning *et al.*, 2018).

The conductivity of CPs is also one of the subjects discussed in this doctoral thesis. The conductivity is correlated with surface properties of CPs, which have subsequently impact on biocompatibility, anti-corrosion or anti-biofouling properties of CPs.

1.1.1 Structure and synthesis of conducting polymers

The electrical properties of CPs are predominantly influenced by the structure of their backbone, present functional groups, morphology, and oxidation state. The conjugated structure of their chain consists of alternating single and double bonds or conjugated segments coupled with atoms providing p-orbitals for continuous orbital overlap. These bonds endow the polymer with metal-like semiconductor properties (Fig. 1) (Dai, 2006).

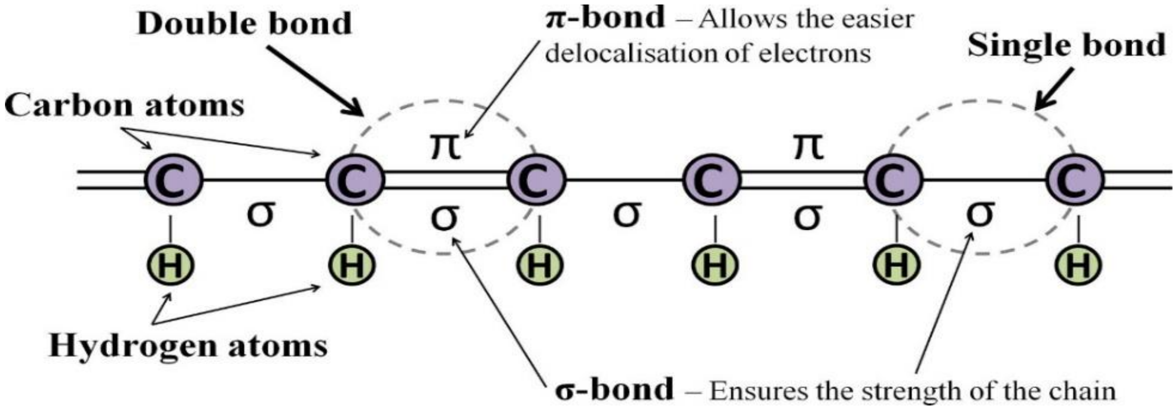

Fig. 1: A simplified schema of a conjugated polymer backbone: a chain containing alternating single and double bonds.
(Balint, Cassidy and Cartmell, 2014)

Except for conjugation, the presence of charge carriers mediating its transport along the chain is the essential factor for high electrical conductivity. The CPs arise from a process called doping. Doping is the process of oxidizing (p-doping) or reducing (n-doping) a neutral polymer (Guimard, Gomez and Schmidt, 2007). CPs are commonly prepared by two different processes, chemical or electrochemical polymerization. The chemical polymerization was used for the preparation of tested polyanilne (PANI) samples used within the researches conducted in this doctoral thesis (Fig. 2). Overall, many studies are focused on the investigation of CPs prepared by the chemical way of synthesis. PANI and polypyrrole (PPy) are certainly the most frequently studied representatives (Kumar and Yadav, 2016; Abu-Thabit, 2016; Apetrei *et al.*, 2018; Yuan *et al.*, 2016; Kausaite-Minkstimiene *et al.*, 2015).

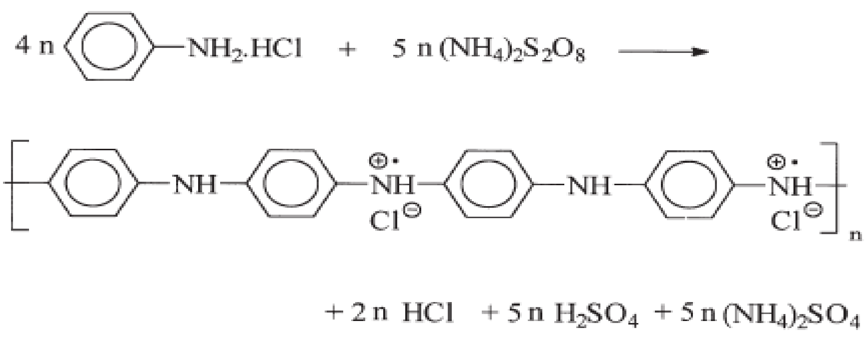

Fig. 2: Chemical synthesis of PANI using the oxidation of aniline hydrochloride with ammonium persulfate. (Stejskal and Gilbert, 2002)

1.1.2 Polyaniline

This CP is typically prepared by the chemical oxidation of aniline with ammonium peroxydisulfate in an acidic aqueous medium (Stejskal and Gilbert, 2002). The oxidation of aniline is an exothermic reaction, and the temperature of the reaction mixture increases during its course (Stejskal *et al.*, 2015). By removing or adding electrons through chemical or electrochemical oxidation and reduction it is possible to obtain forms of PANI with different chemical structures, stability, coloring, and electrical properties as following: the fully oxidized pernigraniline base, half-oxidized emeraldine base, the 75% intrinsically oxidized nigraniline and fully reduced leukoemeraldine base (Fig. 7) (Ghasemi-Mobarakeh *et al.*, 2011). It is also well known that PANI in the emeraldine oxidation state can be reversibly switched between its electrically non-conducting (base) (PANI-B) and conducting (salt) (PANI-S) forms.

In addition to polymer powder, PANI can produce a thin film, which is also an interesting application form. Any surface which is immersed in the reaction mixture used for the PANI preparation becomes coated with a thin film of this polymer (Fig. 3).

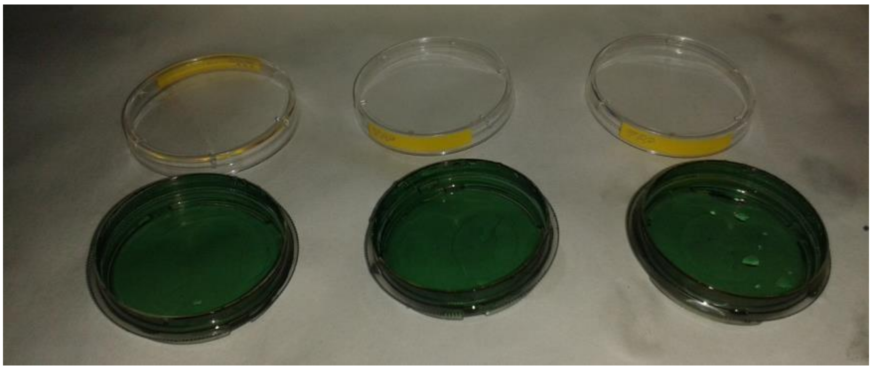

Fig. 3: Conducting PANI films prepared at the laboratory of the Centre of Polymer Systems by Nikola Mikušová.

Recent reports have also shown that PANI possesses antimicrobial effects. (Gottesman *et al.*, 2011) (Nanlin Shi, 2006). Moreover, PANI is also important polymer thanks to its potential anti-corrosive activity (Mengoli *et al.*, 1981; DeBerry, 1985); (Banerjee, Pangule and Kane, 2011).

1.1.3 Poly(phenylenediamine)

Poly(phenylenediamine) (PPDA) is an electroactive polymer of the aromatic diamines family. Phenylenediamines show excellent diversity caused by the main part of these materials – diamines. PPDA can exist in three isomer forms – ortho, meta and para; differing in chemical, physical, mechanical and thermal properties (Stejskal, 2015). PPDA can be prepared by oxidation of respective monomers to oligomers and polymers (do Nascimento, Sestrem and Temperini, 2010).

Nowadays, there is not enough information on PPDA. Reported publications on the antimicrobial and biological activity of this polymer are also sparse. However, PPDA has a potential, similarly to PANI and PPy, to become promising antimicrobial agents and to be successfully used in biomedical applications (Al-Hussaini and Eldars, 2014; Kucekova *et al.*, 2017).

1.1.4 Polypyrrole

Another CP is PPy whose synthesis by chemical oxidation was described already in 1887. The oxidation of pyrrole leading to preparing PPy is governed by the same principles as oxidation of PANI, and their formal chemistry is similar (Blinova *et al.*, 2007). PPy can be also prepared using electrochemical synthesis (Vernitskaya and Efimov, 1997; Parakhonskiy and Shchukin, 2015).

PPy is frequently used due to its good conductivity, excellent environmental stability, *in vivo* and *in vitro* biocompatibility, stability under *in vivo* conditions, redox behavior and reversible protonation (Omastova *et al.*, 2014). Compared to PANI, PPy has reasonably high conductivity in a wide pH range. Due to this fact, PPy provides outstanding corrosion protection abilities (Chamovska, Porjazoska-Kujundziski and Grchev, 2013; Nautiyal *et al.*, 2018; Garcia-Cabezon *et al.*, 2020). PPy has also been considered as a potential candidate for applications involving biofilm detection and control (Cordeiro *et al.*, 2015).

