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Ph.D. thesis

SMART POLYMER GRAFTED BRUSHES FOR BIOMEDICAL APPLICATIONS

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Oświadczenie

Ja niżej podpisany Svyatoslav Nastyshyn (nr indeksu: 1159870) doktorant Wydziału Fizyki, Astronomii i Informatyki Stosowanej Uniwersytetu Jagiellońskiego oświadczam, że przedłożona przeze mnie rozprawa doktorska pt. „Smart polymer brushes for biomedical applications” jest oryginalna i przedstawia wyniki badań wykonanych przeze mnie osobiście, pod kierunkiem dr. hab. Joanny Raczkowskiej i dr. hab. Yuriya Stetsyshyna. Pracę napisałem samodzielnie.

Oświadczam, że moja rozprawa doktorska została opracowana zgodnie z Ustawą o prawie autorskim i prawach pokrewnych z dnia 4 lutego 1994 r. (Dziennik Ustaw 1994 nr 24 poz. 83 wraz z późniejszymi zmianami).

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Kraków, dnia

.....

Dedicated to my family, and friends
that are always with me

'The important thing is to never stop questioning [or learning]'

A. Einstein

Abstract

Biophysics is a branch of physics that applies physical methods to study biological phenomena and aims at the study of bioactive materials for applications in health care sciences, mainly for tissue engineering, drug delivery, bioseparation, and bacteria killing. Bioactive surfaces are promising for such applications since they are capable of promoting or suppressing interactions with biological objects (cells, proteins, and other biomolecules), depending on the special demands. Numerous bioactive surfaces are built on polymer grafted brush coatings, which are macromolecular chains grafted by one end to a solid surface. In particular, great interest is paid to smart polymer grafted brush coatings, which are capable of changing their physicochemical properties reversibly in response to external stimuli (temperature, pH, ionic pressure, light, etc.).

The work aims at the fabrication of novel polymer grafted brush coatings with precisely defined properties, characterization of their physical and chemical properties and the determination of their biological activity, which is crucial for application in biomedicine.

Overview

The thesis considers the potential of novel, smart polymer grafted brushes for specific biomedical applications, such as substrates for cell culture and tissue engineering and antibacterial coatings. The main achievements and challenges in these fields are presented in Section I. The experimental background is provided in Section II. The methods used for the fabrication of smart polymer brushes are described in Section II.1. The techniques used for the characterization of the physicochemical properties of coatings, relevant for their biological activity, are described in Subsection II.2. In turn, experimental techniques that provide information on the impact of coatings on the protein corona, cell growth, and bacteriological activity are presented in Subsection II.3.

In Section III, the novel smart polymer grafted brush coatings designed for the effective culture of cow granulosa cells and oocyte-cumulus complexes are presented.

The impact of CaCO_3 nanoparticles embedded in the noncytotoxic polymer brush on human cells was studied for three different cell lines. The obtained results are presented in the Section IV.

Section V presents novel nanocomposites built on smart polymer brushes with embedded silver nanoparticles, capable of the 'kill-release' strategy, which were developed in frame of this work to prevent transmission of dangerous bacteria by limiting the possibilities of their proliferation. The potential cytotoxicity of such coatings, which limits their applications in health-care sciences, was studied for human skin and melanoma cell lines. The results of these studies are presented in Section VI. The most appropriate conditions for the fabrication of a noncytotoxic nanocomposite, with the highest possible antibacterial activity and preserved thermo-responsive properties were experimentally determined in Section VI.

When the nanoparticle is injected into the blood, it undergoes an interaction with the biological substances. The results of the basic studies of interactions between smart polymer brushes grafted to SiO_2 nanoparticles and proteins using isothermal titration calorimetry are presented in Section VII.

The main results and conclusions are summarized in Summary. The references are provided in the References section, and all abbreviations used in the thesis are listed in the List of abbreviations.

The results presented in the thesis are published in the articles listed below (author contribution is provided):

1. Y. Stetsyshyn, J. Raczowska, K. Harhay, K. Awsiuk, Y. Shymborska, **S. Nastyshyn**, H. Ohar, V. Vasilyev, D. Ostapiv, M. Sharan, O. Sharan, S. Voronov, A. Budkowski. Grafted polymer brush coatings for the growth of cow granulosa cells and oocyte-cumulus cell complexes. *Biointerphases*. 15 (2020) 031006. <https://doi.org/10.1116/6.0000183>.

Author contribution:

The author of the thesis participated in the preparation of the smart polymer brushes and performed the atomic force microscopy measurements of the surface morphology.

2. O. Lishchynskyi, Y. Stetsyshyn, J. Raczowska, K. Awsiuk, B. Orzechowska, A. Abalymov, A.G. Skirtach, A. Bernasik, **S. Nastyshyn**, A. Budkowski. Fabrication and impact of fouling-reducing temperature-responsive pOEGMA coatings with embedded CaCO₃ nanoparticles on different cell lines. *Materials*. 2021, Vol. 14, Page 1417. 14 (2021) 1417. <https://doi.org/10.3390/MA14061417>.

Author contribution:

The author of the thesis selected the materials and prepared smart coatings for the embedding of CaCO₃ nanoparticles, established the lower critical solution temperature of the brushes, studied the morphology of the brush coating with atomic force microscopy, performed ellipsometry measurements to study the thickness of the brush coating, and contributed to the writing of the text.

3. J. Raczowska, Y. Stetsyshyn, K. Awsiuk, M. Brzychez-Włoch, T. Gosiewski, B. Jany, O. Lishchynskyi, Y. Shymborska, **S. Nastyshyn**, A. Bernasik, H. Ohar, F. Krok, D. Ochońska, A. Kostruba, A. Budkowski. “Command” surfaces with thermo-switchable antibacterial activity. *Materials Science and Engineering: C*. 103 (2019) 109806. <https://doi.org/10.1016/j.msec.2019.109806>.

Author contribution:

The author of the thesis contributed to the fabrication of smart antibacterial surfaces, experimentally determined the temperature of transitions in fabricated smart polymer brushes, performed atomic force microscopy experiments, and participated in the writing of the text.

4. **S. Nastyshyn**, J. Raczowska, Y. Stetsyshyn, B. Orzechowska, A. Bernasik, Y. Shymborska, M. Brzychczy-Włoch, T. Gosiewski, O. Lishchynskiy, H. Ohar, D. Ochońska, K. Awsiuk, A. Budkowski. Non-cytotoxic, temperature-responsive and antibacterial POEGMA based nanocomposite coatings with silver nanoparticles. *RSC Advances*. 10 (2020) 10155–10166. <https://doi.org/10.1039/C9RA10874B>.

Author contribution:

The author of the thesis is the first author of the article. The author of the thesis determined appropriate conditions for the fabrication of a smart antibacterial coating built on a pOEGMA grafted brush with embedded silver nanoparticles, studied the temperature response of the antibacterial coating with water contact angles, studied the morphology of the coatings with atomic force microscopy, studied the thickness of the brush with ellipsometry, and contributed to the writing of the text.

5. **S. Nastyshyn**, O. Pop-Georgievski, Y. Stetsyshyn, A. Budkowski, J. Raczowska, M. Hruby, V. Lobaz. Protein corona of SiO₂ nanoparticles with grafted thermoresponsive copolymers: calorimetric insights on factors affecting entropy vs. enthalpy-driven associations. *Applied Surface Science*. 601 (2022) 154201. <https://doi.org/10.1016/j.apsusc.2022.154201>.

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The author of the thesis is the first author of the article. The author of the thesis selected appropriate materials and fabricated the samples for the project, recorded the temperature trends of the ξ -potentials, performed the isothermal titration calorimetry experiments, and prepared the manuscript draft and the figures included in the publication.

6. **S. Nastyshyn**, Y. Stetsyshyn, J. Raczowska, Y. Nastishin, Y. Melnyk, Y. Panchenko, A. Budkowski. Temperature-responsive polymer brush coatings for advanced biomedical applications. *Polymers*. 14 (2022) 4245. <https://doi.org/10.3390/polym14194245>.

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3. I. Bolesta, M. Vakiv, V. Haiduchok, O. Kushnir, A. Demchuk, **S. Nastyshyn**, R. Gamernyk, Optical properties of LiNbO₃-Ag nanocomposites. *Acta Physica Polonica A*. 133 (2018) 860–863. <https://doi.org/10.12693/APHYSPOLA.133.860>.

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I. INTRODUCTION

I.1. Smart polymer brush coatings

Smart polymer brush (SPB) coatings are macromolecular chains grafted by one end to a solid surface that are capable of changing their physicochemical properties reversibly in response to external stimuli (temperature, pH, ionic pressure, light, etc.). [1,2]. The sensitivity of SPBs to external stimuli makes them attractive for various biomedical applications [1–5].

The responsiveness of SPB coatings to external stimuli can be governed by different mechanisms, namely glass-glass [6,7], glass-rubber [8–12] or conformational transitions in the environment [1] (Fig. 1) attributed to intermolecular interactions of macromolecular chains between each other and with the environment.

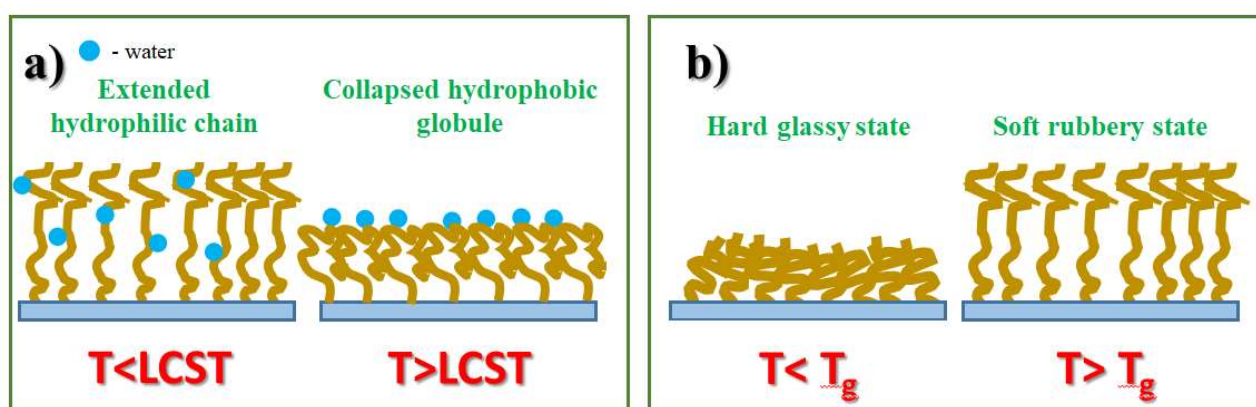


Figure 1. Schematic presentation of the conformational transition of the SPB from the extended hydrophilic chain to the collapsed hydrophobic globule caused by LCST (a) and the transition from the hard glassy state to the soft rubbery state caused by T_g (b).

The glass-rubber transitions of the SPB coatings result in a change of elasticity of the SPB surface. Below the glass-rubber transition temperature (T_g), the SPB coatings are in the hard glassy state; above the T_g , the SPB coatings are in the soft rubbery state [11,13]. In the glassy state, neighboring macromolecules interact strongly, tending to link together. Above the glass-rubber transition, the interactions between neighboring macromolecules are weaker. In turn, glass-glass transitions might have a weak influence on surface elasticity, while showing a considerable influence on its wettability, heat capacity, and refractive index [6,7]. Some of the SPB coatings form orientationally ordered anisotropic liquid crystalline (LC) phases in a well-defined temperature range [14,15].

The conformational transitions of the SPB coatings result in a change in the thickness and hydrophobicity of the SPB coating and can be induced by the change in the temperature or pH of the environment, or by light irradiation [1,2]. The lower critical solution temperature (LCST) stimulates the

conformational change of the macromolecular chain. Below LCST, macromolecular chains are miscible with the solvent, presenting the extended hydrophilic chain conformation [1] (Fig. 1). When the temperature is increased above the LCST, the macromolecules become non-miscible with the solvent and transform into collapsed hydrophobic globules [16]. The thickness of the SPB coatings decreases rapidly upon crossing the LCST. Similar transitions of SPB coatings can be induced by the change of pH [1]. SPB coatings that undergo conformational transitions governed by temperature are applied in thermoresponsive chromatography [17,18], in smart antibacterial surface engineering [19–21], and in drug delivery [22].

The most common SPB coatings that undergo LCST transitions at physiological temperature are based on poly(oligo(ethylene glycol) methyl ether methacrylate) (pOEGMA) and poly(N-isopropylacrylamide) (PNIPAM) [23–26]. An interesting transition was found in poly(4-vinylpyridine) (pVP) SPB coatings [27–29]. When the temperature is raised above the transition temperature, the hydrophobicity of the pVP SPB coating increases drastically. The transition found in pVP shows up also in the change of surface charge [27]. This transition makes pVP-based SPB coatings attractive for thermoresponsive chromatography [30] and other biomedical applications [1–5].

Depending on the desired application, the SPB coatings may be grafted to different surfaces. Usually, SPB coatings are attached to flat surfaces (such as glass plates or silicon wafers) for tissue engineering, LC alignment, and bacteria-killing on daily-used, frequently touched surfaces. If the application requires a large surface area or the ability to disperse the samples in blood (e.g. for drug or gene delivery) or other solvents (e.g. for thermoresponsive chromatography), the SPB coatings may be grafted to nanoparticles, nanotubes, microparticles, etc.

I.2. SPB for cell culture and tissue engineering

SPB coatings are a promising group of materials for tissue engineering and cell culture. Cell proliferation and tissue formation are strongly dependent on the physicochemical properties of SPB with respect to tissue and cells, such as elasticity, roughness, hydrophobicity, and surface charge [31–36].

Optimal physicochemical properties of the substrate for cell culture and tissue engineering may be adjusted by the appropriate selection of the chemical composition of the SPB coatings. Furthermore, SPB coatings exhibit temperature-stimulated transitions from the hydrophilic to the hydrophobic state and, therefore, are capable of thermal tissue detachment [31–36].

SPB coatings for cells should be biocompatible and highly stable (of low biodegradability) in physiological media. Not all SPB coatings are biocompatible, since they kill the cells from tissue (i.e.

they are cytotoxic) or prevent their adhesion. Furthermore, polymers can degrade in physiological media or even during sterilization, and their functionality can be lost [37].

Polymers used for cell culture are divided into two groups: synthetic and natural polymers. Among the natural polymers used for tissue engineering, the most commonly used are collagen, gelatin, elastin, actin, keratin, albumin, chitosan, alginic acid, chitin, cellulose, silk, and hyaluronic acid. In turn, the most widely used synthetic polymers are: poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolic acid) (PLGA), polyhydroxybutyrate (PHB), poly(hydroxyvalerate) (PHV), poly(hydroxybutyratevalerate) (PHBV), poly(dioxanone) (PDS), polycaprolactone (PCL), polyurethanes (PUs), polyphosphazenes, polyanhydrides, polyacetals, poly(propylene fumarate) (PPF) and poly(ethylene glycol) (PEG) [35]. The natural polymers produced by living organisms are highly biocompatible and easily accepted by cells. In turn, synthetic polymers with precisely defined properties can be easily synthesized in a chemical laboratory.

The majority of known SPB coatings, such as pNIPAM or pOEGMA, do not provide sufficient conditions for cell adhesion, and hence, formation of the cellular sheet is restricted. Therefore, to enhance cell adhesion, SPB coatings are frequently modified using various biological substances, such as Arg-Gly-Asp or multivalent choline phosphate - phosphate choline [38].

In turn, Nakayama et al. recently reported improved conditions for cell adhesion and cell detachment through cationization of the pNIPAM brush [31].

An interesting approach was demonstrated in the work [39], in which PNIPAM brushes were charged with the carboxylic group by copolymerizing pNIPAM with 2-carboxyisopropylacrylamide. Cell adhesion was higher on the surface of the copolymer brush at $T < LCST$.

In the work [39], poly(NIPAM-co-N,N-dimethylaminopropylacrylamide-co-N-tert-butylacrylamide) brushes were used to fabricate the SPBs, which allowed temperature-controlled adhesion and detachment of human bone marrow mesenchymal stem cells (hbmMSC). In contrast, other cells derived from human bone marrow (hbm-derived cells) did not adhere to the SPB. Therefore, such SPBs can be used for mesenchymal stem cell separation.

The group of T. Okano developed a commercially available Nunc™ Multidishes with UpCell™ Surface which allows effective cell adhesion from the culture medium, provides appropriate conditions for cell sheet formation, and is capable of releasing the formed cell sheet by temperature stimuli. The device is built from a temperature-responsive SPB coating based on PNIPAM, adhesive for cells at 37°C, which becomes antifouling against cells at a temperature decreased below LCST, resulting in the release of the formed cell sheet. Usually, to detach cells and tissues from scaffolds, trypsin treatment is used, which may affect the integrity of the cellular sheet. In contrast, the approach introduced by the Okanos

group does not require trypsin treatment, as cell detachment occurs spontaneously when temperature decreases [40–42].

X. Shi et al. grafted the pOEGMA brush to surfaces with different roughness and studied cell adhesion to fabricated surfaces [32]. The results revealed that the adhesion of cells is greater on the surfaces with topographical patterns in presence of cell-adhesive proteins such as fibronectin and vitronectin [32]. In the case where the cell-adhesive proteins were absent, cells adhered to the surfaces with different morphologies with quite similar rate. pOEGMA belongs to protein resistant surfaces [43] but the presence of cell adhesive proteins in culture medium enhanced cell adhesion to surfaces with different morphology [32].

I.3. Smart antibacterial surfaces

The polymer-based antibacterial surfaces have great potential for preventing the formation of bacterial biofilm on the daily-used, frequently-touched surfaces. Today, antibacterial surfaces are divided into two groups: (I) non-fouling surfaces that prevent the adhesion of dangerous bacteria and (II) surfaces containing an antibacterial agent that kills bacteria [21]. Unfortunately, both kinds of antibacterial surfaces have some disadvantages. Surfaces containing an antibacterial agent may debilitate eukaryotic cells [44]. In turn, the surfaces that are anti-fouling for bacteria often may be anti-fouling also against some proteins and eukaryotic cells. Therefore, there is a constant need for new materials, with improved properties. High interest is paid to switchable surfaces based on SPB coatings that show their antibacterial activity under external stimuli (light, temperature, pH) [45–49].