1.1.5 Anticorrosive properties of conducting polymers

CPs show very significant feature - the ability to easily coat various surfaces. This process consists in immersion these surfaces in a polymer reaction mixture. Subsequently, the formation of CP films begin (Hashim, 2010). Furthermore, polymeric film technology has a key role in helping to understand the cell-surface interactions. It then leads to the elucidation of how living cells respond to the surfaces.

Despite the popularity of CPs, certain properties of these polymeric materials and practical problems still limit their use (Guimard, Gomez and Schmidt, 2007;

Thomas *et al.*, 2000; Green *et al.*, 2012; Kishi *et al.*, 2012). However, polymeric films can be modified through several techniques to improve their properties such as the antibacterial and antifungal activities which are also equally important (Muthusankar *et al.*, 2018; Zengin *et al.*, 2019).

In addition to the formation of polymeric films, CPs can be used for the preparation of polymeric coatings containing also additives (fillers, pigments, stabilizers). Polymeric coatings can be functional (adhesives), protective (anticorrosion) or decorative (paint) (Kalendová *et al.*, 2015; Francis and Roberts, 2016). Nowadays, coatings containing anti-corrosion agents, such as pigments, are used for corrosion protection (Prokeš and Kalendová, 2007).

Figure 4 shows the appearance of the selected coatings and the metal substrate after the corrosion test.

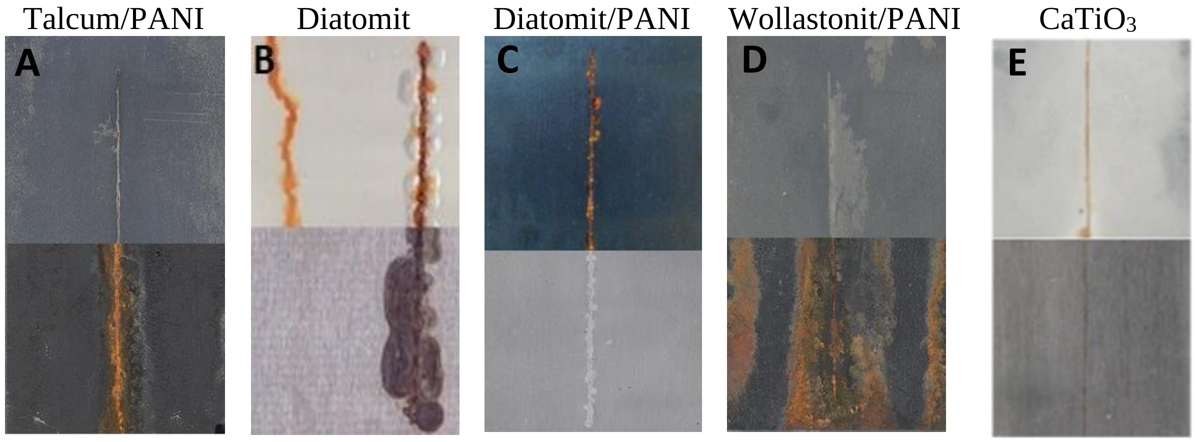

Fig. 4: The appearance of the selected coatings and the metal substrate after the corrosion test. The coatings are shown in the upper half of the panel, steel panel is shown on the lower half. (Department of Paints and Organic Coatings, University of Pardubice)

1.1.6 Modification of conducting polymers

CPs offer many advantageous properties over the other materials. However, the additional modification and optimization of CPs are also required. Optimizing of properties of CPs is important with respect to their targeting to specific applications. The most significant properties of CPs with respect to biomedical fields cover mainly the conductivity, biocompatibility and redox stability (Guimard, Gomez and Schmidt, 2007). Surface modification techniques were also developed to impart antimicrobial properties on polymeric films and coatings. One of the modifications relies on reprotonation with various dopant acids (Stejskal, Prokes and Trchova, 2008), thereby altering their biological properties (Humpolicek *et al.*, 2015; Bober *et al.*, 2015). Modification, overall, can change the physical, chemical, mechanical and material properties, functionality, nanostructure of CPs, etc. Since the polymeric surfaces are often non-reactive, and surface modification may involve chemical alteration of the surface layer, it requires the generation of high-energy species. These high-energy species include radicals, ions, and molecules in an excited electronic state to promote a surface reaction. This is enabled by using modification techniques such as flame, plasma (see Fig. 5), UV, laser, X-ray, electron beam or ion beam (Sharma, Sims and Mazumder, 2002). Modification using sterilization (e.g. moist and dry heat, UV or ethylene oxide) is another possibility of how to change the characteristics of CPs.

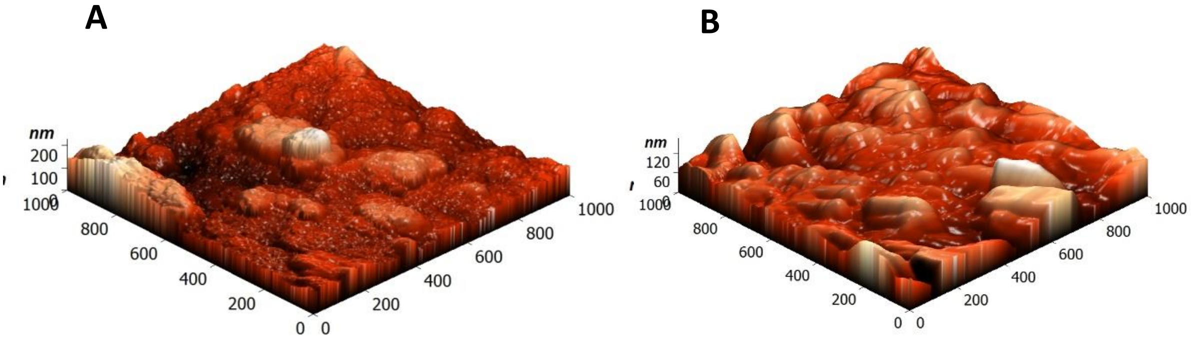

Fig. 5: Atomic force microscopy (AFM) micrographs (1x1 μm^2) of PANI-S films – untreated (A) and oxygen plasma treated for 30 sec. (B) showing surface roughness reduction. AFM evaluation was performed in cooperation with the Laboratory of Surface Engineering and Optoelectronics, JSI in Ljubljana.

1.2 Biological properties of polymeric materials

In coatings and polymeric films, besides other factors, there is an emphasis on the interaction of the material surface with microorganisms. The colonization of the surface by microorganisms can have an unfavorable impact on its functionality. Microbial adhesion can cause corrosion, degradation or failure of the material. Microbial adhesion can further lead to biofilm formation (Fig. 6) if the microorganism species Fs biofilm-forming. Moreover, biofilm may initiate the formation of biofouling. Hence, the best way to control biofouling is to prevent the formation of biofilms before it starts.

Microorganisms (bacteria, fungi) clump together with an intention to build certain protection of their colony, also especially on wetted surfaces. This unwanted microbial adhesion is, therefore, the main reason why a lot of area in industry, biomedicine and many others, endeavor to develop antibiofilm materials. The second mentioned problem, the biofouling[1], causes serious problems worldwide, as the deterioration of materials and the affecting of human health (Bachmann and Edyvean, 2006).

This doctoral study focused on polymeric films and coatings with the aim to influence their antibiofilm properties. Moreover, the surfaces of films and coatings can be subsequently modified to improve properties particularly related to the antibiofilm activity.

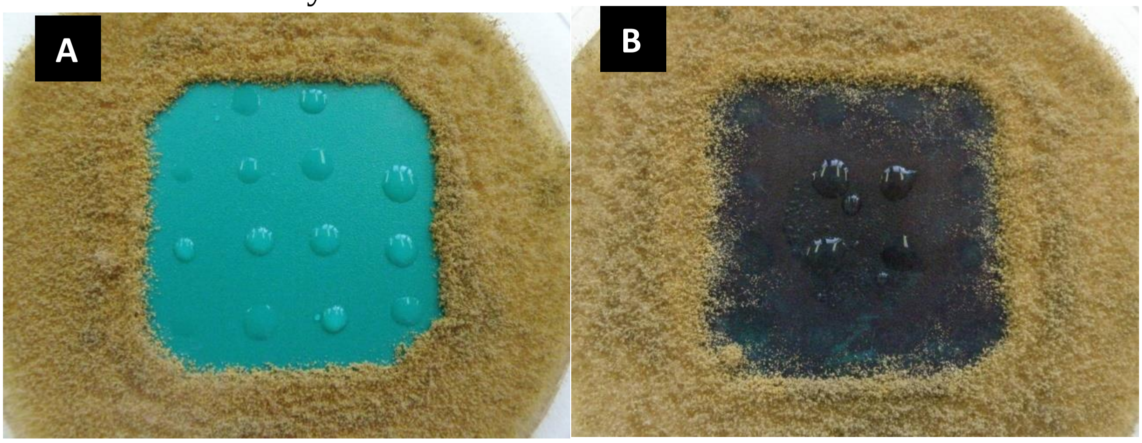

Fig. 6: Fungal biofilm formation: *Aspergillus niger* on polypropylene (A) and *Aspergillus oryzae* on PANI (B). (The images were taken in the laboratory of Microbiology, the Centre of Polymer Systems, TBU in Zlín)

1.3 Surface properties affecting biofilm formation

The attachment of microbial cells onto any surface is the first and critical step of biofilm formation. Thus, the surface properties of materials play a very important role from the beginning (Fig. 7) (Kochkodan and Hilal, 2015). From a material perspective, the surface roughness, topography, surface free energy, surface charge, electrostatic interactions, and surface hydrophobicity are generally known to be relevant parameters for the attachment process (Rummel et al., 2017). To develop some suitable materials, that are applied in biomedical fields or in industry, it is also needed to understand the structure and chemistry of the solid-liquid interface (Pavithra and Doble, 2008). These factors are related to biofilm formation and biofouling mainly because of the determination of the interaction between the surface and the foulants (Kochkodan and Hilal, 2015).