The mechanism of the kill-release strategy that includes the temperature-induced release of antibacterial agents previously immobilized in SPB resulting in killing the bacteria followed by the temperature-induced self-cleaning of the coatings is sketched in Fig. 2. Below the LCST, the SPB is surrounded by the hydration layer and the bacteria adhere weakly to the surface of the SPB. Once the temperature is increased above the LCST, three ways of antibacterial activity are possible. In the first way, bacteria adhere to the SPB and are killed by the embedded antibacterial agent. In the second way, the SPB begins to release the antibacterial agent and kill the non-adhered bacteria. The third way combines both mechanisms described above. The dead bacteria adhered to the brush are released when the temperature of the brush is decreased below the LCST.

Another type of anti-bacterial SPB coatings are the so-called zwitterionic polymers such as poly(carboxybetaine) (PCB), poly(sulfobetaine) (PSB), and poly(phosphataine) (PPB) containing positively and negatively charged units. Zwitterionic polymers are capable of formation of a thick hydrated shell, which prevents bacterial adhesion and biofilm formation. On the other hand, positively

charged units of the zwitterionic polymer may kill the potentially adhered bacteria and release it under conditions controlled by pH [21].

The surfaces containing silver nanoparticles have great potential to prevent the formation of bacterial films [50]. Although the exact mechanisms of antibacterial properties of silver nanoparticles are not specified, several hypotheses describing them exist [51]. The silver nanoparticles may easily anchor to bacterial cell wall and penetrate it, changing cell wall permeability and eventually damaging the cell membrane. Also, silver nanoparticles release the silver ions, which promote overproduction of reactive oxygen species causing the death of the bacteria. Moreover, silver ions are able to form stable complexes with nucleic acid, thus preventing transcription and stopping bacterial activity at the genetic level [52]. Interesting surfaces promising for thermo-stimulated bacteria killing may be designed via incorporation of silver nanoparticles into smart thermo-responsive brushes.

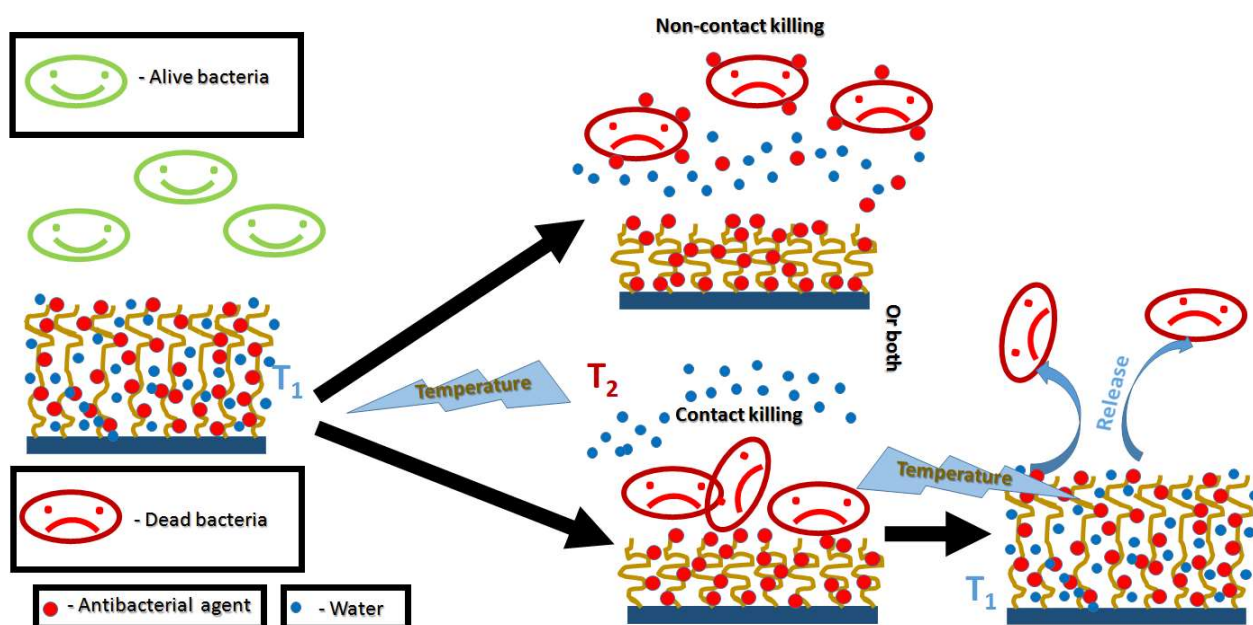


Figure 2. Schema of the thermo-controllable bacteria killing based on SPB.

I.4. Nanoparticles with protein corona

A vast diversity of SPB grafted to various nanoparticles has been designed for the treatment and diagnosis of different diseases [53]. When a nanoparticle enters the blood, it undergoes various interactions, mainly nonspecific, with the proteins [54–56]. Understanding these interactions is crucial for the theoretical prediction and practical realization of nanoparticle functionality in the physiological environment.

Today, many approaches are known for the study of protein corona formation: a study based on surface plasmon resonance (SPR) [56,57], a study based on fluorescent quenching [58], a study through electrophoresis [59] and many others [28,29,57]. Recently, Landfester et al [54] applied a remarkable isothermal titration calorimetry (ITC) method for the 'in situ' study of the interaction between nanoparticles and proteins. This method did not require any additional labeling of the nanoparticles and proteins or specific nanoparticles with surface plasmons. The ITC allows for determination of the binding affinity (K_a), enthalpy (ΔH) and stoichiometry (n) of such interactions.

The ΔH characterizes how much heat was adsorbed ($\Delta H > 0$) or released ($\Delta H < 0$) during the interaction, $K_a = [\text{adsorbed protein}]/[\text{total protein}][\text{adsorption sites}]$ characterizes the affinity of the proteins to the nanoparticle.

Landfester et al [58] declared that the role of hydration water in protein-nanoparticle interaction should not be underestimated in the ITC approach. There are two types of hydration water around the nanoparticle: primary water and secondary water [59,60]. Primary water is the water that forms the h-bonds with the surface of the nanoparticle, secondary water is the water that surrounds the nanoparticle, forming cage-like structures [59,60]. The presence of secondary water is caused by the phenomenon called hydrophobic hydration [60]. The water that is not in contact with the nanoparticle is called bulk water.

When the surface of the nanoparticle is hydrophobic and the nanoparticle is dispersed in water, the surface of the nanoparticle is surrounded by the secondary water. The protein interacting with the hydrophobic nanoparticle causes the release of secondary water from the surface of the nanoparticle followed by the h-bonding of the released secondary water with bulk water. When the h-bond is formed between the released secondary water and the bulk water, 21 kJ/mol of heat is released. The theory used in ITC experiments states that the water that is in contact with the nanoparticles is more organized than the bulk water; however, in the work [61] no differences between the water on the hydrophobic surface and the bulk water were found. Hence, the interactions having negative enthalpy and arbitrary entropy are called enthalpy-driven interactions and are driven by exothermic recombination of the secondary water, which usually occurs between the hydrophobic units of proteins and nanoparticles.

When the surface of the nanoparticle is hydrophilic and the nanoparticle is dispersed in the water, it is surrounded by primary water. When the protein interacts with the nanoparticle, the h-bonds between the nanoparticle and primary water are broken, and the heat is adsorbed. Then, the released primary water forms the h-bonds with the bulk water, and the heat is released. Hence, when the protein interacts with the hydrophilic nanoparticle, the amount of detected heat flux is much lower than in the case of enthalpy-driven interactions that usually occur between hydrophobic nanoparticles and protein. In the case when the ΔH is negative, interactions between hydrophilic surfaces cannot be distinguished from enthalpy-

driven interactions that usually occur between hydrophobic surfaces. Only the interaction with positive enthalpy and entropy gain can be declared as an entropy-driven interaction. Entropy-driven interactions are mostly typical for hydrophilic polar associations. In Fig. 3 the entropy and enthalpy-driven protein-nanoparticle associations are illustrated.

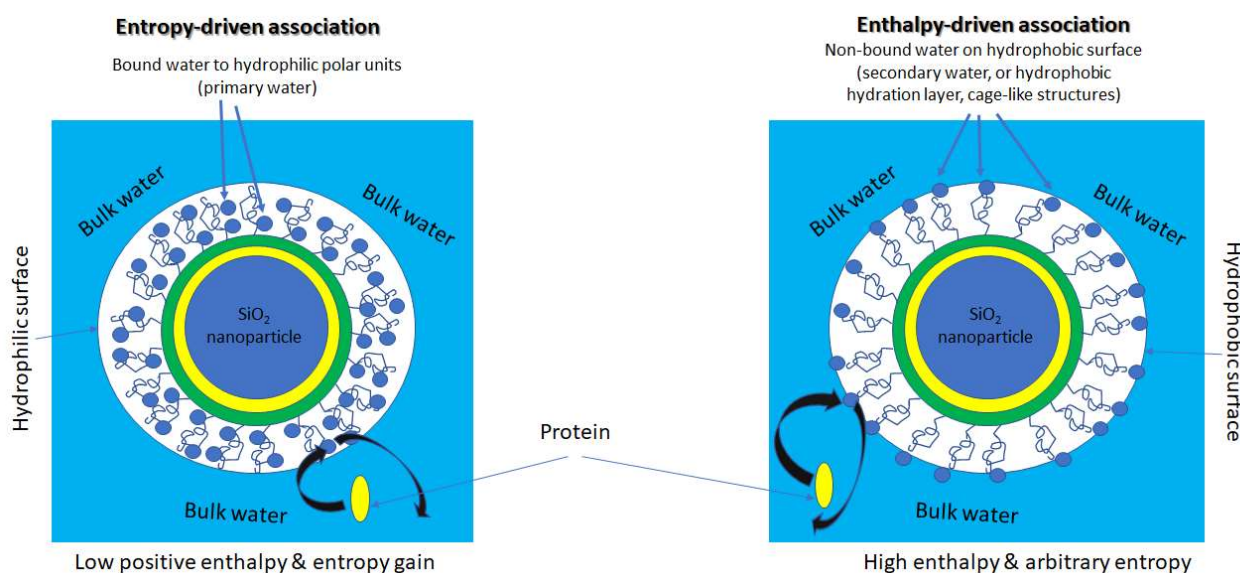


Figure 3. Schematic representation of entropy- and enthalpy-driven protein-nanoparticle interactions.

1.5. Fabrication of smart polymer brush coatings and embedding inorganic nanoparticles

Polymer grafted brushes may be fabricated employing different methods. The methods of brush fabrication can be divided into two groups, called grafting-onto and grafting-from the surface [62]. The grafting-from method implies in situ polymerization of the monomer directly from the substrate surface [65], while the grafting-onto method involves the synthesis of a polymer with a reactive end group attached to the surface.

To achieve a highly monodisperse brush, which is one of the most challenging issues in the fabrication of coatings, the controlled living polymerizations are applied. During controlled polymerization, the growing molecular chain switches from active to dormant states spontaneously. Three methods of controlled living grafting from polymerization are popular nowadays: atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer polymerization (RAFT), and nitroxide-mediated polymerization (NMP).

The ATRP method was developed by the Polish scientist K. Matyjaszewski with colleagues [63]. There are five components required for ATRP: initiator (alkyl halide), metallic catalyst (contains transition metal and halogen), ligand, solvent, and monomer. Polymerization contains three steps: initiation, propagation, and termination. At the initiation step, the initiator generates the radical: the

transition metal from metallic catalysis during the ATRP is oxidized from the lower to the higher oxidation state and takes on the halogen that originally was the part of initiator creating the radical. Once this radical is formed, it can initiate the growth of a polymer chain and polymerize the monomer, forming an active polymer chain. During the ATRP, the growing chain transforms to a dormant state when the halogen from metallic catalyst links to it; once the transition metal takes on the halogen from the growing molecular chain, it transforms to an active state.

Another method, RAFT polymerization [64], is often used for the grafting polymerization of a wide diversity of monomers. The first component of RAFT is the initiator. The second is the so-called RAFT agent, which is a thiocarbonyl thio compound containing two groups: R-group and Z-group. The attachment of the RAFT agent through the Z- group or R-group can considerably influence the outcome of polymer brush grafting [65,66]. In general, the R-group initiates the growth of most polymer chains, and the Z-group stabilizes the intermediate radical species. In this case, R-designed RAFT agents allow the termination of two macroradicals on the surface, resulting in the loss of RAFT agent. In contrast, Z-designed RAFT agents prevent these side reactions. However, the transfer of the macroradical to the RAFT agent takes place close to the surface. With increasing brush length, the thiocarbonylthio group may become less and less accessible, and the growing polymer brush has a shielding effect [65,66].

The third component is the solvent, which is optional, and the fourth is a monomer. The RAFT polymerization contains 6 steps: initiation, propagation, RAFT pre-equilibrium, re-initiation, main RAFT equilibrium, and termination. In the initiation step, the initiator is decomposed, and the radical is formed. Then, at the propagation step, the macromolecular chain grows. At the RAFT pre-equilibrium step, the active polymer chain reacts with the RAFT agent to form the RAFT adduct radical and the polymeric chain containing a part of the RAFT-agent. Then, at the re-initiation step, this adduct radical initiates the growth of new polymer chains. The main RAFT equilibrium step is the most important step in the RAFT polymerization process for a uniform distribution of the polymer chains. At the main RAFT equilibrium step, the growing polymeric chain initiated by the RAFT adduct radical links to the polymeric chain containing the part of the RAFT agent, and the RAFT intermediate radical species are formed. The part of the polymer on the other side of the intermediate radical species detaches and undergoes further polymerization. When the polymeric chain contains the part of RAFT agent, it is in a dormant state; once the chain detaches from the part of the RAFT agent, it transforms to an active state. The termination may occur when the monomer is finished, two active chains link together, or when disproportionation occurs.

The third method, commonly used for the polymerization of monomers, is NMP. The NMP does not require any metallic catalysis and is not affected by contact with air. The key point in controlled polymerization is the transformation of the polymer chain from active to dormant state. In the NMP method, the agents that transform the growing chains from active to dormant state are nitroxides.

Traditionally, the NMP has been considered to be effective exceptionally for the polymerization of styrenics. However, over the last two decades, advances in initiator design, such as N-tert-butyl-N-[1-diethylphosphono-(2,2-dimethylpropyl)] (SG1), 2,2,5-trimethyl-4-phenyl-3-azahexane-3-oxy (TIPNO), and 3-(((2-cyanopropan-2-yl)oxy)-(cyclohexyl)amino)-2,2-dimethyl-3-phenylpropanenitrile (Dispolreg 007) have led to the extension of the list of potential monomers, such as acrylates, methacrylates (either copolymerized or homopolymerized), dienes, vinyl acetate, acrylamides, and many others, making NMP a desirable technique capable of engineering next generation materials for emerging applications [67].

The ATRP and RAFT polymerizations contain toxic agents such as heavy metals or thiocarbonylthio compounds. To resolve this issue, either the entire brush coating after the synthesis should be well-purified, or the polymerization without the toxic agents, such as NMP should be employed. However, as already mentioned, the NMP became capable of polymerizing methacrylates, only very recently and hence it is difficult to employ it. When the application of the coatings does not require high monodispersity of the brush, noncontrollable polymerization, such as oligoperoxide-initiated polymerization [29] may also be employed.

SPB may play a role as a thermoresponsive matrix for different nanoparticles that may provide improved conditions for applications in biomedicine. The nanoparticles may be (I) fabricated on the surface of the brushes via physical vapor deposition [71,72], (II) embedded into the brush during polymerization [68] (Fig. 4), or (III) fabricated inside the brush after polymerization [69] (Fig. 5).

In the method of physical vapor deposition of the nanoparticles, the nanoparticles are deposited on the surface of the previously prepared SPB by thermal evaporation of the bulk metal through a high vacuum followed by its condensation on the surface of SPB. The method of physical vapor deposition is frequently employed for the fabrication of thin films. However, if the conditions for a very thin film (few nanometers) are established, it may lead to the formation of separate nanoparticles [70].

The method of embedding the nanoparticles during the polymerization of SPB involves simultaneous polymerization of the brush and embedding of the previously prepared nanoparticles, which are added to the polymerization mixture of the SPB (Fig. 4).

The method of fabrication of nanoparticles inside the brush (Fig. 5) requires two steps: (I) adsorption of the metal ions from the salt solution and (II) reducing of the metal from the adsorbed salt by reducing agent. This method is used for the fabrication of silver nanoparticles in pOEGMA, pVP, and p(OEGMA-co-VP) brushes in work [71], where it was shown that the nanoparticles fabricated following this method are uniformly distributed on the surface of the SPB and demonstrate the temperature-dependent optical properties. Such a temperature dependence of the optical properties of the fabricated nanoparticles was due to temperature responsiveness of the SPB.

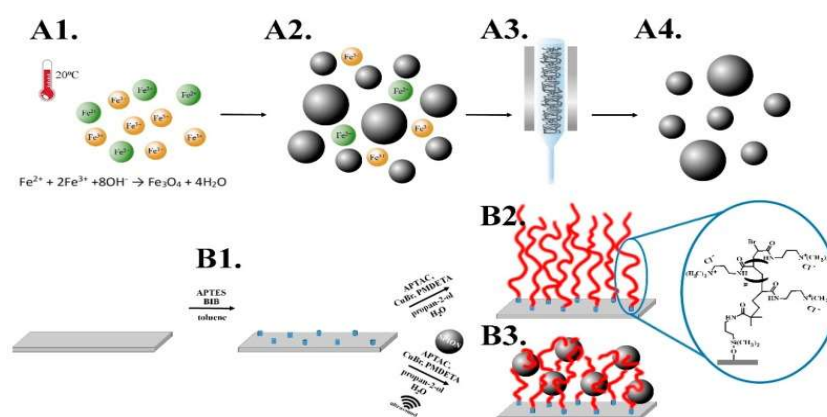


Figure 4. The procedure of the synthesis of SPION nanoparticles (A1), their purification with magnetic chromatography from ions (A2-A3), ATRP of the brush (B1-B2) with simultaneous embedding of nanoparticles (B3) [68].