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[1] Biofouling is fouling of any deposit (e.g. microorganisms, macroorganisms, plants, animals) or the undesirable accumulation of biotic matter onto a surface in contact with liquid, including biofilm formation (Flemming, 2002; Fusetani, 2004).

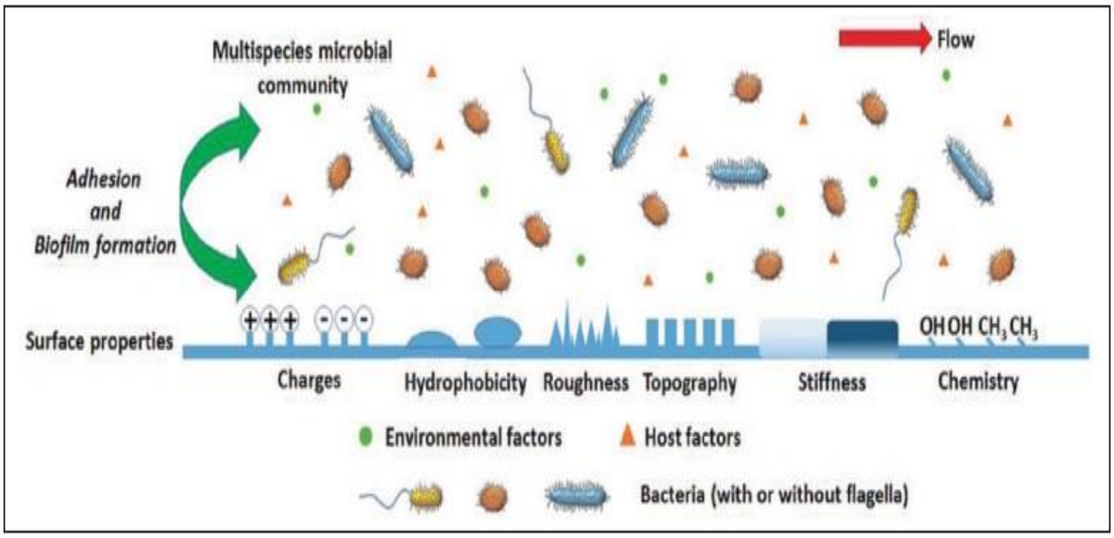

Fig. 7: Bacterial adhesion/biofilm formation and the effects of material properties in complex environments. (Song, Koo and Ren, 2015)

1.3.1 Biofilm prevention

Many scientists deal with microbial biofilm and effort to find new and effective procedures. Especially, they want to find out how to decrease the number of undesirable microbial biofilms onto substrates. The modification of material may achieve the increasing of material biocompatibility and on the other hand the decrease of biofilm formation. The antibacterial or antibiofilm surface can be also modified using antibacterial agents (nanoparticles, polymeric compounds) such as silver, titanium dioxide (TiO_2) or metal oxides (Mg, Zn), to improve antimicrobial activity. (Parashar *et al.*, 2011; Modaresifar *et al.*, 2019; Netala *et al.*, 2015; Manna *et al.*, 2015).

2 AIMS OF DOCTORAL THESIS

The doctoral thesis is focused on the understanding of critical factors influencing the interaction between microorganisms and material surfaces. Special attention is paid to the formation and growth of biofilm-forming bacterial and fungal strains. Followed issues were concerned:

- Preparation of polymeric surfaces using chemical polymerization.
- Modification of surface properties of tested materials (polymeric surfaces, polymeric coatings) and surface structuring of TiO_2 NTs.
- Characterization of material and surface properties of pure and modified surfaces.
- Determination of antibiofilm properties of surfaces using selected strains of biofilm-forming bacteria and filamentous fungi.

3 METHODOLOGY

Within the doctoral study, the experimental part was focused on the determination of antibiofilm activity of various material surfaces. In this chapter, the preparation and modification of polymeric films (such as PANI), polymeric coatings with various fillers and pigments (such as PPY, PPDA, PANI) and TiO_2 NTs are described. The description of the methodology used for the evaluation of the biological properties of tested surfaces is explained below including references to published articles of my research. The antibiofilm effect of individual surfaces is discussed in the context of surface properties such as topography, surface energy, and conductivity. Moreover, new methods were introduced to laboratory practice - comet assay and protein adsorption.

3.1 Preparation and modification of surfaces

3.1.1 Preparation of polyaniline films

PANI-S films was performed *in situ* in accordance with Stejskal & Sapurina (Stejskal and Sapurina, 2005). The PANI-S was further converted to PANI base (PANI-B) through deprotonation with 1 M ammonium hydroxide (Sigma-Aldrich, USA). PANI films were modified using the re-protonation process with different acids, namely 50 wt% aqueous solution of phosphotungstic acid (PTA; PANI-PTA), and 15 wt% solution of poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA; PANI-PAMPSA) (both acids purchased from Sigma-Aldrich, USA) (see published article Mikušová *et al.*, 2017). Modification of PANI films was carried out in the laboratories of the Centre of Polymer Systems at TBU in Zlín. PANI films were also treated by highly reactive oxygen plasma. The treatment was done for two times of duration (5 and 30 seconds) in the glow and afterglow régime (AG) (Fig. 8). This experiment with oxygen plasma was performed in cooperation with JSI in Ljubljana, Slovenia.

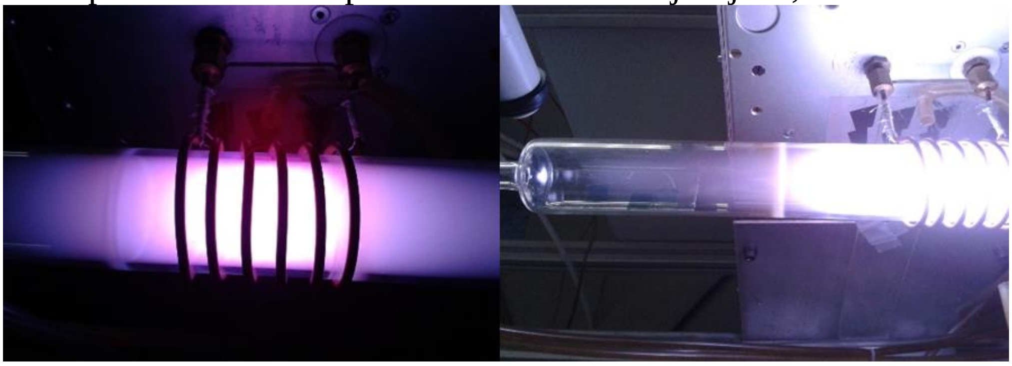

Fig. 8: Glow (left) and afterglow (right) regime of oxygen plasma treatment. (The image was taken by Nikola Mikušová in the laboratory of JSI)

3.1.2 Preparation of polymeric coatings

The commercial epoxy-ester resin Worleédur D46 was used as the binder for preparing the polymeric coating. Inorganic pigments coated with layers of the CP were applied as chemically active pigments. Tungstate and molybdate pigments were prepared by solid-phase reaction (Trojan, Brandová and Šolc, 1987) and included either iron(III) tungstate (Fe_2WO_6) or iron(III) molybdate $Fe_2(MoO_4)_3$. The pigments were modified with the following anti-corrosion agents: PANI, PPy, PPDA, or $ZnFe_2O_4$ mixed oxide ($ZnFe_2O_4$). The final composition of the coatings used is summarized in Tab. 1 (see published article Mikušová *et al.*, 2019).

Tab. 1: Composition of polymeric coatings. (ϕ_i=VCP – Volume concentration of pigment (prepared in cooperation with Department of Paints and Organic Coatings, University of Pardubice)

Diatomite (SiO_2)	Tungstate (Fe_2WO_6)
SiO_2/PANI	Fe_2WO_6/PANI ϕ_i= 1%
SiO_2/PPDA	Fe_2WO_6/PANI ϕ_i= 15%
SiO_2/$ZnFe_2O_4$	Fe_2WO_6/PPDA
Wollastonite ($CaSiO_3$)	Molybdate ($Fe_2(MoO_4)_3$)
$CaSiO_3$/PANI	$Fe_2(MoO_4)_3$/PPy
$CaSiO_3$/PPDA	$Fe_2(MoO_4)_3$/PANI
$CaSiO_3$/$ZnFe_2O_4$	$Fe_2(MoO_4)_3$/PPDA

Reference sample - WorléeDur D 46

The coatings were prepared in cooperation with the University of Pardubice, Faculty of Chemical Technology, Institute of Chemistry and Technology of Macromolecular Materials, Department of Paints and Organic Coatings.

3.1.3 Preparation of nanostructured surfaces based on TiO_2

TiO_2 NTs were fabricated by the electrochemical anodization of Ti foil (Advent Research Materials, England) of 0.1 mm thickness (99.6% purity) (see published article Kulkarni *et al.*, 2017). The NTs were prepared in cooperation with JSI in Ljubljana, Slovenia.

3.2 Characterization of surface properties

Topographic changes of the polymeric surfaces after plasma treatment were monitored with AFM (Solver PRO, NT-MDT, Russia) in the tapping mode in air. The surface roughness has been measured on 1 x 1 μm^2 and 2 x 2 μm^2 AFM images, as this size of the area was the most representative for roughness measurements of samples. This test was performed in cooperation with JSI in Ljubljana, Slovenia.

Contact-angle data were obtained with a Surface Energy Evaluation System (SEE system) from Advex Instruments (Czech Republic). Deionized water, ethylene glycol, and diiodomethane have been used as testing liquids. The volume of droplets was set to 5 μL for all experiments to avoid errors associated with gravity acting on the sessile drop.

Turning to the conductivity of the PANI films, this was measured by the four-point Van der Pauw method. A programmable electrometer with an SMU Keithley 237 current source and a Keithley 2010 Digital Multimeter with a 2000 SCAN 10-channel scanner card (USA) were employed. Measurements were carried out at laboratory temperature. The surface energy evaluation and the electrical conductivity evaluation was performed in the laboratory of the Centre of Polymer Systems at TBU in Zlín (see published article Mikušová *et al.*, 2017).

3.3 Evaluation of biological properties

The strains of gram-positive and gram-negative biofilm-forming bacteria and filamentous fungi obtained from the Czech Collection of Microorganisms (CCM) were used for the research work of the doctoral thesis:

a) Biofilm-forming bacteria:

- *S. aureus* CCM 2022, *S. aureus* CCM 3953, *S. epidermidis* CCM 4418, *S. epidermidis* CCM 7221, *P. aeruginosa* CCM 3955, *E. faecalis* CCM 4224, *E. faecalis* CCM 7000, *E. coli* CCM 3988, *K. pneumoniae* CCM 4415

b) Filamentous fungi:

- *Aspergillus niger* CCM 8155, *Gliocladium virens* CCM 8042, *Paecilomyces variotii* CCM F-398, *Trichoderma viridae* F-486

3.3.1 Bacterial biofilm formation

In the first step, the standard method using crystal violet dye was used for all of the tested samples. However, PANI owns such surface properties which cause that this method is not able to provide reliable results. Therefore, in the next step, the biofilm formation of bacteria was evaluated using a method measuring the ATP amount (see published article Mikušová *et al.*, 2017). The process of quantifying the biofilm with bacteria (linked in chapter 3.3) followed a procedure described by Koutný *et al.* (Koutny *et al.*, 2006). The statistical differences between the individual values were evaluated using Tukey's significant difference test. The evaluation of bacterial biofilm formation was performed in the laboratories of the Centre of Polymer Systems at TBU in Zlín.

3.3.2 Fungal biofilm formation

The filamentous fungal strains, listed in chapter 3.3, were cultivated on malt-extract bouillon broth (MEBB, Himedia, India) solidified with agar (20 g L^{-1}), which was utilized as a nutrient-rich agar, and on nutrient-poor agar (see published article Mikušová *et al.*, 2017). Fungal growth (on the edge) and biofilm formation (in the middle) on the polymeric surfaces was expressed as the percentage of area covered with fungal mycelium. Photographs of the samples were taken, onto which a grid was created (Fig. 9). The individual squares of the grid, which either did or did not contain fungal biofilm, were counted and percentages for growth and biofilm formation were determined. The growth of filamentous fungi on the nutrient-poor agar was also subsequently analyzed in the same way. All tests were performed in duplicates. The statistical differences between the individual values were evaluated using the Sign test. The evaluation of fungal biofilm formation was performed in the laboratories of the Centre of Polymer Systems at TBU in Zlín, in cooperation with master student Kristýna Janů.

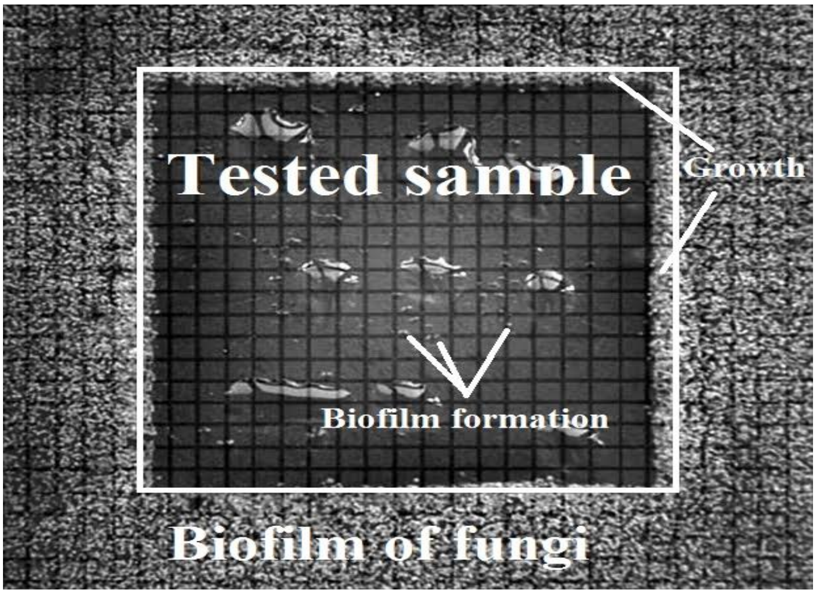

Fig. 9: Grid applied to evaluate biofilm formation and growth of fungi. (The grid was prepared at the Centre of Polymer Systems)

3.3.3 Methods introduced to laboratory practice

3.3.3.1 Comet assay

Within the doctoral study, new methods were introduced to extend the competence of the biological laboratory in the Centre of Polymer Systems. This important step helped to contribute with new knowledge about tested material properties in the laboratories of the Centre of Polymer Systems. Comet assay and protein adsorption are well established methods for the biological characterization of materials for their application in biomedicine fields.

Comet assay was performed according to the procedure within the protocol of Olive and Banath (Olive and Banath, 2006) where is the whole methodology described in more detail.

3.3.3.2 Protein adsorption

Protein adsorption was utilized according to the instruction protocol included within the Micro BCA Protein Assay Kit (Thermo Scientific, USA) where is the whole methodology described in more detail.

4 RESULTS AND DISCUSSION

The results related to the presented topic are summarised in the following sections. Some of the presented results have been published in the journals with impact factor which are mentioned below.
The sections are entitled as follow:

- Biofilm formation on PANI based films
    - Biofilm formation on pure and modified PANI films (published in *Chemical Papers*, 2017, *71(2)*, pp. 505-512)
    - Biofilm formation on plasma modified PANI films (unpublished results)
- Biofilm formation on polymeric coatings (published in *International Journal of Polymeric Materials and Polymeric Biomaterials*, 2019, *68(4)*, pp. 152-159)
- Biofilm formation on nanostructured TiO_2 (published in *Materials Science and Engineering: C*, 2017, *77*, pp. 500-507)
- Methods introduced to laboratory practice
    - Comet assay and Protein adsorption

4.1 Biofilm formation on PANI based films

4.1.1 Biofilm formation on pure and modified PANI films

The present research is the first which explore the behavior of PANI films in contact with selected bacterial and fungal strains, thereby providing crucial data on biofilm formation and the behavior of microorganisms on PANI surfaces. The first determination of the antibiofilm activity of pristine and modified PANI films has been established using crystal violet dye. However, this method proved to be inappropriate because of distorted results. Because of that, the methodology had to be changed to measuring ATP level using a luminometer which provided values being interesting and significant for this research work.