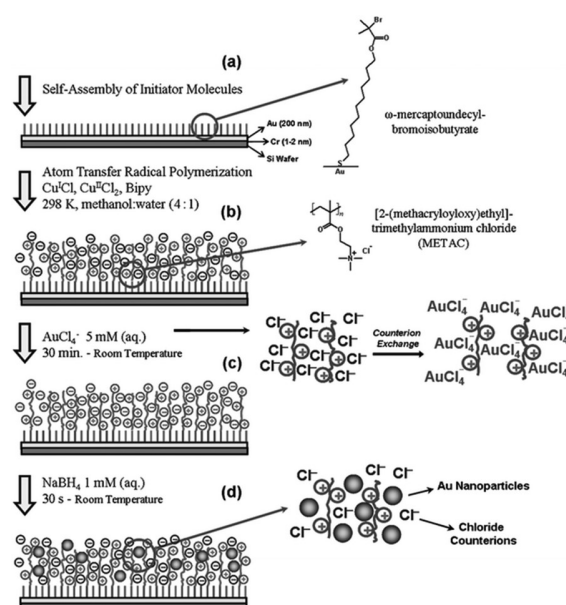


Figure 5. The procedure of the fabrication of gold nanoparticles inside of the brush. The brush is grafted with ATRP (a-b), then the aurum-containing ions are attached to the charged groups of the brush (c), and finally the gold nanoparticles are reduced by NaBH_4 [69].

I.6. Determination of transition temperatures in SPB coatings

SPB coatings may undergo different transitions (see I.1). The methods of their determination depend on the type of transition and the type of surface to which the SPB is grafted. In this work, the transitions stimulated by the temperature will be considered.

SPB grafted onto flat surfaces. The conformational transition governed by LCST of SPB coatings results in a rapid change in thickness [72] and wettability of the brush coating [73]. To determine the temperature of the transition from extended hydrophobic chain conformation to collapsed hydrophilic globule, the water contact angles at different temperatures may be measured. The temperature dependence of the water contact angle is akin to a sigmoid with the transition temperature at its inflection point (middle point). PNIPAM is an example of SPB coatings that undergo LCST between 25-30 °C (Fig. 6) [73].

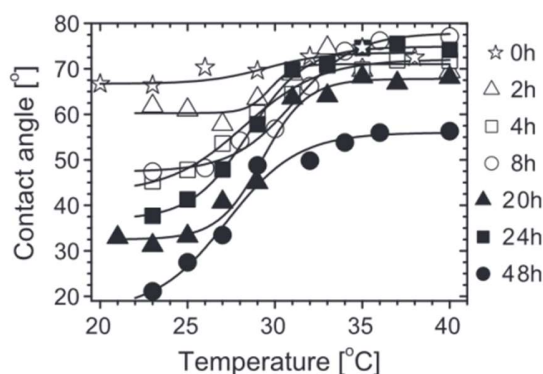


Figure 6. Thermal dependence of the water contact angles of the pNIPAM SPB coatings grafted to the flat glass surface [73].

The LCST of the SPB coating may also be determined from the analysis of the temperature dependence of the thickness of the SPB coatings [74]. One of the techniques that allows one to determine the thickness and refractive index of the polymer brush coatings is ellipsometry. The thickness and refractive index of the pNIPAM SPB coating as a function of temperature are presented in Fig. 7. The thickness of the SPB coating decreases above 30°C significantly due to LCST. Above the LCST the SPB coating transforms into a hydrophobic state and pushes the water out, resulting in an increase in the refractive index.

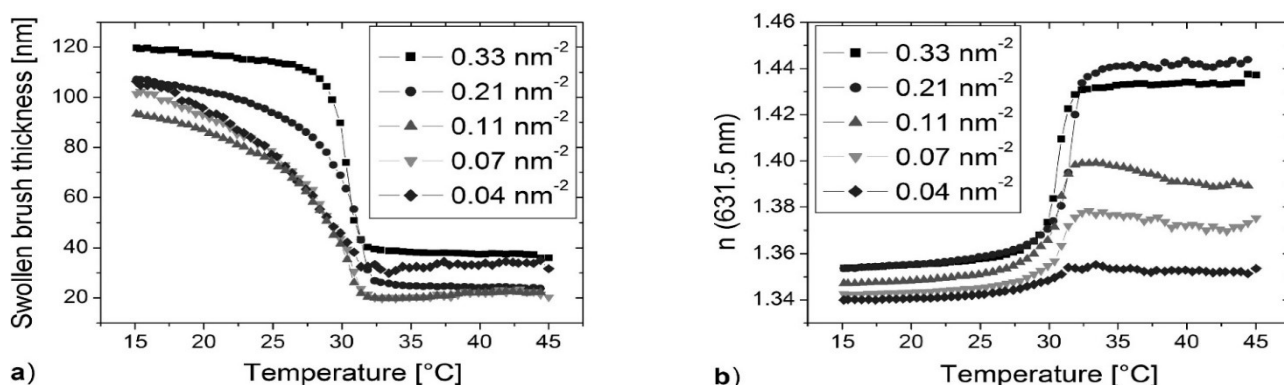


Figure 7. The thickness (a) and refractive index (b) of the pNIPAM brush with different grafting densities immersed in PBS [74].

To determine the T_g of SPB coatings, the surface roughness can be examined with atomic force microscopy (AFM) at different temperatures, and characterized in terms of the root mean square (RMS) roughness [12]. Below T_g , the RMS roughness of the SPB coating is considerably higher than above it [75], as presented for the poly(butyl methacrylate) (pBMA) SPB coating in Fig. 8 [12]. The T_g of the pBMA coating determined from surface roughness is between 15-20 °C depending on the thickness of the SPB coating [12].

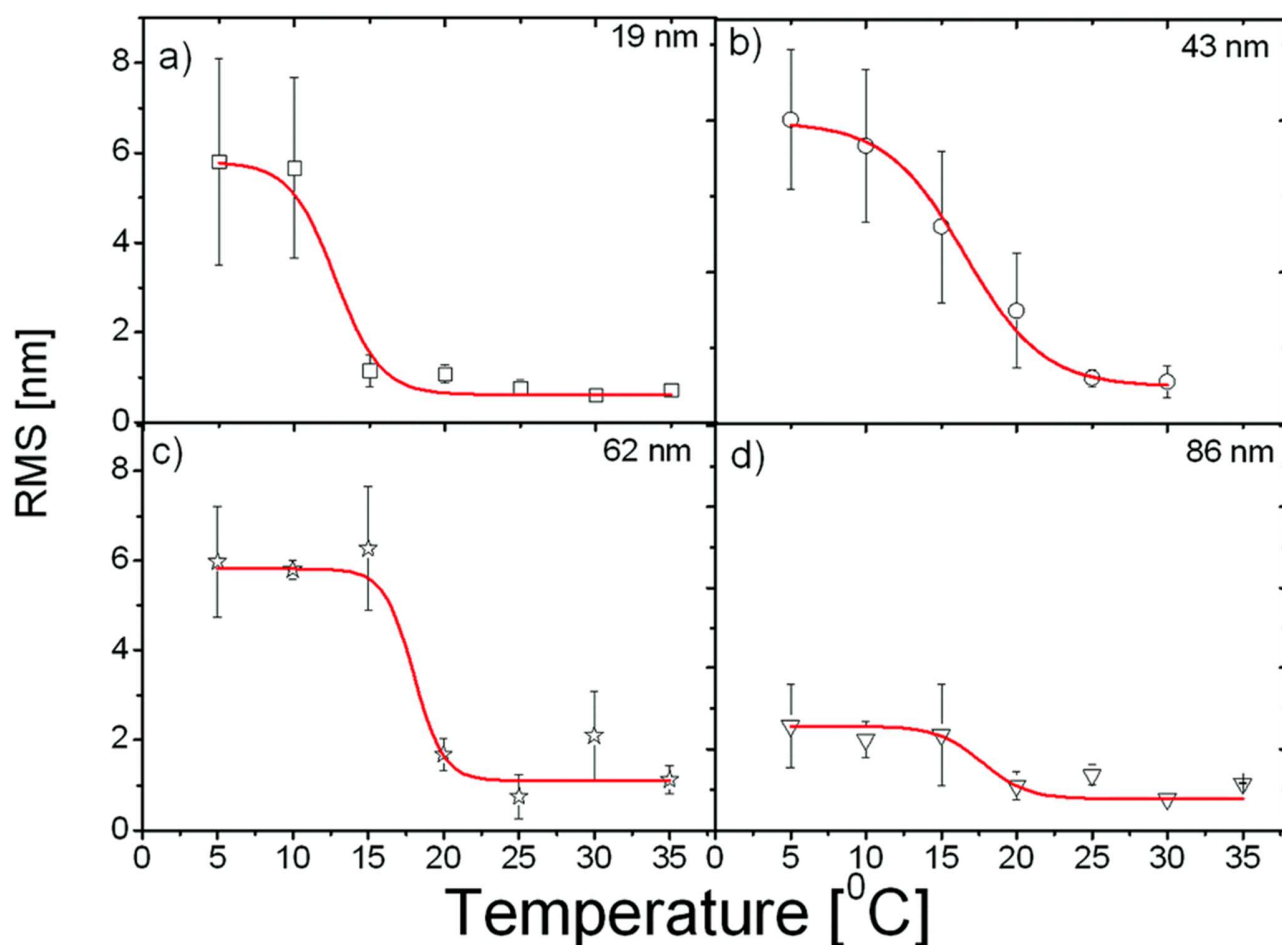


Figure 8. The RMS roughness of the PBMA SPB coating having a thickness of 19 nm (a), 43 nm (b), 62 nm (c), and 86 nm (d)

SPB grafted to the dispersive objects. A commonly used approach to determine the LCST of the SPB coatings grafted onto dispersive objects is based on the dynamic light scattering (DLS) technique [76–78]. The DLS allows the determination of the hydrodynamic diameter of the nano-sized objects through the characterization of their motion in the medium. Sharp and significant change in the thickness of SPB coatings governed by LCST impacts on the hydrodynamic radius (see II.2.5) of the particle [76–78]. The temperature dependence of the hydrodynamic radius of the pOEGMA SPB coating grafted to

SiO₂ particles with different molecular weights of the monomers, presented in Fig. 9 shows that the hydrodynamic diameter of the particles decreases almost twice above the LCST [78].

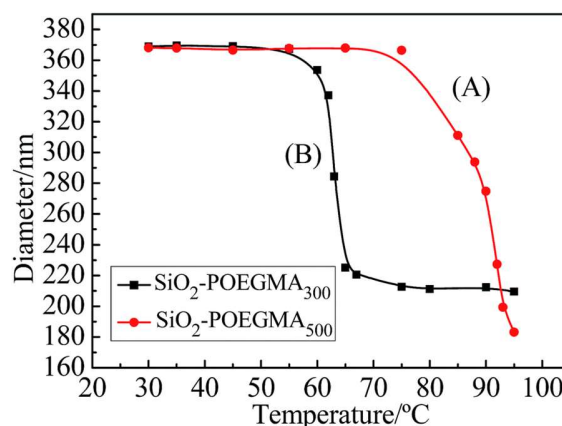


Figure 9. Temperature dependence of the average hydrodynamic diameter for POEGMA₃₀₀¹ and POEGMA₅₀₀¹ measured using dynamic light scattering. [78].

The DLS method allows also the determination of the ξ -potential (see II.2.5). In the case of the pNIPAM and pOEGMA SPB coatings, the LCST transition results in a significant decrease of the ξ -potential (see II.2.5) in water [24,79,80].

To determine the glass-glass and glass-rubber transitions of the SPB coatings attached to dispersive surfaces, nuclear magnetic resonance spectrometry (NMR) [81] and differential scanning calorimetry (DSC) [6] are commonly used.

¹ The index denotes the molecular weight of OEGMA monomer

II. Experimental section

II.1. Fabrication of the coatings

In this work both, controlled grafting polymerization and non-controlled grafting polymerization methods were employed to fabricate SPB coatings. Non-controlled polymerization was performed following oligoperoxide-initiated polymerization [7,29], and controlled polymerization was performed following ATRP [63] (I.5). Furthermore, different types of nanocomposites were developed: nanocomposite built on inorganic nanoparticles embedded in the brush grafted onto glass plates (Fig. 10a) and dispersive nanocomposite of polymer brush grafted to nanosized SiO₂ nanoparticles (Fig. 10b).

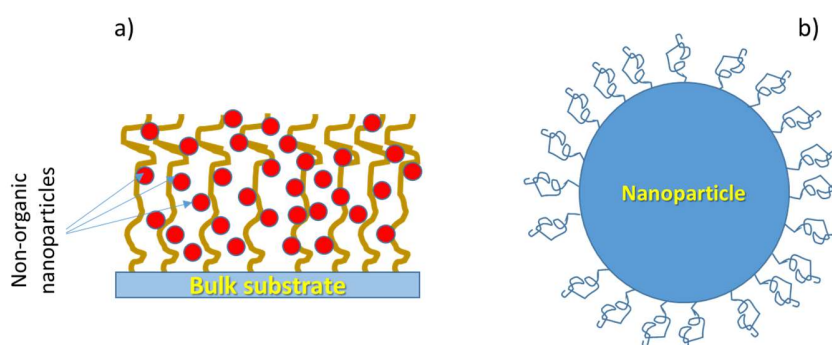


Figure 10. Polymer brushes grafted to bulk substrates containing the embedded non-organic nanoparticles (a), and polymer brushes grafted to non-organic nanoparticle (b)

II.1.1. Preparation of the brushes grafted to glass plates following the oligoperoxide- initiated polymerization

The oligoperoxide-initiated polymerization belongs to the non-controllable polymerizations that is used very often by Stetsyshyn et al. [1,7,28,29] for the fabrication of SPB coatings. Brushes fabricated following this method demonstrated advanced physicochemical properties for the temperature-controlled orientation of liquid crystals [7] and protein adsorption [28,29]. This method of polymerization does not require any heavy metals or thiocarbonylthio compounds that may be cytotoxic. Therefore, the oligoperoxide- initiated polymerization was employed for preparation of the pOEGMA, pVP, and p(OEGMA-co-VP) brushes, dedicated for cell culturing.

The preparation of the brushes following the oligoperoxide-initiated polymerization includes three steps: (I) amination of the glass plates with (3-aminopropyl) triethoxysilane (APTES), (II) binding of the oligoperoxide to the aminated glass plates, and (III) grafting from polymerization of the brushes. The schema of the process is sketched in Fig. 11.

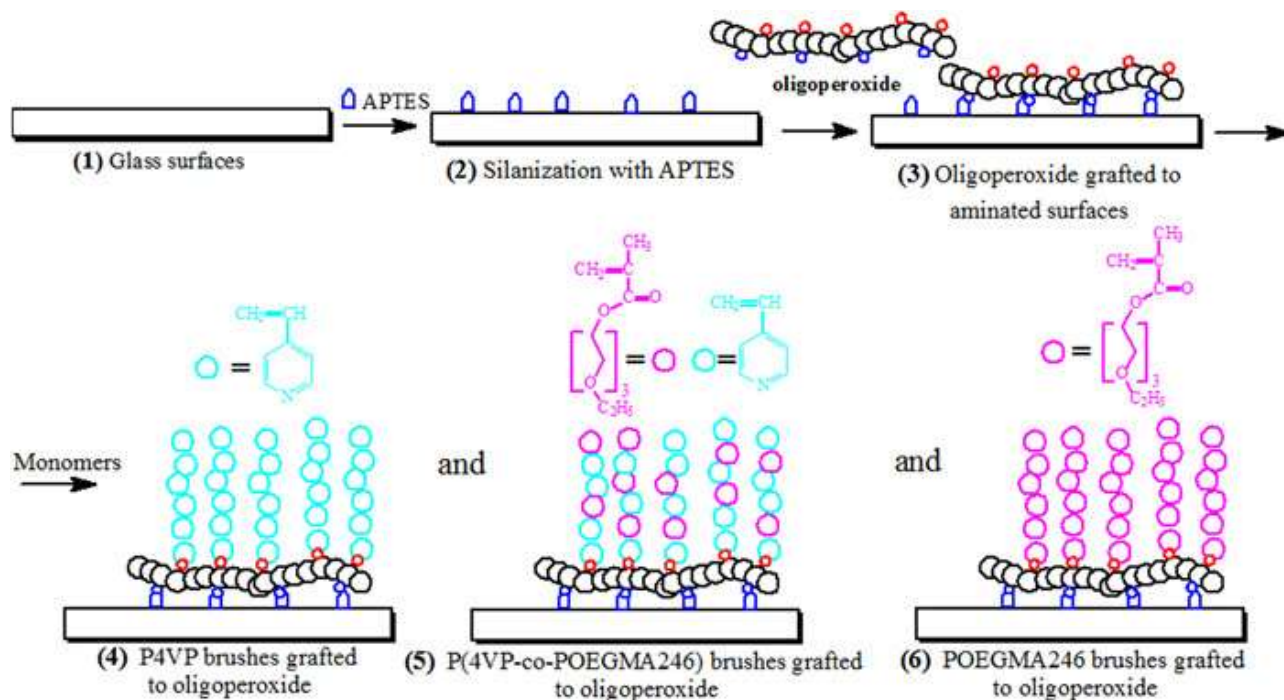


Figure 11. Oligoperoxide initiated polymerization of the pOEGMA, p(OEGMA-co-VP) and pVP.

Amination of glass plates with APTES. The glass plates were purified with ethanol using the ultrasonic bath 3 times for 15 minutes. Subsequently, the glass plates were placed in a 0.2 % (w / w) methanolic solution of APTES for 24 hours. The glass plates were then removed from the solution, washed with methanol three times using an ultrasonic bath, and finally dried at ambient temperature.

Grafting the oligoperoxide to aminated glass plates. The glass plates aminated with APTES were dipped in a 1% (w/w) solution of oligoperoxide in dioxane for 24 hours. Subsequently, the glass plates were removed from the solution and washed with dioxane using the ultrasonic bath 3 times for 15 minutes. Finally, the glass plates were dried at room temperature.

Polymerization of brushes. Glass plates modified with oligoperoxide were placed in test tubes filled with a 0.1M aqueous solution of oligo(ethylene glycol) methyl ether methacrylate (OEGMA), 4-vinyl pyridine (VP), or OEGMA-VP 1:1, heated to 90 ° C, and remained under an atmosphere of argon for 48 hours. The plates were then removed and washed three times with methanol and water using the ultrasonic bath. Subsequently, the plates were dried at room temperature. As a result, polymer brushes composed of pOEGMA and pVP as well as their copolymer were produced.

II.1.2. Atom transfer radical polymerization of the brushes grafted to the glass plates

The ATRP is one of the frequently used approaches for the controlled living polymerization of many different brushes [63]. The method of controlling the polymerization in ATRP is briefly described in Subsection I.5. In this paragraph, the process of the preparation of the brushes is described in detail.