4.1.1.1 Results and discussion

Surface energy and conductivity evaluation

The evaluation of surface energy through contact angle measurement revealed a rise in the total surface energy (γtot) in each PANI film in comparison with the polypropylene and polystyrene references, as can be concluded from values for γLW (disperse componentand γAB (acid-base (polar) component). Indeed, the hydrophilicity or hydrophobicity of the surface is an important characteristic that exerts a major impact on cell attachment and fungal and bacterial growth. The greatest value for surface energy was observed for PANI-B and PANI-PTA films. In contrast, the lowest value measured pertained to PANI-PAMPSA, which demonstrated the most notable inhibition of bacterial biofilm.

Bacterial biofilm formation

PANI surfaces, pure and modified, have reacted differently to resist bacterial adhesion as is seen in Fig. 10. PANI-S failed to exert any significant effect against biofilm formation. The surface of PANI-B slightly inhibited the biofilm formation only in the case of *E. faecalis* CCM 7000 in comparison with the PS reference sample. The other bacterial strains were not significantly reduced on the non-conducting surface. Marginally greater activity against the biofilm formation was observed in PANI-PTA compared with the reference. The most pronounced effect against biofilm generation recorded for all tested surfaces pertained to the PANI-PAMPSA film. This modified surface was capable to inhibit the biofilm formation on all the bacterial strains. The antibiofilm activity of the PANI-PAMPSA film stems from its surface energy being lower than other PANI films. PANI-PAMPSA has the most hydrophilic surface of the tested films. Generally, some strains of bacteria with hydrophobic properties actually prefer more hydrophobic surfaces for their growth and vice versa, which is mainly influenced by surface charge (An, 2000).

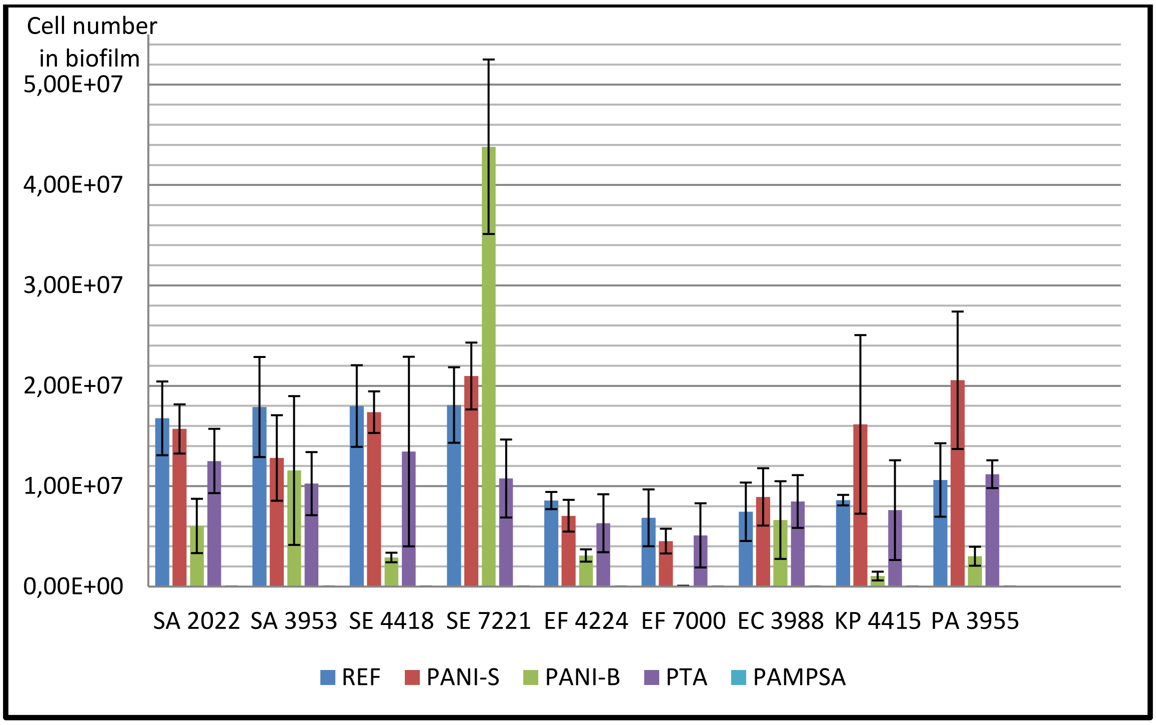

Fig. 10: Growth of bacterial biofilm formation expressed as an average number of cells with SD. (measured in the laboratory of Faculty of technology in TBU in Zlín by Nikola Mikušová)

Fungal biofilm formation

Fungal biofilm formation and mycelia growth were tested on both MEBB and nutrient-poor agar. After 42 days of cultivation, it was apparent that 100 % of the occurrence of fungal mycelia on the outer parts of the samples was observed in nearly all PANI surfaces. As for the biofilm growth on the inner parts of the samples, it can be concluded that the most intensive biofilm formation was recorded for PANI-PTA with *G. virens* (88 %), while the less intensive on PANI-B in *A. niger* (0 %) and PANI-PTA with *A. niger* (4 %). The surface of PANI-S showed the densest biofilm formation with fungi *G. virens* (72 %) and, similarly, with *P. variotii* (67 %). On the surface of PANI-B, the lowest biofilm formation was found in comparison with the other two types of tested samples. Generally, *G. virens* formed the densest biofilm, while the biofilm formation with *T. viridae* and *A. niger* was the lowest.

Comparing results from all the surfaces when utilizing nutrient-rich and nutrient-poor agar demonstrated that, in general, a higher percentage of the fungal growth was achieved with complete agar. It was the same in case of biofilm formation. Therefore, the employed strains needed complete agar to facilitate their growth and biofilm formation due to the utilization of nutrients, as nutrient-poor agar lacked sufficient nutrients for the same to take place. The use of nutrient-poor agar led to the releasing of mycelia occurrence on the tested samples in most cases, except for *G. virens* on the surface of PANI-S.

The filamentous fungi are not intensively studied compare to bacterial strains (Lugauskas, 2003; Binkauskiene, Lugauskas and Bukauskas, 2013).

4.1.1.2 The outcome of the research work

Within this research, the knowledge about mutual interactions of polymeric surfaces and microbial attachment was significantly advanced. Pristine and modified PANI surfaces were subjected to the determination of their antibiofilm activity. Overall, the reprotonated surface of PANI-PAMPSA demonstrated antibiofilm activity against all tested strains of biofilm-positive bacteria. Whereas, the other tested PANI films did not show any significant antibiofilm activity. Concerning the anti-fungal activity, used filamentous fungi were able to form mycelia to a greater extent on PANI films, mainly on nutrient-rich agar compared to nutrient-poor agar. These findings and surface properties have practical importance for various antimicrobial surface treatments.

This study was summarized into the manuscript which was subsequently submitted and published in the journal with impact factor as *Formation of bacterial and fungal biofilm on conducting polyaniline*, *Chemical Papers*, 2017, *71(2)*, pp. 505-512. Moreover, the study was already cited in three articles.

4.1.2 Biofilm formation on plasma modified PANI films

The surface energy is crucial when the interaction with proteins of physiological fluids are considered. The surface energy can be modified by either chemical reaction (Queffélec *et al.*, 2012; Mikušová *et al.*, 2017) or by plasma treatment (Slepička *et al.*, 2012). Plasma provides diverse possibilities to refine a polymer surface, enabled by the adjustment of parameters like gas flows, power, pressure and treatment time. Due to the numerous ways, a plasma interacts with the polymer surface. The gas type (Ar, He, N_2, O_2) and plasma treatment also influences the wettability of materials, respectively increases hydrophilicity. Thus, it does not allow to most bacterial cells, which prefer less wettable, hydrophobic materials, to adhere and proliferate on the treated surfaces. In cooperation with JSI, in Ljubljana, Slovenia, PANI films treated by oxygen plasma were prepared and subsequently their properties were tested.

4.1.2.1 Result and discussion

In the present research, PANI-S and PANI-B films were treated by radiofrequency coupled oxygen plasma and subsequently its wettability, morphology and chemical composition using AFM, X-ray photoelectron spectroscopy (XPS) and secondary ion mass spectrometry (SIMS) were investigated. Further, the conductivity of treated films was determined. To reveal the impact of plasma treatment on living organisms the study of bacterial biofilm formation and eukaryotic cell adhesion were performed.

From measuring conductivity it can be concluded that the value of untreated PANI-S (10 S cm^{-1}) was decreased after 5 s plasma treatment of glow regime approximately to 6 S cm^{-1} and the film was stable almost for the whole time of measuring of material aging. In the case of the afterglow regime, the conductivity of PANI-S was about 5 S cm^{-1} and during 21 days of testing, it was decreased even to 3.5 S cm^{-1}. Thus, the glow regime seems to be more effective, the value was reduced only by one order. Plasma treated PANI-B film has stayed still nonconducting.