First, the 20x20mm glass plates were aminated by APTES, then the aminated glass plates were modified by the ATRP initiator 2-isobutyryl bromide (IBB), and finally, the SPB coatings were grafted to glass plates modified with the ATRP initiator (Fig. 12).

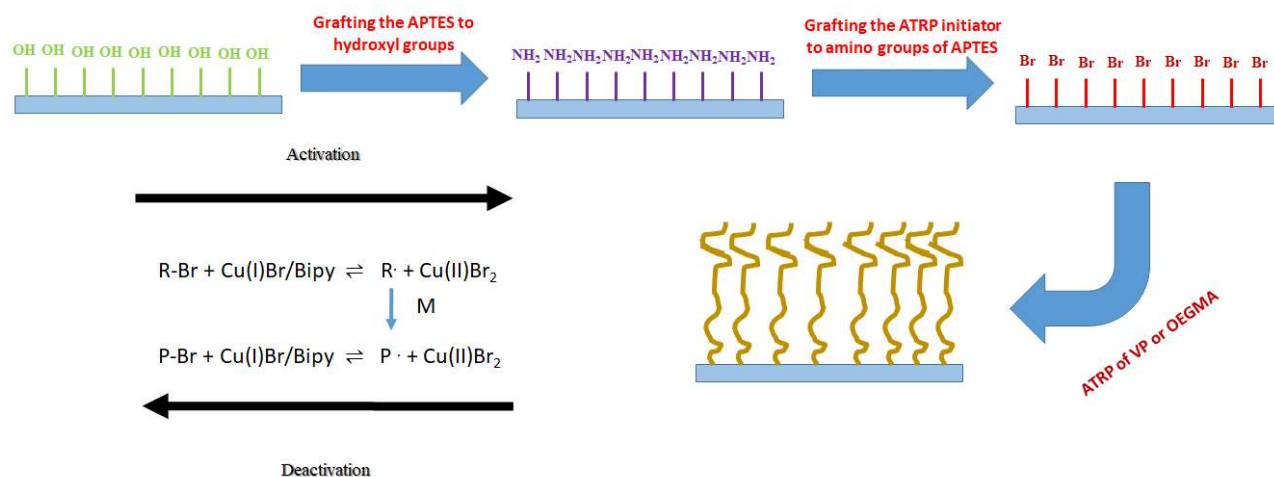


Figure 12. The procedure of atom transfer radical polymerization of OEGMA and VP.

Amination of glass plates with APTES. The glass plates (20x20 mm) were cleaned with ethanol by using the ultrasonic bath 3 times for 15 minutes. The glass plates were then placed in a vacuum desiccator with a vial containing approximately 10 drops of APTES. Then, the vacuum desiccator was pumped to <1 mbar, isolated from the pump, and left for 30 minutes. Finally, the glass plates were removed from the vacuum desiccator, cleaned with ethanol by employing an ultrasonic bath and annealed for 20 minutes at 120⁰C. After annealing, the glass plates were ready for further modification.

Modification of aminated glass plates with an ATRP initiator. 0.25 mL of α - bromoisobutyl bromide and 0.3 mL of trimethylamine were mixed in 10 mL of anhydrous tetrahydrofuran filled in a Petri dish. The aminated glass plates were then immersed in the solution for 40 minutes. The plates were then removed and cleaned with tetrahydrofuran (THF) in an ultrasonic bath.

ATRP of VP and OEGMA. The VP and OEGMA were passed through a column filled with aluminum oxide to remove the stabilizers. Subsequently, 19.5 g of VP or 38.4 g of OEGMA were mixed with 16 ml of methanol and filled into a Schlenk flask. The solution was then bubbled with nitrogen for 15 minutes and 7.4 mg of CuBr₂, 51.5 mg of bipy, and 65.3 mg of sodium L- ascorbate were added and bubbled for the next 15 minutes. The glass plates with the grafted initiator were placed in test

tubes and the entire solution was filled into them and remained for 12 hours. Finally, the glass plates were removed from the tubes and washed with methanol in an ultrasonic bath.

II.1.3. Atom transfer radical polymerization of the brushes grafted to the nanosized SiO₂ nanoparticles

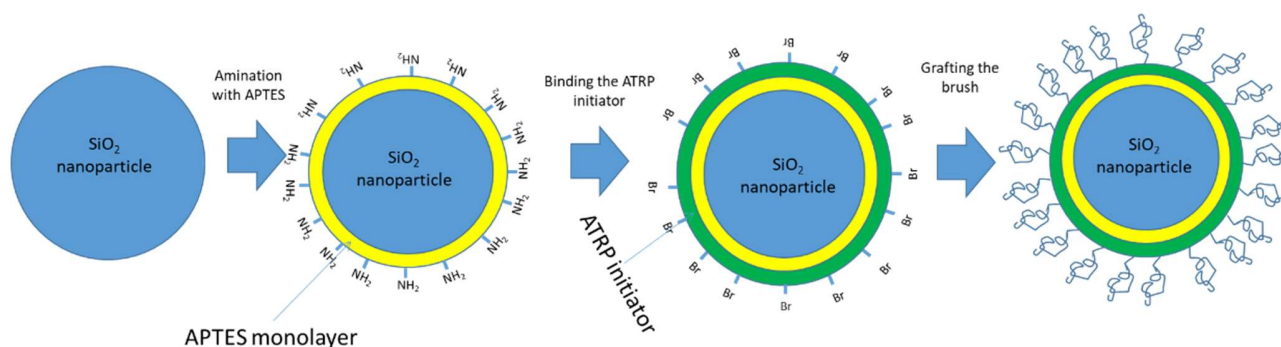


Figure 13. The procedure of the ATRP grafting of polymer brushes to SiO₂ nanoparticles

The pOEGMA, pVP, and p(OEGMA-co-VP) SPB coatings were grafted to nanosized SiO₂ spherical nanoparticles employing ATRP (see I.5 and Fig. 13). Firstly, the SiO₂ nanoparticles were aminated with APTES, then IBB was attached to amino groups of APTES, and finally the brushes were polymerized. The SiO₂ nanoparticles were fabricated using the Strobber [82,83] bottom-up method.

Preparation of SiO₂ nanoparticles (diameter of ~70 nm). Ethanol (49.5 mL) and miliQ water (0.64 mL) were mixed in the Erlenmeyer flask equipped with the magnetic mixer. After, ammonia hydroxide (2.7 mL) and triethylorthosilicate (TEOS) (4 mL) were added dropwise. The reaction was carried out for 24 hours at room temperature under mixing. Next, the entire mixture was centrifuged and washed 3 times in ethanol. Subsequently, the purified silica nanoparticles were dried under a hood at room temperature for 4 hours.

Amination of SiO₂ with APTES. The silica nanoparticles (500 mg) were dispersed in 150 mL of ethanol. After, 1.5 mL of APTES was added dropwise under the ultrasonication for 15 minutes. Next, the mixture was mixed for 30 minutes at room temperature. Then the entire mixture was centrifuged and washed 3 times with ethanol. Subsequently, the nanoparticles were dried in the hood for 4 hours at room temperature.

ATRP of VP and OEGMA. The silica nanoparticles with the grafted initiator (60 mg) were dispersed in (10 mL) of methanol under ultrasound and the mixture was transferred to a Schlenk flask. The inhibitors from the monomers were removed in the column. The purified monomers were then added to the mixture at the concentrations presented in Table 1. Next, the solution of methanol (2 mL) and

water (3 mL) was bubbled with argon, and then, bipy (322 mg), sodium L-ascorbate (278.5 mg), and copper bromide (155.7 mg) were added to it. Subsequently, both solutions were mixed in a Schlenk flask and bubbled with argon for the next 15 minutes and remained for the polymerization.

The polymerization of pOEGMA was conducted for 24 hours; polymerization of p (OEGMA-co-VP) and pVP was conducted for 60 hours. After polymerization, the samples were washed 3 times with methanol and water. Next, the samples were dispersed in a 1% aqueous solution of ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA) and stirred for 3 days. The aqueous solution of EDTA was changed daily. Subsequently, the nanoparticles were washed with water in an ultrasonic bath, dispersed in the water, frozen, and finally freeze-dried.

Table 1. The concentrations of monomers in reaction mixture, and expected fractions of polymers in the chain.

Sample	Amount of OEGMA, g/mol	Amount of VP, g/mol	Expected fraction OEGMA	Expected fraction of VP
POEGMA	0.05	-	1	-
P(OEGMA-co-VP) 3:1	0.038	0.12	0.56	0.44
P(OEGMA-co-VP) 1:1	0.025	0.025	0.37	0.63
P(OEGMA-co-VP) 1:3	0.01	0.04	0.22	0.78
PVP		0.05	-	1

The expected ratio of the OEGMA and VP in the chain was calculated with the Mayo-Lewis equation [32]:

$$F_{VP} = \frac{r_{VP}f_{VP}^2 + f_{VP}f_{OEGMA}}{r_{VP}f_{VP}^2 + 2f_{VP}f_{OEGMA} + r_{OEGMA}f_{OEGMA}^2},$$

$$F_{OEGMA} = 1 - F_{VP},$$

where F_{VP} and F_{OEGMA} are the fraction of the VP and OEGMA segments in the single molecular chain of p(OEGMA-co-VP), r_{VP} and r_{OEGMA} are the reactivity of VP and OEGMA respectively, f_{VP} and f_{OEGMA} are the molar concentration of the VP and OEGMA in the reaction mixture, respectively.

II.1.4. Embedding of CaCO_3 nanoparticles in pOEGMA brush coating

To embed the CaCO_3 nanoparticles in the pOEGMA SPB coatings, the method of nanoparticle fabrication inside the brush (see Subsection I.5) was applied using biomineralized precipitation from solutions of CaCl_2 and Na_2CO_3 (Fig. 14).

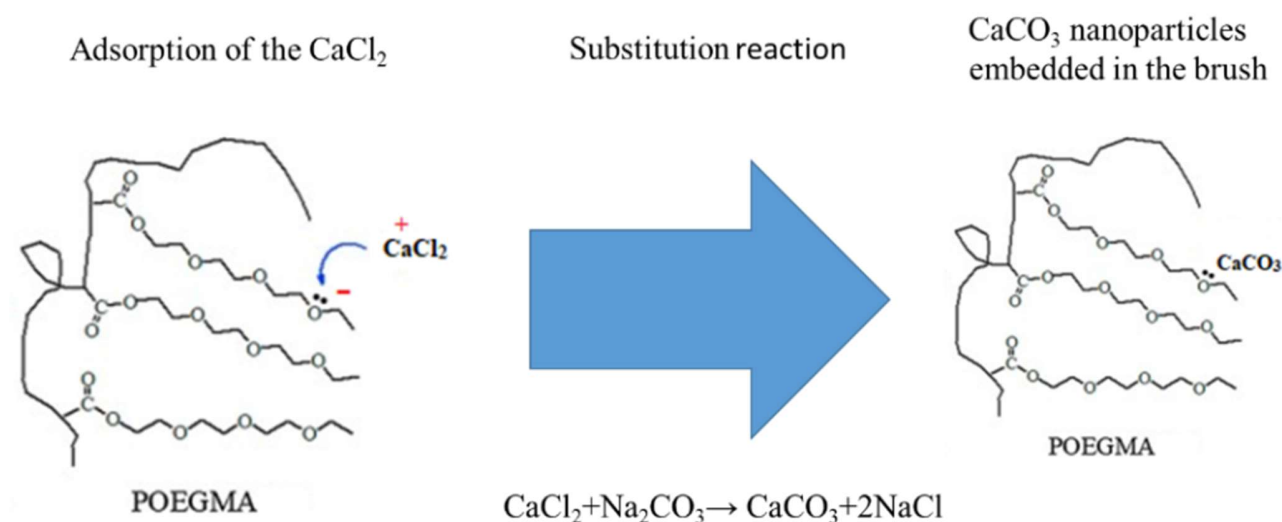


Figure 14. Schema of the fabrication of the CaCO_3 nanoparticles inside the pOEGMA brush

Glass plates coated with pOEGMA were immersed in a 1M aqueous solution of CaCl_2 for 10 minutes and then removed and quickly immersed in Na_2CO_3 for 5 minutes. Finally, the entire surface was washed by a distilled water stream and dried.

II.1.5. Embedding the silver nanoparticles into the brushes

To embed the silver nanoparticles in the pOEGMA or pVP SPB coatings, the method of fabrication of nanoparticles inside the brush (see Section I.5) was used with the reduction of Ag from AgNO_3 [69,71] by NaBH_4 .

The glass plates with grafted SPB coatings were immersed in an aqueous solution of AgNO_3 for a while. Subsequently, the plates were quickly removed and immersed in a 0.01M aqueous solution of NaBH_4 for 12 hours. Finally, the plates were removed and purified with water. Detailed concentrations of substances and durability of the reactions are provided in the description of the obtained results. The schema of the procedure for fabricating the silver nanoparticles in the brushes is presented in Fig. 15.

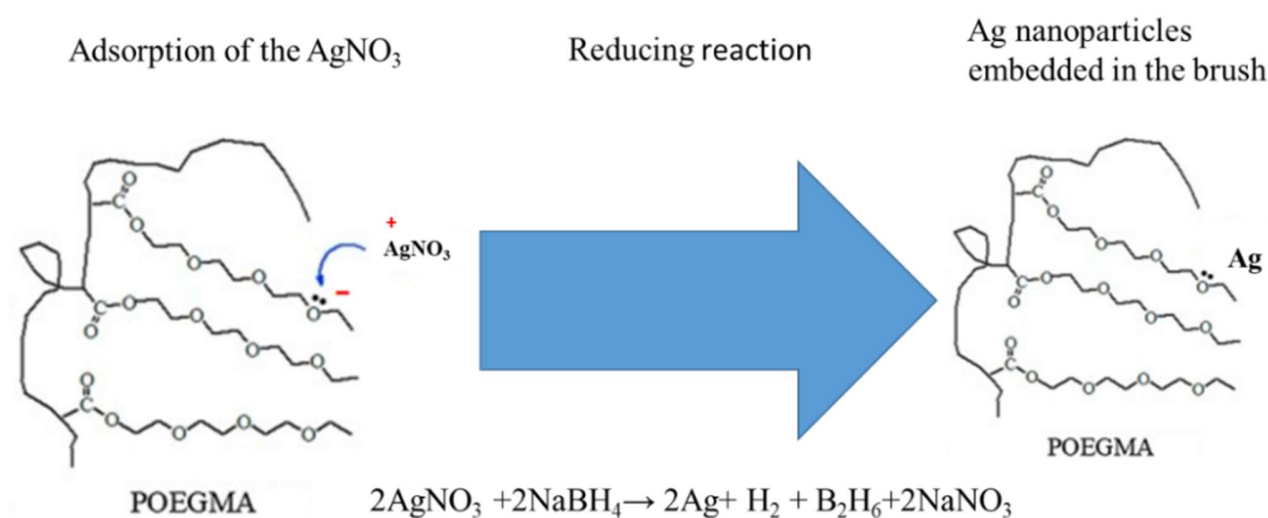


Figure 15. The procedure of embedding the silver nanoparticles into the brush

II.2.Characterization of the physical and chemical properties of the coatings

After the smart polymer brushes were prepared, detailed studies of their physicochemical properties were performed. The detailed analysis of the fabricated sample requires advanced experimental techniques and their appropriate setups. The techniques employed in this work to characterize the chemical composition of the samples are time-of-flight secondary ion spectrometry (ToF-SIMS) and X-ray photoelectron spectrometry (XPS). In turn, the thickness and refractive indices of the SPB were determined by ellipsometry, while their topography was investigated by AFM. The brush grafting density was determined from the refractive index of the coating determined from ellipsometry. To confirm the thermo-responsiveness of the coatings, their wettability was traced using water contact angle measurements as a function of temperature.

In turn, to determine the size, shape, and distribution of inorganic nanoparticles embedded into the polymer brushes grafted to bulk glass plates, scanning electron microscopy (SEM) was employed. To trace the size, shape, and structure of nanocomposites built on polymer brushes grafted to SiO₂ nanoparticles, transmission electron microscopy (TEM) was used. To determine the weight fraction of the polymer brushes grafted onto SiO₂ nanoparticles, thermogravimetric analysis (TGA) was employed followed by calculations of the thickness of the brush coating. The hydrodynamic diameter and the ξ -potential of the polymer brushes grafted onto SiO₂ nanoparticles were determined with dynamic light scattering (DLS).

II.2.1. Time-of-flight secondary ions mass spectrometry (ToF-SIMS)

The ToF-SIMS method enables the determination of the chemical composition of the sample. The examined sample is irradiated by the primary ions beam of a given energy. When the primary ion strikes the surface of the studied sample, its kinetic energy is dissipated through collisional cascades within the near-surface region. At the end, the primary ion is buried below the surface of the sample, the neighboring bonds are broken, and the atoms, ions and molecules are ejected from the surface of the studied sample. The ejected charged fragments (ions) having the charge ze are accelerated by electrical field U of several thousand of volts to time of flight mass analyzer. In ToF analyzer the ions travel to the detector passing the way of length L during the time t . The heavier ions travel with the lower velocity than the lighter ones and the m/z of the ejected ions are determined from the equation [84]:

$$\frac{m}{z} = \frac{2eU}{L^2} t^2$$

Based on measured time we are able to identify the ions extracted from sample surfaces.

The ToF-SIMS technique provides information about chemical composition of the sample related to the intensities of peaks recorded for a given ion weight to its charge ratio. The ToF-SIMS may work in imaging mode mapping the spatial distribution of the representative ions, characteristic for a given component, on the surface of the sample. Using additional ion beam enables layer-by-layer sputtering of the sample and determination of chemical composition of its deeper regions.

The chemical composition of the fabricated polymer-grafted brush coatings was examined with the TOF.SIMS 5 apparatus (ION-TOF GmbH) apparatus (see Fig. 16). The Bi_3^+ 30 keV ions were selected as the analysis beam and Cs^+ 500 eV ions were selected as the sputter beam. The area of 500 squared microns was irradiated by a Cs^+ ion beam, and the central region of 100 squared microns was analyzed.



Figure 16. The ToF-SIMS apparatus.