Wettability has significantly increased. PANI-S films after treatment increased their hydrophilicity of the surface. However, it was found that 5 s plasma treatment is not practical and appropriate in this case. The surfaces were nonuniform and therefore it is necessary to lengthen the time of plasma treatment. On the contrary, PANI-B films showed excellent wettability for 10 days. Wettability can positively contribute to preventing microbial adhesion.

The AFM of the tested surface showed that the morphology of PANI films is not homogenous. The values of Ra are only informative. However, how it is seen from micrographs in Fig. 11, the surface roughness was slightly decreased and the PANI films became smoother. So, plasma treatment has just gently changed the morphology. The roughness of surfaces stayed still high.

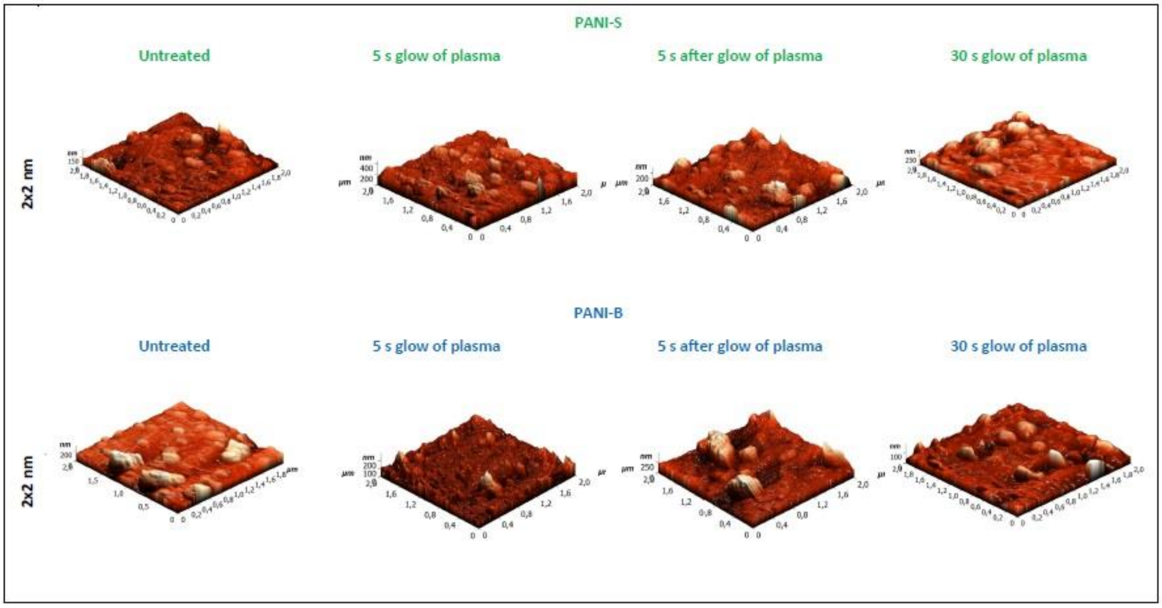

Fig. 11: AFM micrographs (2x2 nm²) of PANI films untreated and treated by oxygen plasma. Average surface roughness (Ra) with SD. (The images were taken in cooperation with the laboratory of JSI)

Subsequently, XPS of PANI films untreated and treated by oxygen plasma were performed and the obtained results confirmed the increased amount of oxygen onto the surface due to the oxygen plasma treatment. The rest of the values were not significantly influenced, only the presence of atom C was slightly decreased after the plasma.

Concerning the biological response of plasma-treated surfaces, the growth of bacterial biofilm was observed, *S. aureus* representing gram-positive strain and *P. aeruginosa* representing gram-negative strain. Further, it was found that bacterial attachment was decreased compared to the reference (PP without PANI films) due to plasma treatment and probably high wettability of tested surfaces. However, the different times of plasma treatment did not show any significant influence on bacterial biofilm formation. The same finding was concluded for the aging time of PANI films. The effect of plasma treatment on eukaryotic cells (mouse embryonic fibroblast cell line ATCC CRL-1658 NIH/3T3, USA) did not prove any significant biocompatible activity. Cell adhesion was almost to the same extent compared to treated samples.

Despite the fact, the plasma treatment has contributed to the improvement of the surface properties of PANI films, it is still necessary to upgrade the operation process of oxygen plasma, to choose the appropriate time of treatment and to stabilize methodologies of measurements. However, observed wettability of the treated surface can be further developed for preparing biocompatible surfaces.

4.1.2.2 The outcome of the research work

The surface of microorganisms is usually hydrophobic, as well as the surfaces of CPs. These polymeric surfaces may be an appropriate substrate with antibiofilm activity. Due to this fact, the doctoral study was focused to obtain significant results which will be beneficial for broad scientific purposes. Within research, the oxygen plasma treatment was used for modification of polymeric surfaces to make them hydrophilic and to preclude the attachment of biofilm-forming bacterial cells.

4.2 Biofilm formation on polymeric coatings

The main reason why the following study was introduced in our laboratories was the fact that biofilm formation on the surface can lead to the change of various surface properties (transparency, surface energy, conductivity, degradation of material resulting in a change of its composition). These undesirable changes in surface properties of polymeric coatings commit significant problems in many fields. Bacterial/fungal biofilm is also one of the crucial aspects of corrosion processes. The interesting properties of CPs are their ability to work as anti-corrosive agents (Kalendová *et al.*, 2008), for instance in the coating. The most extensively studied CPs are PANI, PPy, and poly(p-phenylenediamine), which have been applied with success in certain applications, e.g. active anti-corrosion agents for metallic materials by means of organic polymeric coatings (Armelin *et al.*, 2007; Deshpande *et al.*, 2012; Sathiyanarayanan *et al.*, 2005).

A solvent-type epoxy ester resin was selected as a binder for the preparation of polymer coatings due to excellent flexibility, long life, good adhesion, ease of handling, rapid air drying, good film toughness (Oil and Association, 1993). Moreover, it has excellent corrosion protection for aluminum and zinc pigments (Müller and Fischer, 2006). The ability of polymers is also to be as an “anti-biofouling” coating (Au *et al.*, 2013).

The coatings were prepared in cooperation with the University of Pardubice, Faculty of Chemical Technology, Institute of Chemistry and Technology of Macromolecular Materials, Department of Paints and Organic Coatings. At the laboratory of CPS, the biological tests and surface energy evaluation were performed.

4.2.1 Result and discussion

Evaluation of surface properties

The scanning electron microscopy (SEM) analysis was used for evaluation of the surface and morphology of pigment particles and polymeric coatings separately because at low concentration of pigments the SEM analysis remained unchanged. The magnification of individual present figures depended on particle size given by its structure and type of use. The surface modification using CPs did not change the morphology or particle size and the pigments tended to form clumps, which had to be separated during dispersion in a polymeric binder.

Surface energy plays a very crucial role. Based on obtained results, it obvious that pigmentation increased the surface energy of polymeric coatings. If pigments are treated with CPs, the effects of the individual components are synergistic. In actual fact, the pigments largely become more porous, showing a complex surface and exhibiting binding sorption properties. The differences in surface energy of each coating pertain to the nature of the pigment (chemical composition, particle shape), as well as to the presence of the CP. The highest values for surface energy of the polymeric coatings were primarily observed in samples where the pigment surface had been modified with PPDA, except for the sample $CaSiO_3$/PPDA. The lowest surface energy was observed for the reference material WorléeDur D 46.

Bacterial biofilm

The antibiofilm effect of the tested coating was described using two biofilm-forming bacteria species. The number of bacterial cells and related biofilm formation on the tested surfaces were affected by the composition of the polymeric coating. Generally, a methodology using ATP measurement did not show any significant differences or decreasing of bacterial adhesion. Only the weakest biofilm formation was observed on surfaces containing the pigment $Fe_2(MoO_4)_3$, especially in modification with PPy in the case of *P. aeruginosa* or modification with PPDA in case of *B. cereus*. Polymeric coatings with modified

$CaSiO_3$ also reduced biofilm formation with regard to *P. aureginosa*, especially in the case of $CaSiO_3$/ $ZnFe_2O_4$.

Filamentous fungi biofilm

The polymeric coatings were evaluated for antifungal activity as well. The observed results contribute more information to knowledge about the anti-fungal activity of CPs. Herein, the authors mimicked real conditions by inoculating a mixed culture of four fungal species (listed in chapter 3.3).

On MEBB, it was determined that the greatest degree of growth occurred in the case of $CaSiO_3$/PPDA, while the least growth was seen on the surface with the $CaSiO_3$. More intensive biofilm formation on MEBB – compared with the reference sample – was observed almost on all surfaces, except SiO_2, SiO_2/PANI, and SiO_2/$ZnFe_2O_4$. The effect of SiO_2 is not connected to the surface energy of the final material as they were similar to the reference. The test performed on incomplete agar should reveal if the fungi were able to use any of the tested materials as a source of nutrients. Based on the results obtained in this work we can conclude that biofilm overgrowth and formation on all tested polymeric coatings were lower than on reference. The fungi were not therefore able to use the polymeric coatings as a source of nutrients. However, the polymeric coatings have not been tested yet on biofilm formation. The published studies are rather focused on testing of physicochemical features (Kalendová *et al.*, 2015).