II.2.2. X-ray photoelectron spectroscopy (XPS)

XPS is based on the external photoelectric effect, which consists of the release of electrons from the atom under irradiation by X-rays [84,85]. The phenomenon of an external photoelectric effect was discovered by Hertz in 1887 [88,89] and described after 18 years by Einstein by an equation relating the kinetic energy of photoelectron E_k with its binding energy in atom E_B , and the frequency of X-ray ν [84,86]:

$$E_k = h\nu - E_B - \Phi,$$

where the h is the Planks' constant, ν is the frequency of the irradiating X-rays, and Φ is the correlating factor accounting the work function of the spectrometer. The value of the energy $h\nu$ of irradiating X-rays has to be greater than $E_B + \Phi$, otherwise the photoelectron will not release the atom and will not be detected. The X-ray photoelectron spectrometer measures the kinetic energy of the photoelectrons followed by the determination of their binding energy in the atom and enabling to record the intensities of the emitted photoelectron as a function of its binding energy. Electron binding energies are characteristic not only for the atoms from which the electrons originate but also for the chemical state (e.g. oxidation state) of the atoms. The intensities of the emitted photoelectrons are related to the

concentration of the atoms from that they were released. The concentration of each atom C_i in the studied sample may be determined from the empirical equation relating them to the intensities of characteristic peaks I_i [84]:

$$C_i = \frac{I_i/S_i}{\sum_i I_i/S_i} 100\%,$$

where the S_i is the sensitivity factors that are different for each atom.

XPS analysis of the SPB coatings grafted to bulk glass plates was carried out with a PHI VersaProbe II apparatus (Physical Electronics, MN, USA). The samples were irradiated with a focused monochromatic Al K_α ($E = 1486.6$ eV) X-ray beam with a diameter of $100\ \mu\text{m}$, and the beam was rastered over an area of $400 \times 400\ \mu\text{m}^2$. The pass energy of the analyzer was set at 46.95 eV, and double neutralization with electrons and low-energy monoatomic Ar^+ ions was used to avoid charging effects. The spectra were referenced to the neutral carbon C1s peak (C-C), at a binding energy of 285.0 eV.

XPS analysis of SPB coatings grafted to SiO_2 nanoparticles was carried out with a K-Alpha+ spectrometer (Thermo Fisher Scientific, East Grinstead, UK). The spectra were measured on nanoparticles spread from water solutions on freshly cleaned gold substrates. All reported XPS spectra were referenced to the C1s peak of sp^3 hydrocarbons at 285.0 eV.

II.2.3. Atomic force microscopy (AFM)

AFM is a technique that allows the study of the topography of the surface in the nanoscale range. The atomic force microscope is equipped with a sharp tip attached to a cantilever. When cantilever moves above the examined surface, the distance between tip and surface changes due to the surface topography and either far attractive or close repulsive inter-atomic forces occur [87,88], leading to the deflection of cantilever or change of its oscillations. The atomic force microscope may work either in contact mode or non-contact mode depending on the distance between the tip and the sample.

When the tip is in region A (Fig. 17) the AFM works in contact mode and the repulsive forces from the sample are detected by the deflection of the cantilever, which is traced by the reflection of the laser beam [87,88]. Contact AFM is frequently used for the study of solids [70,89] as for soft materials such as polymers, the tip may damage the topography [12,57].

When the tip is in region C (Fig. 17) the AFM works in frequency modulation mode, in which the tip oscillates over the sample with its mechanical resonance frequency. The attractive forces between the sample and the tip shift the resonance frequency when the distance between the tip and the surface changes. The topography of the sample is determined in this regime by analyzing the shifts of the resonance frequency. When the tip is in region B (Fig. 17) [87,88], the AFM works in amplitude modulation mode with the fixed frequency of the tip oscillations. The amplitude of the oscillating tip

changes, since in this mode the tip may approach the sample close enough to interact with it via repulsive forces, changing the amplitude of the tip oscillations [87,88].

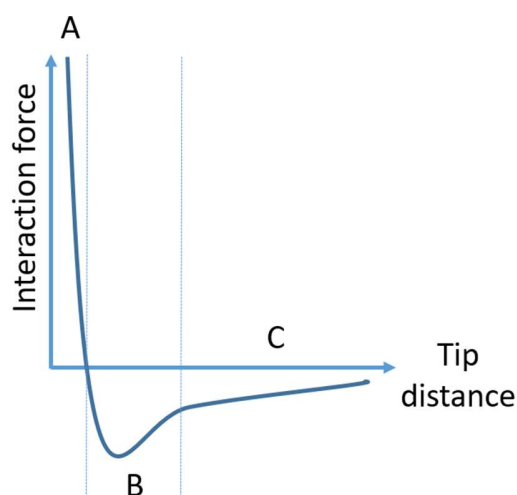


Figure 17. Schematic illustration of the interaction force between the tip and the substrate as a function of the distance between the tip and the sample. Three regions (A, B, C) are indicated. Different modes of imaging are used when the tip is positioned in each region.

Atomic force microscopy working in Lateral Force or Phase Contrast mode may provide additional information about the differences in the sample composition.

The morphologies of the pOEGMA, pVP, and p(OEGMA-co-VP) coatings in this work were studied with the Agilent 5500 system (Keysight) working in non-contact mode (position B in Fig. 17, amplitude modulation mode). The 1x1 micron AFM images were recorded in three randomly chosen positions on the sample and characterized using the RMS roughness, calculated using the PicoImage software provided along with the AFM. The RMS value quantitatively characterizes the roughness of the sample.

II.2.4. Transmission and scanning electron microscopy

The objects studied in this work are much smaller than the wavelength of visible light and cannot be pictured by an optical microscope. Electron microscopy allows one to picture objects smaller than 10 nanometers using electrons instead of the photons. There are two types of electron microscopy used in this work: TEM and SEM.

For TEM, the specimens are fixed on a solid support grid, and then the electron beam accelerated by an electrical field is passed through the specimen. Certain structures within the specimen, called electron-dense regions, absorb more electron energy than others. This phenomenon is used for the

visualization of nano-objects. The electrons that passed through the specimen are registered by the detector.

TEM analysis was performed on a Tecnai Spirit G2 transmission electron microscope (TEM; FEI Brno, Czech Republic). Few (1-2) milligrams of the nanoparticles were dispersed in 1 mL of ethanol and a drop of the dispersion was placed on a carbon-coated copper grid and dried under ambient conditions. The diameters of 100 SiO₂ cores were counted using Image J 1.53n software (USA), then the cumulative particle size distributions were plotted and differentiated with Origin 9 (OriginLab Corporation, MA, USA). The weighted particle size distribution was fitted with lognormal function

$f(x) = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{(\ln x - \mu)^2}{2\sigma^2}}$ the mean particle diameter was represented by the median value $d \pm SD_v$, where $d = e^\mu$, and $SD_v = \sqrt{e^{2\mu+2\sigma^2}(e^{\sigma^2} - 1)}$ is the standard deviation.

The SEM visualizes the nano-objects by detecting the secondary electrons (SE) that are emitted from the specimen after its irradiation by the electron beam. There are two types of detectors in scanning electron microscopy: the backscattered electron detector (BE) and the secondary electron detector (SE). The secondary electrons originate from surface regions and result from the inelastic interactions between the beam and the sample and provide information about surface topography. In turn, backscattered electrons come from the deeper regions of the specimen as a result of the elastic interactions and their number is proportional to the atomic number. Additionally, SEM working in energy dispersive x-ray analysis mode (EDX) recording the characteristic X-rays emitted under the irradiation of the sample by the electrons, enables determination of the chemical composition of the specimen. To visualize biological objects that could be destroyed in high vacuum, measurements in the presence of water vapor in low vacuum mode are applied.

SEM measurements in low vacuum mode (LVSEM) were performed on the Quanta 3D SEM FEG microscope produced by the FEI Company (USA) equipped with the EDAX Ametek EDX Silicone Drift Detector (SDD). The SEM micrographs of SE and BSE were collected by the LVSED (SE) and BSED (BSE) detectors using an electron beam of 10 keV energy. During the measurements, the sample was kept at 100 Pa of water vapor at room temperature. EDX measurements were performed at electron beam energy of 6.5 keV.

II.2.5. Determination of the hydrodynamic diameter and ξ -potential of nanoparticles with dynamic light scattering

The DLS technique determines the sizes of nanoparticles dispersed in a solvent by characterization of their continuous random Brownian motion in the solvent. The motion of the

nanoparticles is characterized by the diffusion coefficient D that is related with hydrodynamic radius R_H through the Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta R_H},$$

where T is the temperature of the solvent, $k = 1.380649 \times 10^{-23} \frac{J}{K}$ is the Boltzmann's constant, and η is the viscosity of the solvent. The hydrodynamic radius R_H of the particle is defined as the radius of the particle that could move in the same solvent with the velocity of the sphere of radius R_H . The smaller nanoparticles move with a higher velocity than the larger ones.

The random Brownian motion of the particles causes fluctuations of the intensity of the scattered light in time (in the range of nanoseconds or microseconds). The frequency of the fluctuations of the light intensity depends on the size of the particles. The intensity of scattered light fluctuates more frequently and more quickly for the smaller particles than for the larger ones.

During the dynamic light scattering experiment the dispersion of the nanoparticles is irradiated by rapid snapshots one after another, always comparing the signal back to the original one. Between the consecutive snapshots in a scale of micro- or even nanoseconds the intensity of signals are quite similar, but for longer time the similarity decreases and eventually the signal changes completely and there is no correlation with the original signal. This process of comparing the signals is called autocorrelation. The autocorrelation $G(\tau)$ of the function $I(t)$ is defined as:

$$G(\tau) = \lim_{t_m \rightarrow \infty} \frac{1}{t_m} \int_0^{t_m} I(t) I(t - \tau) dt$$

The larger particles move slowly and the time required to lose the correlation is longer, while the smaller particles move faster and the loss of correlation occurs faster. The software fits the recorded correlation function to the corresponding model and determines the hydrodynamic diameter of the particle. With dynamic light scattering, the sizes of several nanometers and up to ten microns can be determined.

When the particle is dispersed in the solvent, it is surrounded by the layer of ions called the diffusion layer. The potential of the diffusion layer is quantitatively characterized by the ξ -potential. The ξ -potential of the particles can be determined with dynamic light scattering through the study of the electrophoretic mobility of the particles. To determine the ξ -potential of the particles dispersed in the corresponding solvent with dynamic light scattering it should be filled into the special cell equipped with electrodes, then the voltage should be applied between the electrodes and then the electrophoretic mobility of the particles should be studied by dynamic light scattering. The ξ -potential of the particles is strongly dependent on the chemical composition of the nanoparticles and the solvent. ξ -potential of

the particles plays one of the critical roles in the interactions between nanoparticles and biological objects such as proteins, cells, and bacteria.



Figure 17. Picture of the Nano-ZS Zetasizer ZEN3600 apparatus

Measurements of hydrodynamic diameter and temperature trends of ξ -potential of the fabricated particles and proteins were carried out with the Nano-ZS Zetasizer ZEN3600 equipped with the heater (see Fig. 17)

II.2.6. Thermogravimetric analysis

The TGA is a method in which the weight of the sample is recorded as a function of temperature. It is known that most of the organic compounds lose their weight between 300-600 °C, while the thermo-tolerant materials such as gold, silver, iron, and silicon lose their weight at significantly higher temperatures. According to this, the weight of the organic compounds in the composite that contains the thermo-tolerant and organic compounds can be determined assuming that around 300°C both compounds contribute to the weight of the sample, between approximately 300-600°C the organic compounds lose their weight, and above approximately 600°C the organic compounds lose their weight totally and do not

contribute to the weight of the sample. The formula for calculating the weight of organic compound is the following:

$$m_{organic} = m_{total} - m_{remained}$$

where $m_{organic}$ - is a weight of organic compound, $m_{remained}$ - is the weight of the sample at the temperature when the organic compounds stop losing weight (around 600-700 °C), and m_{total} - is the weight of the sample at the temperature at which the organic compound started to lose weight (around 300°C). Usually, it is more convenient to talk about the composition of the sample in terms of the weight fraction of the components. The weight fraction of the organic component can be calculated from the formula:

$$\theta_{organic} = \theta_{total} - \theta_{remained}$$

where $\theta_{organic}$ - is the weight fraction of the organic compounds, $\theta_{remained}$ - is the fraction of weight that remains at temperature when the organic compounds stop to loss their weight (around 600-700 °C), and θ_{total} is the weight fraction of the sample at the temperature when the organic compound started to lose its weight (around 300°C). It is worth noticing that the nanocomposite may lose weight below 200 °C that may be attributed to the weight loss of the adsorbed water to the sample.

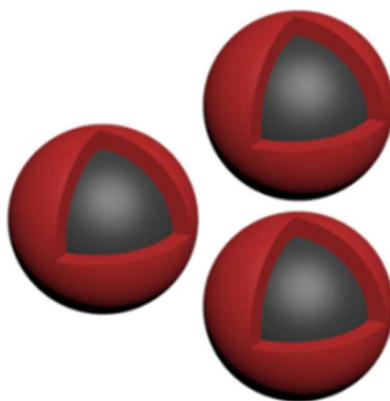


Figure 18. Schema of core-shell spherical particles. The core is marked by black, and the organic shell is marked by red.

The weight fraction determined from TGA allows calculation of the sizes of the compounds of nanocomposites. Let us consider the nanocomposite that is built on the spherical core uniformly coated by an organic coating (Fig. 18). Let us assume that the temperature at which the core begins to lose its weight is significantly higher than the temperature at which the organic compound begins to lose its weight. The thickness of the organic shell of such an object may be determined from equation [83]:

$$d_{organic} = \left(\sqrt{\frac{\theta_{organic}(\rho_{core} - \rho_{organic})\rho_{organic}}{\rho_{organic}(1 - \theta_{organic})}} - 1 \right) r_{core}$$

where $d_{organic}$ is the thickness of the organic shell, $\theta_{organic}$ is the weight fraction of the organic shell, ρ_{core} and $\rho_{organic}$ are the bulk densities of the core and shell respectively, and r_{core} is the radius of the core [83]. This equation is used to calculate the thickness of the polymer brushes grafted onto spherical metallic or other inorganic materials such as SiO₂ nanoparticles.

In this work, the thermal weight loss of the samples was recorded from 20°C to 700°C with the Thermogravimetric Analyzer (Norwalk, CT, USA) under air with a heating rate of 20°C/min.

II.2.7. Ellipsometry

Ellipsometry is a method of characterization of the coatings grafted to the solid. During the experiment, the sample is irradiated with the light beam of known polarization. The beam is reflected from the sample and its polarization is changed by the interactions with the sample material (Fig. 19). Each polarization state of the polarized light beam may be presented as the sum of p- and s- components. The p- component is the component that oscillates in the plane of the incident and reflected light beams, the s- component oscillates in the plane that is perpendicular to the plane of incident and reflected light beams. The ellipsometer determines the changes in the polarization of the light beam in terms of the ellipsometric parameters Ψ and Δ , where the Ψ corresponds to the amplitude ratio and the Δ to the phase shift between p- and s- components of the reflected light beam [90,91]. There are several types of ellipsometry: transmission ellipsometry, reflectance ellipsometry, null ellipsometry, and spectroscopic ellipsometry [90,91]. In this work, spectroscopic ellipsometry was employed.

The schema of the spectroscopic ellipsometer is sketched in Fig. 19. The light from the light source passes through the polarizer and compensator to create the circularly polarized light beam. The light beam that is reflected from the sample passes through the compensator and analyzer and, finally, is detected by the detector. The scrolling polarizer allows to determine the ellipsometric parameters Ψ and Δ of the polarization ellipse.

After the Ψ and Δ are determined, they are fitted to the model, which allows one to determine the thickness and the refractive index of the coating. In this work, the non-conductive and transparent coatings are characterized with ellipsometry. For such samples, the Cauchy model was selected. All ellipsometric measurements were performed using the SpectraRey/3 spectroscopic ellipsometer, SENTECH Instruments GmbH, Berlin, Germany. The angle of incident light beam was 60°, and the Ψ , and Δ were measured for lengths of light beams between 400 and 800 nm.

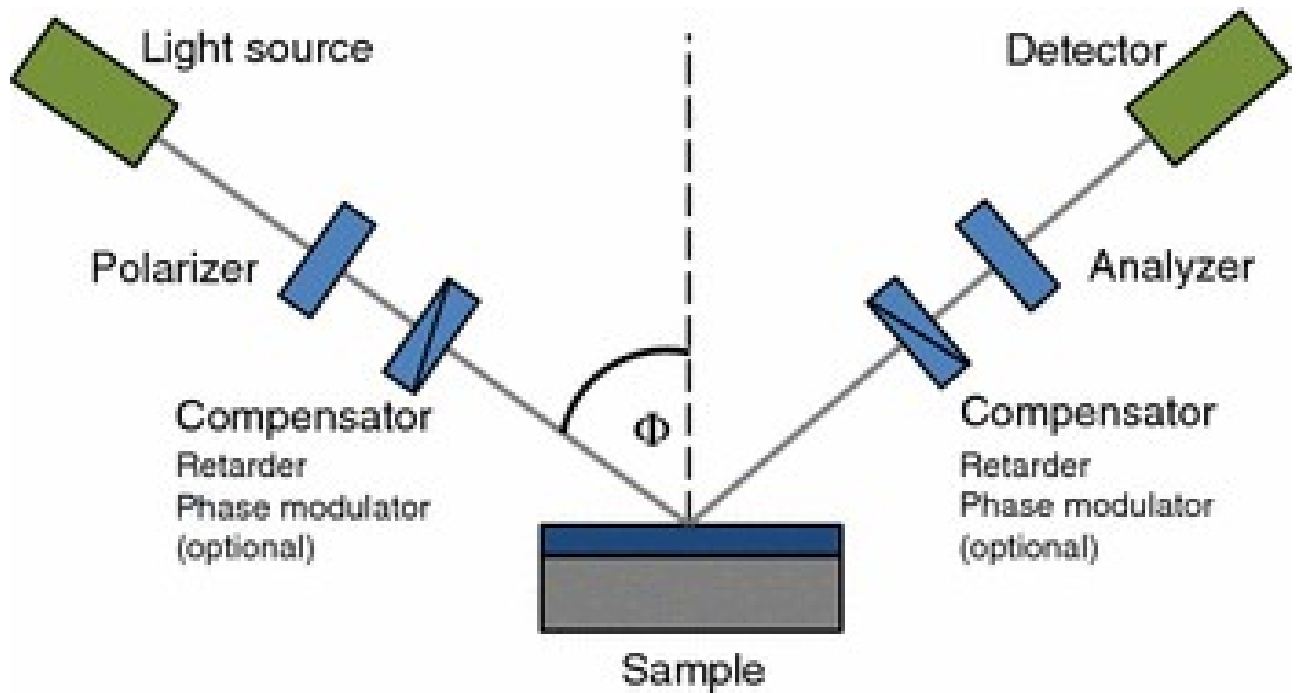


Figure 19. Schema of the spectroscopic ellipsometer.