4.2.2 The outcome of the research work

Tested anticorrosion coatings and their composition can lead to the formulation of a new anti-biofouling surface using in many fields of industry. The application of surface coatings is one of the most frequently used methods for the fabrication of antibacterial surfaces. Moreover, it expands the information about CPs and their modification with pigments. Results contribute to the understanding of the relation between CPs, polymeric coatings, their modification and microbial biofilms, especially filamentous fungi which was not previously studied. In conclusion, the current success of the antibacterial activity of CPs offers great potential for mitigating and preventing biocorrosion in the future.

This study was summarized into the manuscript which was subsequently submitted and published in the journal with impact factor as *The effect of the composition of a polymeric coating on the biofilm formation of bacteria and filamentous fungi, International Journal of Polymeric Materials and Polymeric Biomaterials*, 2019, *68(4)*, pp. 152-159. Moreover, the study was already cited in two articles.

4.3 Biofilm formation on nanostructured TiO_2

Next cooperation with JSI (Slovenia) was focused not on polymeric surfaces, but on TiO_2 NTs differing in their nanostructure. Ti and its alloys are commonly employed in medical implants, but progress in nanotechnologies together with new findings indicating the importance of nanoscale morphology on biofilm formation. Thus, biofilm formation, which is highly relevant for all implantable materials, was studied. Due to the rise of antibiotic-resistant bacterial strains, it is also of primary importance to inhibiting the growth of biofilms on implantable materials by influencing their surface properties.

4.3.1 Result and discussion

Nanoscale morphology and surface chemistry of TiO_2 NTs

The surface properties of both Ti foil and TiO_2 NTs were characterized in terms of surface morphology (using SEM and AFM techniques), wettability, and surface chemistry (using XPS).

The analysis revealed that pristine Ti foil had no special morphological features, while a uniform nanotubular structure was observed for electrochemical anodized TiO_2 surfaces (Fig. 12). TiO_2 NTs were evenly distributed on the surface and that anodization potentials of 10 V, 20 V, and 58 V led to the formation of TiO_2 NTs of 15, 50 and 100 nm in diameter, respectively (Fig. 12).

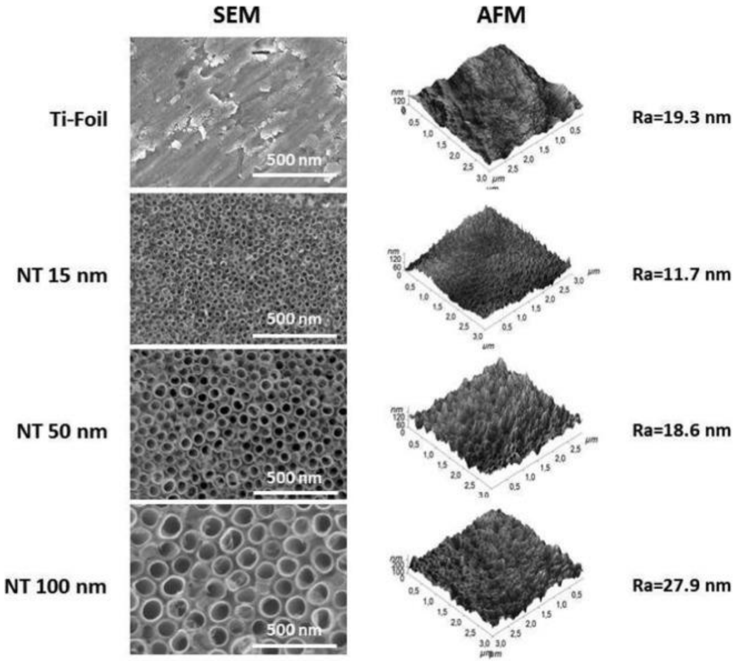

Fig. 12: Surface morphology of Ti foil and TiO_2 NTs determined from images taken by SEM and AFM. (measured by JSI)

Another important aspect of a biomaterial surface is its wettability, which could, together with other surface properties, influence on the biological response. Water contact angle (WCA) measurements conducted on Ti foil and freshly fabricated TiO_2 NTs indicate that Ti foil is poorly wettable (a water contact angle of about 780), while TiO_2 NTs are all hydrophilic (a water contact angle of less than 50). However, it should be noted that fabricated TiO_2 NT surfaces tend to age if exposed to the atmosphere and become hydrophobic. Thus, to ensure the hydrophilic character of the surfaces, all biological experiments were conducted one week after fabrication.

Bacterial biofilm

Within this research two bacteria, *B. cereus* and *P. aeruginosa*, representing both gram-positive and gram-negative biofilm-forming bacteria, were studied. Both bacteria formed their biofilms with slightly higher amounts on TiO_2 NTs compared to Ti foil (Tab. 2). In addition, slightly higher amounts of formed biofilm were observed for *B. cereus* compared to *P. aeruginosa*.

The study has been extended to the determination of eukaryotic cell behavior. Three different cell lineages – mesenchymal stem cells, embryonic stem cells, and cardiomyocytes – were seeded on nanostructured surfaces of Ti. Especially, differences in their behavior were observed. Results showed that mesenchymal and embryonic stem cells were able to adhere and grow on the tested surfaces. Whereas the adhesion of cardiomyocytes was minimal. Moreover, a correlation between the NT diameter and cell behavior was observed. The 100 nm NTs appear to present a crucial diameter for, which NTs, critically influence mesenchymal stem cells behavior, while the 15 nm NTs appear to present a critical diameter for embryonic stem cells.

Overall, it can be concluded that bacterial strains are able to grow on various surfaces to the same extent in comparison with eukaryotic cells where significant growth difference for individual cell lines is observed.

Tab. 2: Biofilm formation expressed as the number of bacterial cells on the area 28.3 mm^2 after 48 hours incubation. (measured in the laboratory of the Faculty of technology in TBU in Zlín by Nikola Mikušová)

Sample	*Bacillus cereus*	*Pseudomonas aeruginosa*
Ti foil	1.24×10^6	2.52×10^6
15 nm	66.5×10^6	7.10×10^6
50 nm	16.7×10^6	12.5×10^6
100 nm	52.5×10^6	2.42×10^6

4.3.2 The outcome of the research work

Nanostructured biomaterials create an artificial microenvironment influencing cell adhesion, proliferation and differentiation together with antimicrobial activity. Among all metals, Ti is the material of choice clinically due to its mechanical strength and a relatively high degree of biocompatibility. The findings have contributed to knowledge about the behavior of both gram-positive and gram-negative biofilm-forming prokaryotic cells on TiO_2 NTs. The well-defined nanostructured Ti surfaces within this study have provided new essential knowledge about its interaction with bacteria and eukaryotic cells which may be significant for developing novel nanostructured implantable devices.

This study was summarized into the manuscript which was subsequently submitted and published in the journal with impact factor as *Interaction of nanostructured TiO_2 biointerfaces with stern cells and biofilm-forming bacteria, International Journal of Polymeric Materials and Polymeric Biomaterials*, 2018, pp. 1-8. The article has even contributed to other research studies and it has been cited in seven articles dealing mainly with the improvement of the morphology of Ti NTs for significant application as medical implants.

4.4 Methods introduced to laboratory

4.4.1 DNA damage induced by conducting polymers

Damage of DNA is one of the critical issues when any redox-active materials are studied. There exists a variety of methods to study DNA damage. Within my study, the comet assay was introduced into the set of experimental methods in the biological laboratories of the Centre of Polymer Systems.

Comet assay

Extracts of PANI and PPy powders, as well as CPs based colloids, were used for evaluation of their effect on DNA damage of tested mouse embryonic fibroblast cell (ATCC CRL-1658 NIH/3T3, USA). The testing was performed according to the protocol containing the precise procedure of comet assay. All tests were conducted in duplicates. The DNA damage of tested eukaryotic cells by prepared extracts was observed. The obtained results showed that in the case of PANI-S and PANI-B the nuclei did not prove any damage, in comparison with the reference sample (pure gel without extract seeded by fibroblast cells). Whereas, PPy salt (PPy-S) and PPy base (PPy-B) had an even higher occurrence of DNA damage compared to sample with H_2O_2 (a reference compound).

Protein adsorption

Detection of protein adsorption was introduced in our biological laboratory through my Ph.D. study. The test was performed by the microplate procedure according to the protocol which was part of the Micro BCA Protein Assay kit. The colorimetric detection and quantitation of the total protein method were used for the evaluation of protein adsorption (using DMEM, bovine serum albumine) on the PANI-S and PANI-B surfaces (taken from the instruction protocol included in Protein Assay kit).