The thickness and refractive index determined from the ellipsometry measurements were used to calculate the grafting density of the polymer brushes. The molecular weight of the fabricated polymer brush coatings was determined from the equation:

$$M = \frac{C_p}{C_0(1-e^{-\kappa\tau})n},$$

where M is the molecular weight of the polymer brushes, C_p , and C_0 are concentration of polymer and peroxide groups on the surface, respectively ($C_0 = 2.38 \times 10^{-6} \frac{\text{mol}}{\text{m}^2}$), κ is the first-order constant of the initiation at 90°C ($\kappa = 8.1 \times 10^{-6} \text{ s}^{-1}$), n is the polymerization effectiveness $n = 0.1$ and τ is the polymerization time [29].

The grafting density of the polymer was calculated from the following equation.

$$\sigma = \frac{h\rho N_a}{M},$$

where the σ is the grafting density of the polymer grafted brushes, h is the dry thickness of the polymer grafted brush coating, N_a is the Avogadro number, M is the molecular weight of the polymer brush and ρ is the bulk density of the corresponding polymer. To determine the swelling ratio of the fabricated polymer coating, the thickness was measured in the dry state (dried on the plate for a certain period), and in the wet state after immersion of the coating in distilled water for 15 minutes. The swelling ratio is determined as the ratio of thickness in the wet state to thickness in the dry state.

II.2.8. Water contact angle measurements

When the drop of water is placed on the surface, it may spread or bead into a drop, depending on the surface properties. This different behavior of the water on various surfaces is due to hydrophobic/hydrophilic interactions between the molecules of the water and molecules of the surface. When the water is placed on the hydrophobic surface, it tends to have as low a contact area as possible with such surface. The wettability of the surface depends on its physicochemical properties. The hydrophobicity of the surface plays one of the key roles in the interaction of biomolecules with the surfaces [55,58].

To evaluate the wettability of the surface, static water contact angles were measured. The water contact angle is the angle that is formed between the tangent to the water surface and the leaf of the surface at the interface between the solid and liquid phases (Fig. 20). It is declared that for hydrophobic surfaces the water contact angle is higher than 90° , while for hydrophilic surfaces it is less than 90° [92].

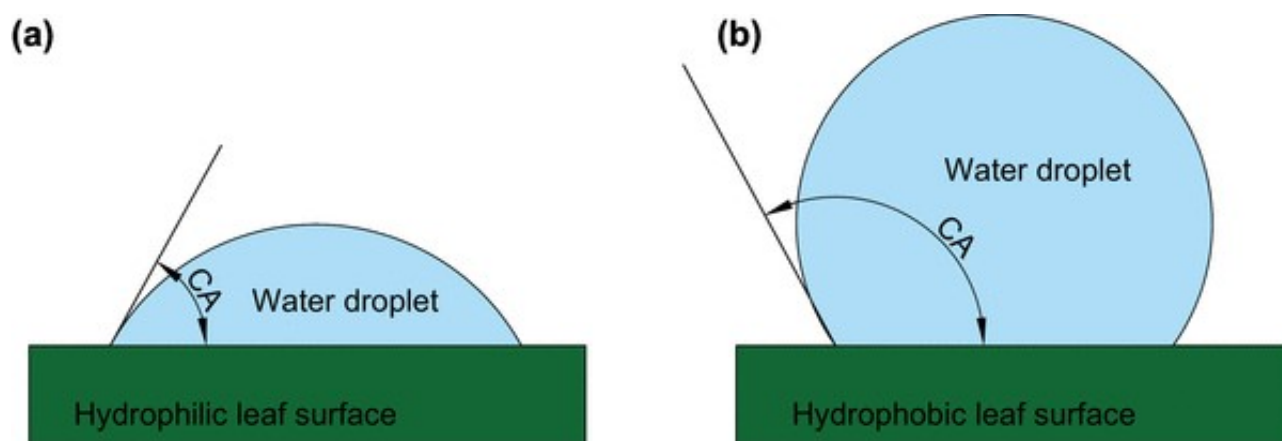


Figure 20. Shape of the water droplet and CA for (a) a hydrophilic and (b) a hydrophobic surface.

In sections I.1 and I.6, it is described that the transition based on the LCST of SPB results in a significant change of the water contact angle of the SPB coating. Therefore, to determine the LCST of the coatings, the water contact angles at different temperatures were studied.

The static water contact angles were measured with the KrussEasyDrop DSA15 instrument (KRÜSS GmbH, Hamburg, Germany) equipped with the Peltier temperature-controlled chamber at temperatures ranging from 5 to 40 °C. SPB coatings were placed on the plate of the apparatus heated to the corresponding temperature and equilibrated for 7 minutes. Then, at least three water droplets were placed on the surface of the brush coating and recorded by an LCD camera, and the water contact angle was determined using the software provided with the equipment. Finally, the samples were dried by nitrogen steam and the procedure was repeated for an elevated temperature.

II.3.Biological activity of the SPB coatings

To study the biological activity of the fabricated SPBs, adequate methods and techniques are required. To evaluate the growth of the cells on the fabricated polymer brushes, optical and fluorescence microscopy were employed. For fluorescence microscopy, cells were labeled with fluorescent dyes, enabling visualization of their nuclei and actin filaments. The metabolic activity of the healthy cells results in the oxygen uptake that was characterized by polarography. In turn, antibacterial properties of the fabricated SPBs were evaluated using a serial dilution method and fluorescent staining. The interaction between the proteins and nanoparticles was studied with ITC.

II.3.1. Cell culture

Cow granulosa cells and the oocytes obtained from ovarian cow follicle were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) medium (obtained from Sigma-Aldrich Ltd) supplemented with the 8-12% of estrus cow serum, and 10-12% of follicular fluid. The sterilized substrates coated with polymer grafted brushes and glass plates were placed in Petri dishes and the cell solution was introduced and remained for 24, 48, and 96 hours in a CO₂ atmosphere at 37 °C. The development of the cells was studied with an optical microscope. After 48 and 96 hours of culture, cells were completely washed from the substrates with cold water and their amount was counted in the camera grid. The experiments were repeated three times.

Human skin HaCaT cells and melanoma WM35 cells were cultured in EMEM (Eagle's minimum essential medium; LGC Standards, Teddington, United Kingdom), and RPMI-1640 (cell culture medium developed at Roswell Park Memorial Institute in 1966 by Moore and his co-workers, Sigma-Aldrich, Darmstadt, Germany) mediums, respectively, in culture flasks placed in a CO₂ incubator providing an atmosphere of 95% air / 5% CO₂. Each medium was supplemented with 10% fetal bovine serum and 1% antibiotics (Penicillin–Streptomycin–Neomycin Solution Stabilized, Sigma-Aldrich, Darmstadt, Germany). Pre-osteoblastic MC3T3-E1 cells were cultured in MEM-alpha glutaMAX-1™ medium (Cat. No. 32561-029, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 µg/mL penicillin/streptomycin. Each cell medium was changed every three days and cells were kept in a humidified incubator at 5% CO₂ and 37 °C.

The fabricated coatings and pure glass were sterilized in ethanol for 15 minutes and placed in a Petri dish, then the WM35 or HaCaT cell solution was added to the Petri dishes (cell density: 8×10^4 cells/mL). Subsequently, the Petri dishes were placed in a CO₂ incubator and remained for 24, 72, and 144 hours. The substrates coated with the fabricated coatings and pure glass for pre-osteoblastic MC3T3-

E1 cells were placed in 6-well cell culture plates and cells were seeded on the substrates and incubated in a 5% CO₂ atmosphere for 24, 72, and 144 hours.

Cell culture experiments were performed in cooperation with National Academy of Agrarian Sciences of Ukrainian and Lviv National University of Veterinary Medicine and Biotechnologies (cow granulosa cells, oocytes), Institute of Nuclear Physics Polish Academy of Sciences (WM35, HaCaT) and Department of Biotechnology at the Ghent University (MC3T3-E1).

II.3.2. Study of the cellular metabolic activity by oxygen uptake with polarography

Polarography is the electrochemical method for the quantitative analysis of the solutions of reducible or oxidizable substances. During the polarography experiment, the plot of the current as a function of applied potential, called the polarogram, is recorded. Then, the concentration of the studied substance dissolved in the solution is determined. To study the concentration of oxygen in the solution of cells, the Clark electrode is usually used (see Fig. 21). The Clark electrode consists of a glass tube filled with an electrolyte, platinum cathode, silver chloride anode, teflon membrane, and O ring. During the experiment, the Clark electrode is immersed in the solution and the potential is applied between the cathode and the anode. The oxygen molecules from the studied solution penetrate through the teflon membrane and react with the anode, and a small amount of current is produced through the chemical reaction. The amount of oxygen in a solution is proportional to the current that is produced in the device. A similar study of cell metabolism was conducted in the works [93,94].

For the study of the metabolic activity of cells, they were dispersed in PBS buffer (pH 7.4). The metabolic activity of the cells was studied in PBS buffer and, also, with the presence of inhibitors: sodium fluoride (NaF, 10^{-3} M), amytal (5×10^{-3} M), sodium azide (NaN₃, 5×10^{-2} M), and disodium ethylenediaminetetraacetic acid (Na₂EDTA, 0.6×10^{-3} M).

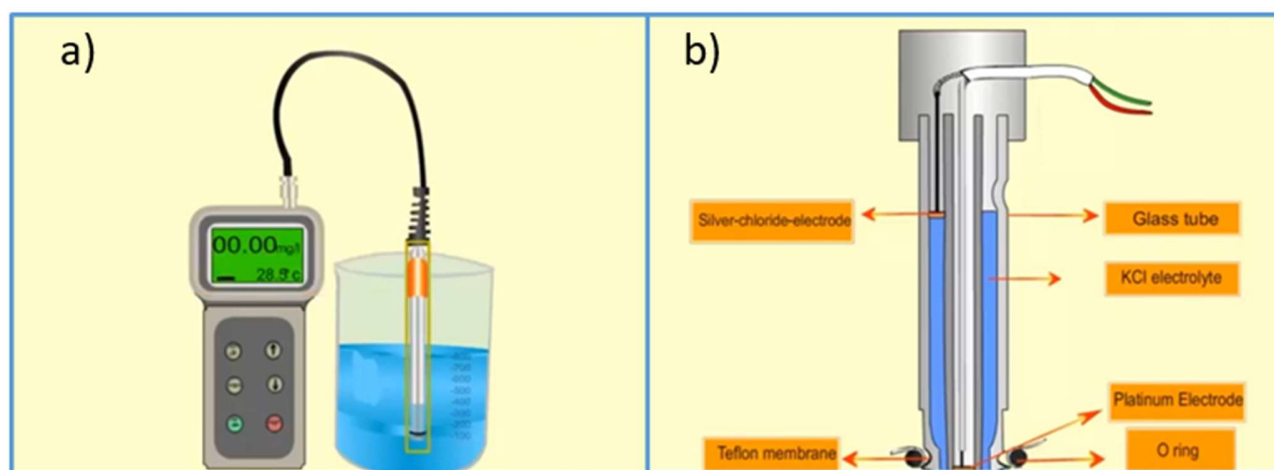


Figure 21. Clark electrode immersed in solution (a) and its schema (b).

II.3.3. Cell staining

To perform a quantitative analysis of the cell growth, they were stained with the fluorescence dye. For this purpose, the substrates with the cells after 24, 72 or 144 hours of culture were removed from the culture medium. For WM35 and HaCaT cells, the fixing procedure was performed prior to staining, using 3.7% paraformaldehyde. After that, 0.2% Triton X-100 (Sigma-Aldrich, Darmstadt, Germany) was added for 4 min, followed by washing the samples with PBS buffer for 2 min. The cells were then incubated with phalloidin conjugated with a 1:200 Alexa Fluor 488 dye solution (Invitrogen, Carlsbad, CA, USA) for 40 min and subsequently the cell nuclei were marked with a 1:5000 solution containing Hoechst dye (Sigma) for 14 min. After this, the cells were ready for fluorescence analysis.

II.3.4. Fluorescence microscopy and statistical analysis of cell viability

The fluorescently labeled cells were visualized with the Olympus IX51 fluorescent microscope equipped with mercury light source (U-LH100HG, Olympus, Tokyo, Japan), U-MWIG2 filter ($\lambda_{\text{exit}} = 530\text{--}550\text{ nm}$, $\lambda_{\text{emit}} = 590\text{ nm}$, Olympus, Tokyo, Japan), and one filter ($\lambda_{\text{exit}} = 470\text{--}490\text{ nm}$, $\lambda_{\text{emit}} = 520\text{ nm}$, Olympus, Tokyo, Japan)). The U-MWIG2 filter was used to visualize the actin filaments, and the U-MNB2 one filter was used to visualize the cell nucleus. Finally, the obtained pictures of the cell nuclei and actin filaments were merged. To obtain quantitative information about cell growth, cells were counted for at least five fluorescent micrographs recorded for a given experimental set. All statistical analyzes were performed by multivariate ANOVA, using Origin (OriginLab, Northampton, MA, USA). Statistical significance between groups was determined by performing Bonferroni's post hoc analysis. Statistical significance was achieved for $p < 0.05$.

II.3.5. Test of antibacterial properties of polymer brush coatings

(serial dilution method)

Representative strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Escherichia coli* ATCC 25922) were selected to study the antibacterial properties of polymer brush coatings. The turbidity of the suspension of two bacterial strains was adjusted to 0.5 McFarland standard, which is equal to 5×10^6 colony-forming units (CFU)/ml. Next, the 16 mL of the suspension was incubated with the polymer brush coatings grafted to the glass for 24 hours at 4 °C and 37 °C separately. Subsequently, the suspension was diluted following the serial dilution method, then the bacteria were inoculated on Mueller-Hinton agar and cultured for 24 hours at 37 °C. The colony-forming units (CFU) were then counted to quantitatively analyze the antibacterial activity of the coatings.

Antibacterial tests were performed in cooperation with the Department of Molecular Medical Microbiology at the Jagiellonian University Medical College.

II.3.6. Differentiation of live and dead bacteria

To differentiate the live and dead bacteria on the SPB coating, they were labeled with the commercially available LIVE/DEAD™ BacLight™ Bacterial Viability Kit. The chemical reagents in this kits change the wavelength of fluorescence because of the metabolic activity of the bacteria. The fluorescence light observed for dead or dying bacteria is red, whereas the fluorescence of alive bacteria is green, enabling to differentiate live and dead bacteria. Labeled bacteria were pictured with the fluorescent microscope BX51 (Olympus).

II.3.7. Isothermal titration calorimetry for the study of nanoparticle-protein interactions.

The ITC is a technique that allows determination of the binding affinity K_a , enthalpy change ΔH , and stoichiometry n of the nanoparticle protein interaction. Subsequently, the changes in entropy ΔS , and Gibbs energy ΔG changes can be calculated from the equations:

$$\Delta G = -RT \ln K_a,$$

$$\Delta G = \Delta H - T\Delta S,$$

where T is the temperature at which the measurement was conducted, $R = 8.31 \frac{\text{J}}{\text{K} \cdot \text{mol}}$ is the universal gas constant. When the equilibrium between the proteins and nanoparticles is reached, the binding affinity K_a is defined as a ratio:

$$K_a = \frac{[\text{PN}]}{[\text{P}][\text{N}]}$$

where $[\text{PN}]$ is the molar concentration of the proteins interacting with the nanoparticle, $[\text{P}]$ and $[\text{N}]$ are molar concentrations of non-interacting proteins and nanoparticles, respectively. K_a characterizes how strong the interaction between the proteins and nanoparticles is. The ΔH determines the amount of heat that is absorbed or released by the system during the association of one mole of the protein and the nanoparticles. The n determines how many proteins interact with one nanoparticle. The traditional ITC is applicable only for spontaneous interactions that undergo with negative ΔG .

The isothermal titration calorimeter is composed of the reference and sample cells, and the syringe combined with the stirrer, which is immersed in the sample cell (Fig. 22). The syringe usually is filled in with the dispersion of the proteins, and the sample cell is filled in with the nanoparticles. During the experiment, the dispersion of proteins is injected dropwise into the sample cell. The temperature of

the sample cell and reference cell are monitored. When the dispersion of proteins is injected into the sample cell and the interaction between proteins and nanoparticle occurs, the heat is released or adsorbed and the temperature in the sample cell is changed and the temperature controller heats or cools the sample cell until the temperature in reference cell and sample cell is the same. The apparatus records the electric power per second applied by the temperature controller and calculates the heat flux, according to the calibration during the interaction. After the measurement, the heat flux per second versus time is plotted (titration curve), and then it is integrated by the software, and the amount of heat per mole of injectant versus the ratio of the concentration of nanoparticles to the concentration of proteins is recorded (isotherm).

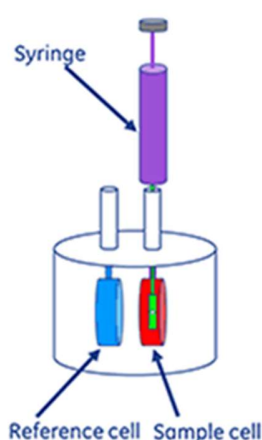


Figure 22. Schema of the isothermal titration calorimeter

Finally, the isotherm is fitted to the appropriate binding model and the binding affinity K_a , enthalpy change ΔH , and stoichiometry n of the nanoparticle protein interaction are determined. Usually it is more convenient to use the terminology of binding site concentration instead of the concentration of nanoparticles. To switch from the nanoparticle concentration to the binding site concentration, the concentration value for the nanoparticles must be adjusted to achieve $n=1$. The concentration of nanoparticles at which n is equal to 1 will be the concentration of the binding sites.

It is worth noticing that the isothermal titration calorimeter measures the global heat from the interaction of the dispersion of proteins and dispersion of nanoparticles. To exactly determine the heat of the interactions between proteins and nanoparticles, (I) proteins and nanoparticles should be diluted in the same solvents, (II) the heat of the dilution of the protein dispersion in the solvent should be subtracted [58]. It is also advised to control the pH during the titration. The pH of all dispersions must be the same before and after measurement.