5 SUMMARY OF WORK

Microorganisms attaching a material surface with subsequent biofilm formation are still a huge threat for biomedical and industrial applications. Moreover, microbial resistance against various antimicrobial compounds is higher compared to planktonic species. This fact forced an effort to find an appropriate modification of the surface of materials to provide intrinsic antimicrobial properties. The aim of this thesis is therefore related to this topic, concretely to reveal the antimicrobial properties of CPs and other materials in their native or modified form. To be more concrete, we wanted to reveal how the microbial attachment is connected with surface properties of materials and subsequently to modify these properties to minimalize biofouling.

As insufficient knowledge about the interaction between the CPs and biofilm-forming bacteria was found by the literature review at the beginning of my Ph.D. study. One of the tasks was to study the behavior of biofilm-forming species of bacteria on CP‘s surfaces. Within the practical part, the presented doctoral thesis is divided into four studies.

Firstly, biofilm formation on PANI films and thein surface properties were determined. Pure PANI surfaces and modified PANI surfaces with biological active acids were prepared. Those surfaces were subsequently subject to testing of antimicrobial effect using biofilm-forming bacteria and filamentous fungi. Briefly, PANI-S film did not show any inhibition of bacterial biofilm formation compared to the reference. In contrast, the PANI film doped with PAMPSA had a very significant antibacterial effect against all of the tested bacterial strains. This finding correlated with surface energy results where the lowest surface energy was measured in PANI-PAMPSA film. Concerning fungal biofilm formation, the target idea was to find if filamentous fungi are able to use compounds from tested surfaces as nutrients, for their growth and biofilm formation. The obtained knowledge about surface properties of CPs is of practical importance for various types of antimicrobial coatings, where PANI can be effectively applied both as an anti-corrosion agent and against pollution. Further, the study work at JSI in Ljubljana (Slovenia), was focused on the effect of oxygen plasma treatment on PANI surfaces. Plasma can change chemistry, morphology and other properties of a surface. Superhydrophilic surfaces prepared thanks to plasma attract water to create a thin layer which may consequently affect the bacterial attachment. Within this study the improved wettability of plasma-treated PANI films was confirmed, especially the significant observation was in PANI-B film. The roughness of the treated surfaces was decreased. It can positively influence biofilm formation of microorganisms which mostly prefer rougher substrate. Biological testing, unfortunately, did not prove the significant antimicrobial activity of plasma-treated PANI films and biocompatibility. Anyway, the obtained findings have extended knowledge about surface properties of CPs after plasma treatment in contact with prokaryotic and eukaryotic cells.

The next study dealt with the evaluation of anticorrosive protection of metallic materials - protective coatings based on organic binders and corrosion-inhibiting pigments prepared in cooperation with Department of Paints and Organic Coatings, University of Pardubice. There was an effort to meet the ecological requirements as substitution of classical anticorrosive pigments by some other environmentally friendly pigments. The main goal was however to improve not only the anticorrosive activity but also the biological activity against bacterial and fungal strains.

The last research work studied TiO_2 NTs in contact with prokaryotic cells. This study was also performed in cooperation with JSI. From the experimental point of view, our contribution lies in the current promising nanomorphology of materials and in the comparison of the different sizes of NTs. TiO_2 NTs were uniformly distributed on the surface compared to pure Ti foil. One of the main contributions of this work is to understand more how Ti nanostructure and the size of NTs can influence bacterial behavior and attachment.

6 CONTRIBUTIONS TO SCIENCE AND PRACTICE

At the beginning of the doctoral study, there was insufficient information about the interaction between the biofilm-forming microorganisms and polymeric materials generally, and CPs especially. Presented work was therefore focused on the preparation, modification, and characterization of materials, predominantly composed of CPs. To fulfill the aim of the thesis, the studies focused on the modification of surface properties of CPs, incorporation of CPs into the polymeric coating, and on the impact of nanostructure prepared on TiO_2 surfaces were performed. An important novelty of all of these studies was the utilization of biofilm-forming species of bacteria and fungi. Newly acquired knowledge is of practical importance for various antimicrobial surface treatments.

The main contribution to the science of the reported work within the doctoral study course of TBU in Zlín, Technology of Macromolecular Compounds, can be found in the preparation and characterization of modified polymeric surfaces to increase their antimicrobial activity, together with maintaining or even improving biocompatibility. The findings related to PANI-S and PANI-B surfaces were summarized in the article published in *Chemical Papers* (Mikušová *et al.*, 2017). Further, the second published article in *International Journal of Polymeric Materials and Polymeric Biomaterials* was focused on polymeric coatings with pigments prepared in cooperation with Department of Paints and Organic Coating, University of Pardubice (Mikušová *et al.*, 2019). The presented thesis together with published articles brings novel approaches for modification of CPs and a deepening of knowledge about their surface properties in reaction with bacterial and fungal cells. In addition to that, during the Ph.D. study, the third article based on TiO_2 NTs was published in *Materials Science and Engineering: C* (Kulkarni *et al.*, 2017); Mikušová as a co-author) in cooperation with JSI

(Slovenia), Ljubljana. The fourth article regarding adhesion, proliferation, and migration of NIH/3T3 cells on modified PANI surfaces was published *International Journal of Molecular Sciences* (Rejmontová *et al.*, 2016; Mikušová as a co-author).

Besides, during the study, new methodologies were introduced to the laboratories in the Centre of Polymer Systems at TBU in Zlín, concretely the cultivation of various biofilm-forming bacteria and filamentous fungi, quantification of biofilm formation on the surfaces, determination of DNA damage using comet assay, and evaluation of protein adsorption on prepared polymeric surfaces.

Overall, the achieved outputs of applied research were already summarized in 4 articles and published in journals indexed in Web of Science. The results were also presented at 2 conferences (posters and contributions). To conclude, the field of Technology of macromolecular compounds has been thus enriched by new insights focused on the influence of material, especially CPs and their surface properties on microbial biofilm formation.

7 FUTURE PROSPECTIVE

In this Ph.D. thesis, we studied the antibiofilm effect of material related to the influence of microbial attachment using modified surface properties. This presented research has extended the knowledge about the interaction between pure and modified surfaces of CPs, and TiO_2 NTs with biofilm-forming bacteria, and mainly with filamentous fungi whose behavior has not been previously defined. This doctoral work may be further developed in several ways. Firstly, the micro and nanostructured surfaces may be prepared and subsequently, they can be functionalized using CPs to get material with unique biological properties. Further, the other possibility of how to extend biological testing of polymers is to use not only biofilm-forming strains but also the strains commonly occurring in a hospital environment. Moreover, within the biological testing of CPs, it would be beneficial to be focused on microorganisms occurring in an environment where they are applied, such as water or soil.

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LIST OF FIGURES

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LIST OF SYMBOLS AND ABBREVIATIONS

AFM	Atomic force microscopy
AG	Afterglow regime
CCM	Czech Collection of Microorganisms
CP/CPs	Conducting polymer/Conducting polymers
JSI	Jožef Stefan Institute
MEBB	Melt-extract bouillon broth
NT/NTs	Nanotube/Nanotubes
PAMPSA	Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
PANI	Polyaniline
PANI-B	Polyaniline base
PANI-S	Polyaniline salt
PPy	Polypyrrole
PPDA	Poly(phenylenediamine)
PPP	Poly(p-phenylene)
PPV	Poly(phenylenevinylene)
PPy-S	Polypyrrole salt
PPy-B	Polypyrrole base
PTA	Phosphotungstic acid
Ra	Average surface roughness
SEE system	Surface energy evaluation system
SEM	Scanning electron microscopy
SIMS	Secondary ion mass spectrometry

VCP	Volume concentration of pigment
WCA	Water contact angle
XPS	X-ray photoelectron spectroscopy
γ_{tot}	Total surface energy
γ_{LW}	Disperse component
γ_{AB}	Acid-base (polar) component

LIST OF PUBLICATIONS

During the doctoral study, the results and new findings were published in four articles in journals with impact factors (two as the first author, two as co-author). The list of published articles is given below.

Author's publication activities

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Mikušová, N., Nechvilová, K., Kalendová, A., Hájková, T., Capáková, Z., Humpolíček, P. *et al.* The effect of composition of a polymeric coating on the biofilm formation of bacteria and filamentous fungi. *International Journal of Polymeric Materials and Polymeric Biomaterials,* 2019, *68(4),* pp. 152-159. DOI: 10.1080/00914037.2018.1429435. ISSN 0091-4037.

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Mikušová, N. The Influence of Surface Properties of Materials on Biofilm Formation. ISSN 1948-5948. DOI: 10.4172/1948-5948-C1-035.
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The influence of surface properties of materials on biofilm formation

Vliv povrchových vlastností materiálů na tvorbu biofilmu

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