The amount of the binding sites determined through the normalization of n can be defined via geometry assuming that the interacting proteins form monolayers around the nanoparticles. The amount of proteins in monolayer formed around the nanoparticles may be approximately determined by determining the ratio between the surface area of the nanoparticle and the projection of the protein area. The isothermal titration calorimetry was carried out on MicroCal ITC200 (Malvern Panalytical Ltd, UK) (Fig. 23).

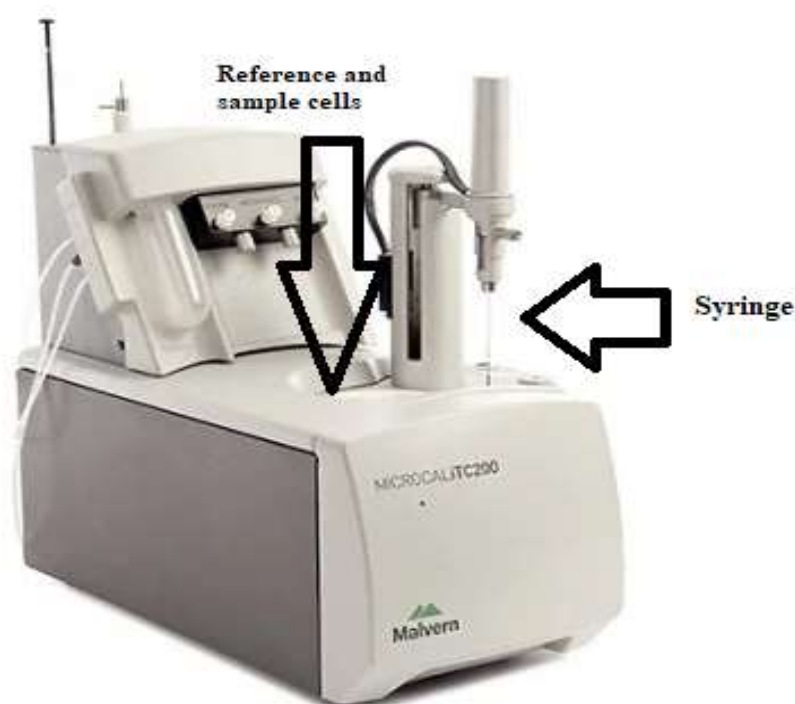


Figure 23. The picture of MicroCal ITC200 (Malvern Panalytical Ltd, UK)

III. Polymer grafted brush-coatings for cell culture

The influence of polymer grafted brush-coatings on cells is strongly dependent on the physicochemical properties of the brush-coating [32, 38 - 41] and cells. Some of the polymer grafted brushes may provide appropriate conditions for the development of some cell lines, and others not. To provide the appropriate conditions for cell growth, first of all, the coating should be capable of cell adhesion, and second, the coating should promote cell proliferation (see I.2).

X. Shi and others studied the adhesion of cells to surfaces of different roughness coated with pOEGMA and showed that the adhesion is higher on surfaces of higher roughness [32]. On the other hand, adhesion of the cells to the surfaces depends on the surface charge and surface hydrophobicity (see I.2).

The appropriate morphology, charge, and hydrophobicity of the polymer brush coatings may be improved by selecting the appropriate homopolymer or copolymerization of several monomers [28]. The surfaces of pOEGMA and pVP are relatively smooth, while the morphology of the p(OEGMA-co-VP) is rough [28]. Furthermore, in the literature, it has been shown that pOEGMA is not toxic to many different cells [95–97]. Both pOEGMA and pVP contain chemical groups capable of hydrogen binding that may increase cell adhesion similarly to cationized pNIPAM [31,39]. The hydrophobicity of the surface may also play a critical role in cell adhesion. The pOEGMA contains more groups that are capable of the formation of hydration layers than the pVP, and, hence, the hydration layer of pVP should be thinner than that in pOEGMA. pOEGMA, pVP, and p(OEGMA-co-VP) undergo the transitions of wettability at physiological temperature and are promising materials for cell detachment (see I.2).

The surfaces for the culture of cow granulosa and oocyte-cumulus complexes are of great interest today, since cow granulosa and oocyte-cumulus complexes play a critical role in follicular development [95,98,99]. Due to the properties of pOEGMA, pVP, and p(OEGMA-co-VP) SPB coatings, they were selected as potential novel coatings for cell culture of cow granulosa and oocyte-cumulus complexes.

The SPB coatings were grafted to the glass plates by the non-controllable oligoperoxide initiated grafting polymerization (II.1.1) [28,29] .

The chemical composition of the brushes was studied with ToF-SIMS (see II.2.1). The thickness and swelling ratio of the brushes were studied with ellipsometry (see II.2.7). The morphology of the surface was studied with AFM (see Subsection II.2.3). To study the development of the cells, they were cultured and examined with optical microscopy (see Subsection II.3.1). The metabolic activity of the cell complexes was characterized by oxygen uptake with polarographic technique (see Section II.3.2). Moreover, the rate of the metabolic activity of the cell complexes in the presence of the four different inhibitors: NaF , amytal-5-ethyl-5-isoamyl-barbituric acid (amytal), NaN_3 , and disodium ethylenediaminetetraacetic acid (Na_2EDTA) were studied (see Subsection II.3.2).

III.1. Characterization of the coatings

ToF-SIMS. To study the chemical composition of the fabricated coatings, the ToF-SIMS technique was employed. The recorded ToF-SIMS spectra of the pOEGMA, p(OEGMA-co-VP) and pVP are presented in Fig. 24. In the spectrum of pOEGMA, the peaks corresponding to CH_3O^+ , $\text{C}_2\text{H}_5\text{O}^+$, $\text{C}_4\text{H}_9\text{O}^+$, and C_2H_5^+ , typical for pOEGMA [26] are observed. The ToF-SIMS spectra of pVP depict the presence of $\text{C}_5\text{H}_6\text{N}^+$ and $\text{C}_7\text{H}_8\text{N}^+$ [100]. In the spectra of p(OEGMA-co-VP), the peaks that are typical for both pOEGMA and pVP are presented. Obtained results confirm the successful fabrication of all coatings using the oligoperoxide initiated polymerization.

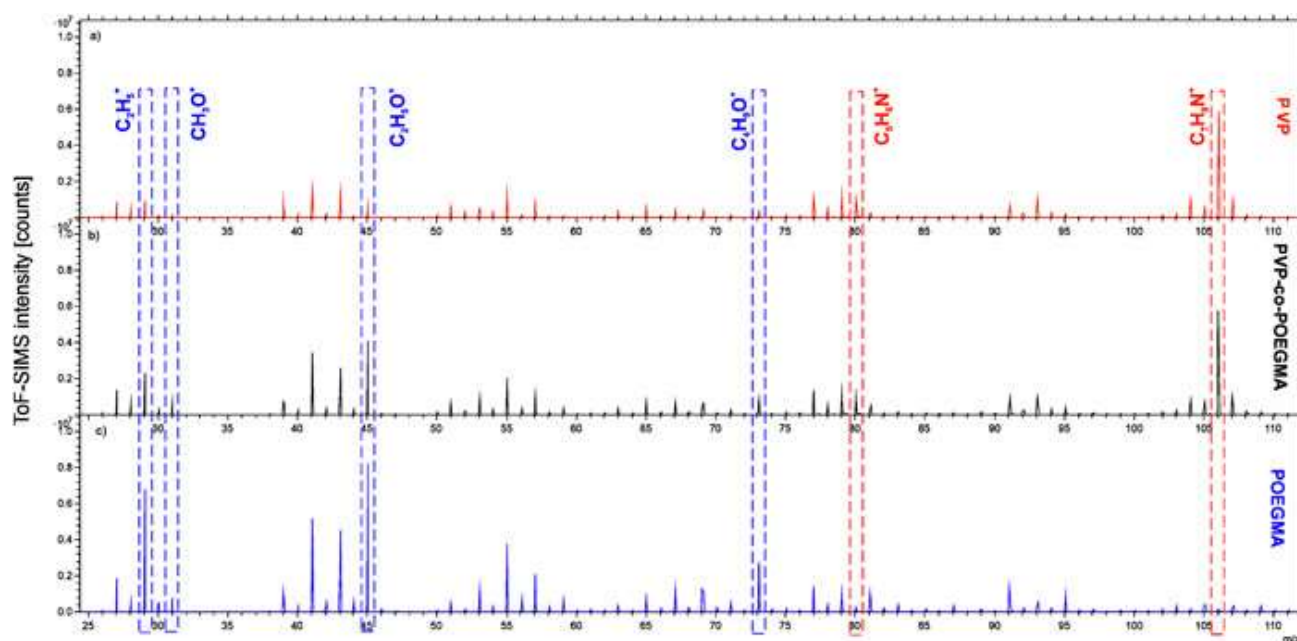


Figure 24. The ToF-SIMS spectra of pVP (a), p(OEGMA-co-VP) (b), and pOEGMA (c).

AFM. Surface topography may impact cell adhesion [32]. To study the topography of the brush coatings, the AFM working in non-contact mode was applied (see Subsection II.2.3). The morphologies of the pOEGMA, p(OEGMA-co-VP) and pVP coatings are presented in Fig. 25. The surfaces of pOEGMA and pVP are relatively smooth, while the morphology of p(OEGMA-co-VP) is rough, which accords with the literature data [28]. It should be noted that the morphology of fabricated surfaces is strongly dependent on environmental conditions, namely, ions concentration, pH and temperature [26,101].

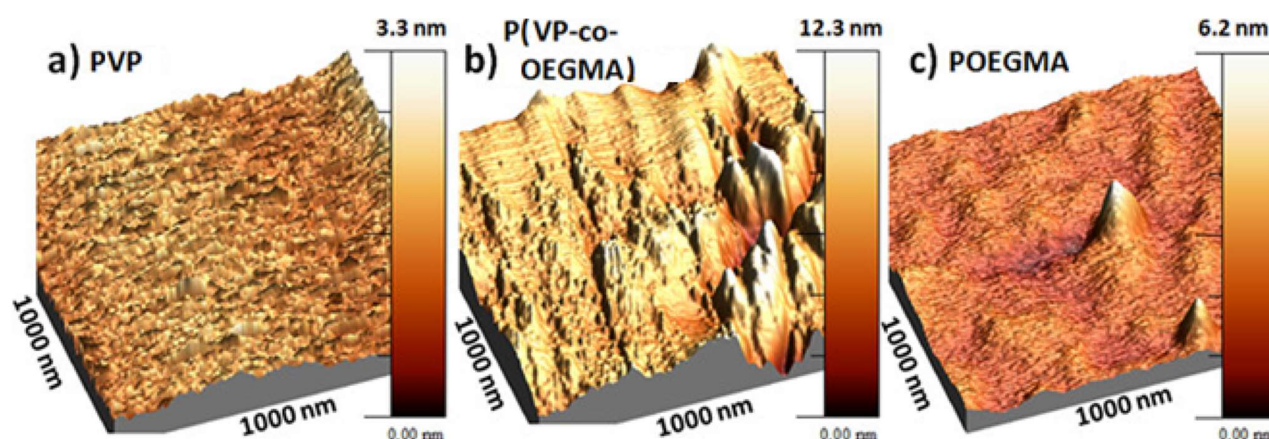


Figure 25. AFM pictures of the topography of pVP (a), p(OEGMA-co-VP) (b) and pOEGMA (c).

Ellipsometric study of the brush. Some polymers, such as pNIPAM, pOEGMA, and others, swell in the aqueous environment, which can change the morphology of the brush coating [26,28,101]. To study the phenomenon of swelling, the ellipsometry was employed (see subsection II.2.7). The thickness of the brush coatings was first measured in the dry state and then in the swollen state (after immersion in water for 15 minutes) and the swelling ratio, defined as the ratio of thickness in the wet state to thickness in the dry state, was calculated for all coatings. The results presented in Table 2 revealed that pOEGMA expands almost 3 times in water. In turn, pVP shows a low swelling in the water. The p(OEGMA-co-VP) swells almost 1.3 times in water. Therefore, the morphology of the coatings can change in the physiological medium [26,28,101].

Table 2. The average thickness, refractive indices, swelling ratio, molecular weight and grating density of the fabricated brush coatings

Characteristic of the grafted polymer brushes	pVP	p(OEGMA-co-VP)	pOEGMA
Thickness in dry state (nm)	32	40	40
Thickness in the swollen state (nm)	35.3	50	108.3
Refractive index in the dry state	1.508	1.484	1.48
Refractive index in the wet state	1.501	1.479	1.389
Swelling ratio	1.1	1.25	2.7
Molecular weight	174 000	232 000	245 000
Grafting density chains per nm ²	0.1	0.1	0.1

III.2. Development and metabolic activity of cow granulosa cells and oocyte-cumulus complexes on polymer grafted brushes

To study the development of granulosa cells on the examined coatings, they were cultured on pOEGMA, p(OEGMA-co-VP) and pVP coatings (see Subsection II.3.1) and on the control glass sample.

The pictures of the granulosa cells growing on different substrates and the results of their quantitative analysis are presented in Fig. 26, and Table 3, respectively.

Granulosa cells form large colonies on the control glass sample, pOEGMA, and pVP coatings. In turn, on p(OEGMA-co-VP) coatings, cells form smaller but numerous colonies suggesting that p(OEGMA-co-VP) provides better conditions for the development of the granulosa cells (Fig. 26).

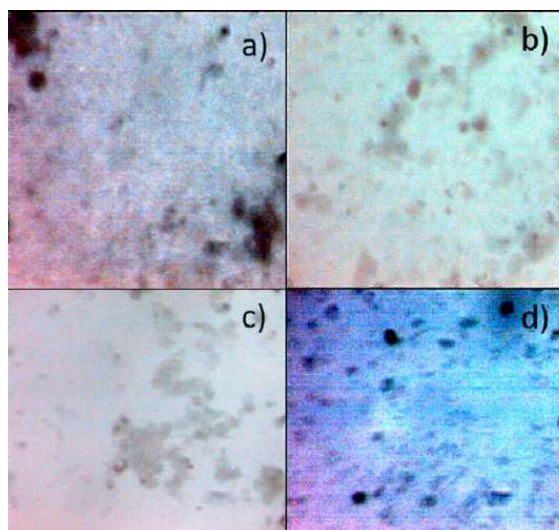


Figure 26. Pictures of granulosa cells developing on the control sample (a), pVP (b), pOEGMA (c) and p(OEGMA-co-VP) (d) after 24 hours of culturing.

Quantitative analysis (Table 3) of the growth of granulosa cells implies that the amount of granulosa cells decreases after more than 48 hours of culturing on pVP and pOEGMA. In turn, on the p(OEGMA-co-VP) the amount of the cells increased, suggesting appropriate conditions for the cell growth provided by this coating.

Table 3. Quantitative analysis of granulosa cells on the control sample, pVP, pOEGMA and p(OEGMA-co-VP)

Surface	Number of cells, $\times 10^3$ cells/ml at different times of incubation, (h)		
	0	48	96
Glass surface (control sample)	4.5	8.2 ± 2.0	10.4 ± 4.0
pVP	—	18.5 ± 5.0	8.3 ± 1.0
pOEGMA	—	.	5.7 ± 1.1
p(OEGMA-co-VP)	—	11.0 ± 4.0	14.4 ± 1.4

The oocyte-cumulus complexes, consisting of the oocyte surrounded by the cumulus granulosa cells, are presented in Fig. 27. It is seen that the cell complexes are quite small and non-uniformly

distributed on the pOEGMA and pVP coatings. In turn, on the p(OEGMA-co-VP) the cell complexes are expanded and uniformly distributed on the surface of the brush. The expansion of the oocyte-cumulus complexes on p(OEGMA-co-VP) suggests normal cell maturation and appropriate conditions for the development of the oocyte-cumulus complexes.

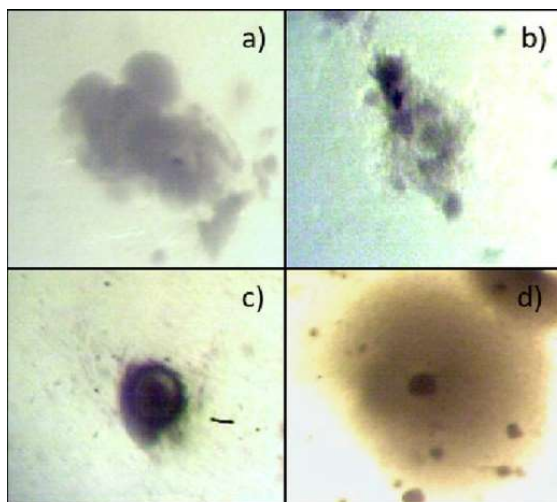


Figure 27. Oocyte-cumulus complexes cultivated on the control glass surface (a) as well as polymer coatings: pVP (b), pOEGMA (c) and p(OEGMA-co-VP) (d).

To evaluate the metabolic activity of the granulosa cells, polarography with a Clark electrode (see Subsection II.3.2) was employed. Oxygen uptake is due to re-synthesis of adenosine 5'-triphosphate (ATP), which plays the key role in the metabolism of the cells. High oxygen uptake is due to the viability of the healthy cells, in turn, low oxygen uptake suggests abnormal cell maturation.

The general oxygen uptake (without the inhibitors) of the cells and the oxygen uptake with the presence of four inhibitors: NaF, amytal, NaN₃, and Na₂EDTA were studied. The first of the mentioned inhibitors, NaF, is a glycolysis inhibitor, inhibiting enolase, which is far downstream in the glycolytic pathway. The next one, amytal, is an inhibitor of complex I (NADH: ubiquinone oxidoreductase), an enzyme complex of the electron-transfer pathway, a proton pump across the inner mitochondrial membrane, responsible for the electron transfer to ubiquinone from NADH formed in the mitochondrial matrix. In turn, NaN₃, is an inhibitor of Complex IV (cytochrome C oxidase), which in mitochondria is the terminal oxidase of the mitochondrial electron transfer pathway, reducing oxygen to water, with reduced cytochrome C as a substrate. Finally, Na₂EDTA is an inhibitor of free radical oxidation of unsaturated fatty acids.

The highest oxygen uptake was observed for cells cultured on the surface of the control sample (see Table 4). Slightly lower oxygen uptake was recorded for cells cultured on the polymer brushes. Cellular oxygen uptake decreased in the presence of inhibitors for cells cultured on the control and pVP surfaces. In turn, for cells cultured on pOEGMA and p(OEGMA-co-VP) coatings, the presence of NaF

increased oxygen uptake, suggesting activation of another pathway of cellular metabolism. All other inhibitors decreased cellular oxygen uptake of cells cultured on these surfaces, indicating the usual functionalization of the corresponding metabolic pathway.

Table 4. Cellular oxygen uptake of the granulosa recorded after 96 hours of culturing.

Sample	Oxygen uptake rate (ng-atoms $O_2 \times \min^{-1} \times \text{ml}^{-1}$ cell suspension)				
	General oxygen uptake rate	In the presence of inhibitors			
		NaF	amytal	NaN ₃	Na ₂ EDTA
Control (glass surface)	1.7 ± 0.1	1.4	0.8	0.1	0.1
PVP	1.5 ± 0.3	1.2	1.0	0.1	0.5
POEGMA	1.5 ± 0.1	1.6	1.3	0.8	0.6
p(OEGMA-co-VP)	1.5 ± 0.2	2.3	1.4	0.9	0.2

III.3. Conclusions

Polymer grafted pOEGMA, pVP, and p(OEGMA-co-VP) brushes were successfully grafted to the glass plates following the oligoperoxide-initiated polymerization. The morphology of fabricated coatings was studied with AFM showing that the surfaces of pOEGMA and pVP are relatively smooth, while the morphology of p(OEGMA-co-VP) is rough. Cell culture is performed in aqueous media, which can influence the topography of the samples as the brush may swell. Therefore, the swelling ratio of the coatings was studied with ellipsometry in aqueous media, and it showed that only pOEGMA swells significantly.

The development of granulosa cells and oocyte-cumulus complexes on the fabricated coatings was studied, and the best conditions for the culturing of those cells were provided with p(OEGMA-co-VP) brushes. Furthermore, the metabolic oxygen uptake of granulosa cells was analyzed with and without the presence of 4 inhibitors: NaF, amytal, Na₂EDTA and NaN₃. In the presence of NaF oxygen uptake by cells cultured on p(OEGMA-co-VP) increased. The results obtained show that p(OEGMA-co-VP) is a promising material for culturing granulosa cells and oocyte-cumulus complexes that could be used for applications in zoology.

IV. Influence of CaCO_3 nanoparticles embedded in pOEGMA brush coating on cells

SPBs and their nanocomposites are promising materials for cell culture and tissue engineering (see I.2). Polymers containing negatively charged units are capable of embedding inorganic nanoparticles [69,71] (see I.5) that can impact cell proliferation.

CaCO_3 -based materials are commonly used in biomedicine for drug delivery [102–104]. In the literature, it is frequently claimed that the pOEGMA brush is not cytotoxic and provides weak conditions for cell adhesion [105–108]. In this section, the influence of CaCO_3 nanoparticles embedded in a low-fouling, non-cytotoxic pOEGMA brush on human cells is explored experimentally.

The pOEGMA brush was attached to the flat glass substrate via surface-initiated ATRP (see II.1.2). The CaCO_3 nanoparticles were embedded into the brush using biomineralized precipitation from CaCl_2 and Na_2CO_3 solutions (see Section II.1.4).

The chemical composition of pOEGMA with embedded nanoparticles was studied with ToF-SIMS (see II.2.1) and XPS (see II.2.2), the surface morphology was studied with AFM (see II.2.3), the embedded CaCO_3 nanoparticles were imaged with SEM (see II.2.4) and the thermal response of the coatings was studied with measurements of water contact angles at different temperatures (see II.2.8).

The influence of CaCO_3 nanoparticles on three different human cell lines was studied, namely keratinocytes (HaCaT), melanoma (WM35), and osteoblasts (MC3T3-e1). Cells were cultured on a control glass substrate, a pOEGMA grafted brush, and a pOEGMA grafted brush with embedded CaCO_3 nanoparticles (see II.3.1). For quantitative analysis, the cells were fluorescently labeled and visualized using fluorescent microscopy (see II.3.3 and II.3.4).

The influence of CaCO_3 nanoparticles embedded in the pOEGMA brush was studied in terms of cell adhesion, proliferation index, and cell viability. The initial amount of cells, recorded on the surface after 24 hours of incubation, may be related mainly to the ability of the surfaces for cell adhesion. Further incubation for 72 and 144 hours characterizes the proliferation activity of the cells. The proliferation index is defined as the ratio of the number of cells on the surface in the given period of incubation to the number of adhered cells. Cell viability is defined as the ratio of the amount of cells on the surface to the amount of cells on the control sample for the given period of incubation.

IV.1. Characterization of the surface of native pOEGMA and pOEGMA with embedded nanoparticles

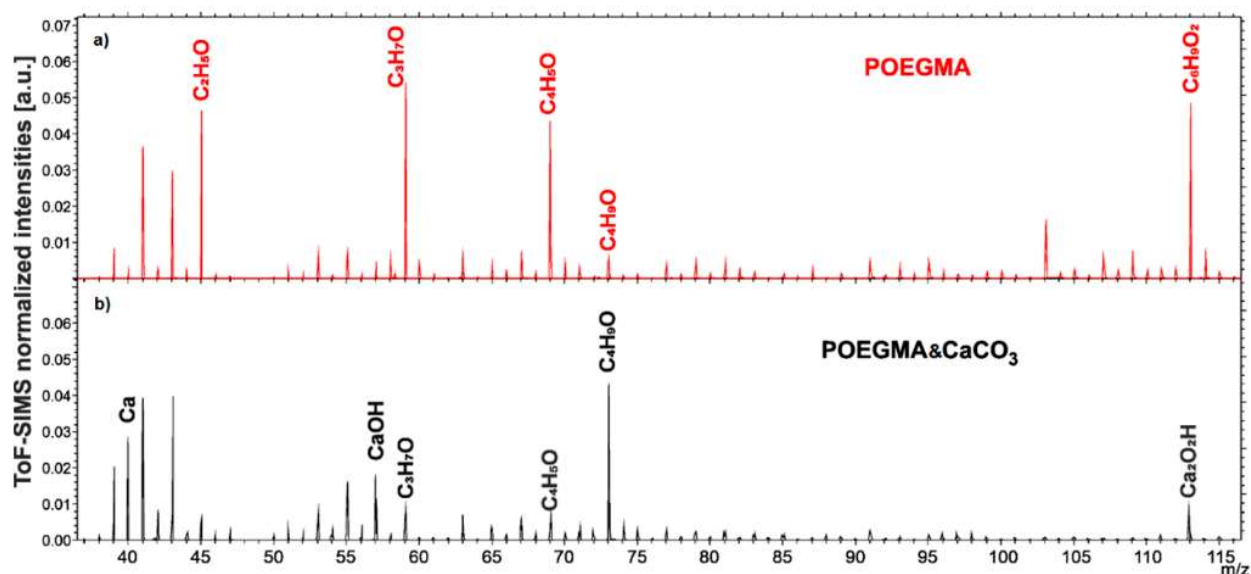


Figure 28. Normalized ToF-SIMS spectra of the pOEGMA brush (a) and the pOEGMA brush containing CaCO₃ nanoparticles (b).

The chemical compositions of pOEGMA and pOEGMA with embedded CaCO₃ nanoparticles were studied with ToF-SIMS and XPS (see Subsections II.2.1 and II.2.2). The ToF-SIMS spectra of pOEGMA and pOEGMA with embedded CaCO₃ nanoparticles are presented in Fig. 28. They contain the peaks corresponding to $C_2H_5O^+$, $C_3H_7O^+$, $C_4H_9O^+$ and $C_6H_9O_2^+$ characteristic of pOEGMA (Fig. 28a) and the peaks corresponding to Ca^+ , $CaOH^+$, $Ca_2O_2H^+$ of CaCO₃ nanoparticles (Fig. 28b), which confirm both, the successful fabrication of pOEGMA brush and incorporation of CaCO₃ nanoparticles.

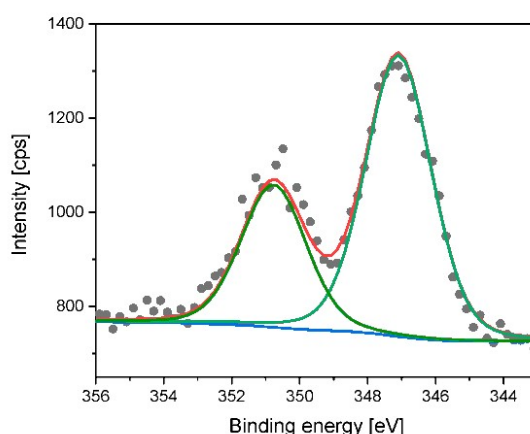


Figure 29. Ca 2p XPS spectra recorded for pOEGMA brush coating with embedded CaCO₃ nanoparticles

The Ca 2p spectra resulting from XPS measurements is presented in Fig. 29, showing the peak for the binding energy equal to 347.2 eV, which corresponds to the Ca 2p 3/2 and may be related to the CaCO₃ compound. Detailed analysis of XPS spectra revealed that the concentration of CaCO₃ is $1.88 \pm 0.53\%$.

Ellipsometry. The thicknesses of POEGMA-grafted brush coatings and pOEGMA with CaCO₃ nanoparticles in a dry state were 110.6 ± 9.3 nm and 117.4 ± 14.1 nm, respectively. In turn, the refractive index of the POEGMA coatings was 1.52 ± 0.02 , whereas for pOEGMA films with CaCO₃ nanoparticles, it equals 1.61 ± 0.05 . These results imply that the incorporation of CaCO₃ nanoparticles into the pOEGMA polymer matrix has a very weak impact on the thickness of the coatings, contrary to their optical properties, which change significantly.

The morphology of the coating was studied with SEM (see Fig. 30) and AFM (see Fig. 31). Squared and homogeneously distributed nanoparticles with a size not exceeding tens of nanometers were observed in SEM images of the surface of the pOEGMA brush with embedded CaCO₃ nanoparticles (Fig. 30). AFM analysis of the surface topography revealed that the presence of CaCO₃ nanoparticles in the coatings increased the surface roughness of the pOEGMA (see Fig. 31), but observation of individual nanoparticles was not possible.

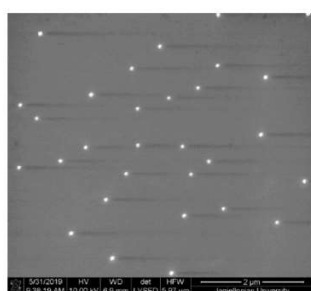


Figure 30. SEM image of CaCO₃ incorporated in POEGMA grafted brush coatings.

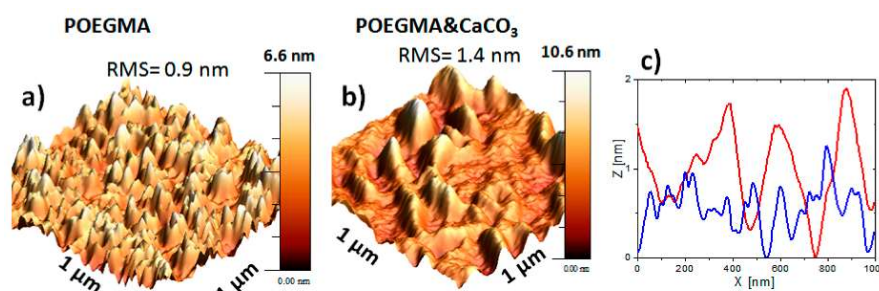


Figure 31. Images of the pOEGMA-grafted brush (a) and pOEGMA-grafted brushes with incorporated CaCO₃ (b) recorded using AFM working in topography mode (a, b). The cross-line analysis corresponds to the topography (c) of the samples without (blue) and with CaCO₃ nanoparticles (red).

The wettability of the fabricated brush coatings and LCST. To study the wettability and temperature response of the fabricated brush coatings, their water contact angles were studied at temperatures between 5 and 40 °C (see Subsection II.2.8). The temperature dependence of the water contact angles of the pOEGMA brush with embedded CaCO₃ nanoparticles and the native pOEGMA brush is presented in Fig. 32. The pOEGMA brush with embedded CaCO₃ nanoparticles is more hydrophilic than the native pOEGMA coating. The temperature dependence of water contact angles of both, the pOEGMA brush with embedded CaCO₃ nanoparticles and the native pOEGMA brush is akin

to sigmoid with the inflection point at 20 °C. According to Subsection I.6, the sigmoidal shape of the curve of the temperature dependence of the water contact angle is typical for the polymers that undergo the LCST transition, with the LCST corresponding to the inflection point of the curve. Hence, the LCSTs of pOEGMA and pOEGMA brush with embedded CaCO_3 nanoparticles are the same and equal to 20 °C.

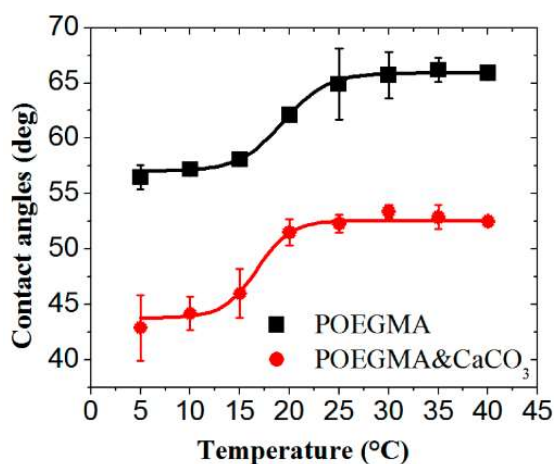


Figure 32. Temperature dependence of water contact angles of the pOEGMA grafted brush with embedded CaCO_3 nanoparticles (red) and the native pOEGMA grafted brush (black)

IV.2. Cell proliferation on pOEGMA grafted brush with embedded CaCO_3 nanoparticles

To study the influence of CaCO_3 nanoparticles embedded in the pOEGMA brush on human skin (HaCaT), melanoma (WM35) and osteoblastic cells (MC3T3-e1), they were cultured for a given time on fabricated coatings and glass as reference, then stained with fluorescence dyes and visualized with a fluorescent microscope. Finally, quantitative analysis was performed to determine cell growth (see Subsections II.3.1 and II.3.3). Furthermore, the proliferation index and cell viability were defined and calculated for a detailed analysis of cellular behavior.

HaCaT skin cells. Pictures of the labeled HaCaT cells cultured on the control glass sample, glass plates coated with pOEGMA and pOEGMA with embedded CaCO_3 nanoparticles are presented in Fig. 33; the quantitative analysis is presented in Fig. 34. Surprisingly, the amount of cells adhered to potentially non-adhesive pOEGMA [105–108] is almost the same as on the control sample (see Fig. 34). The presence of CaCO_3 nanoparticles in the pOEGMA brush slightly reduced the amount of adhered cells (see Fig. 34). The viability of HaCaT cells calculated for coatings with embedded CaCO_3 nanoparticles is reduced compared to the control sample, but does not drop below 80%, hence according to the ISO 10993-5 standard, the coating can be considered non-cytotoxic [109]. For HaCaT cells cultured on pOEGMA coatings, a similar trend is observed for 24 and 72 h, while for 144 hours of

incubation an improved cell viability is found compared to the control sample. The calculated proliferation index suggests an approximately equal growth of HaCaT cells on all surfaces for 72 h of incubation and slightly increased on POEGMA coatings for 144 h.

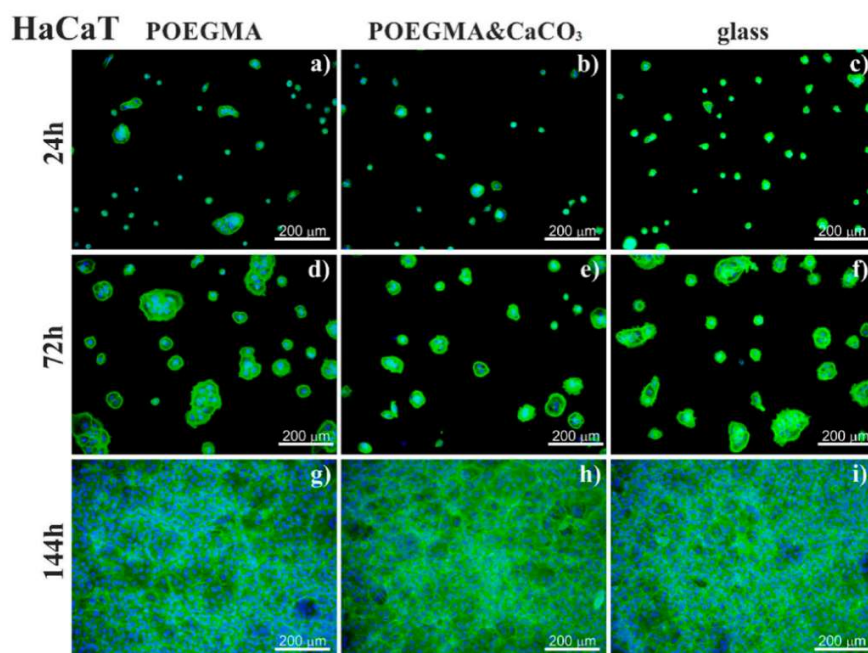


Figure 33. Fluorescence images of HaCaT cells after 24 (a–c), 72 (d–f) and 144 h (g–i) of culture on the glass surface (c,f,i), pOEGMA (a,d,g) and pOEGMA-grafted brush coatings with incorporated CaCO_3 nanoparticles (b,e,h)

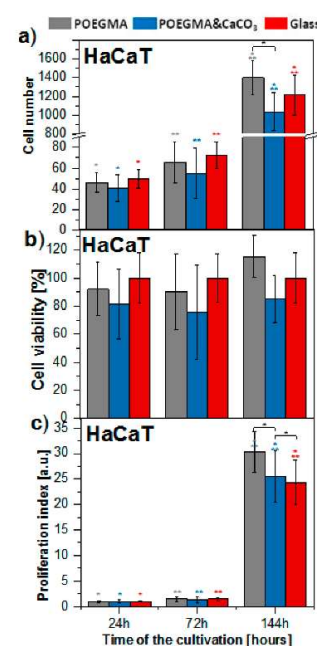


Figure 34. Quantitative analysis of the impact of CaCO_3 nanoparticles embedded in pOEGMA brush

WM35 melanoma cells. The fluorescence images of WM35 melanoma cells on control sample and on fabricated coatings after 24, 72, and 144 hours of incubation are presented in Fig. 35. After 24 hours of incubation, a low amount of the cells is observed on the coating with CaCO_3 nanoparticles, suggesting low adhesiveness of the WM35 cells to this coating (see Fig. 36).

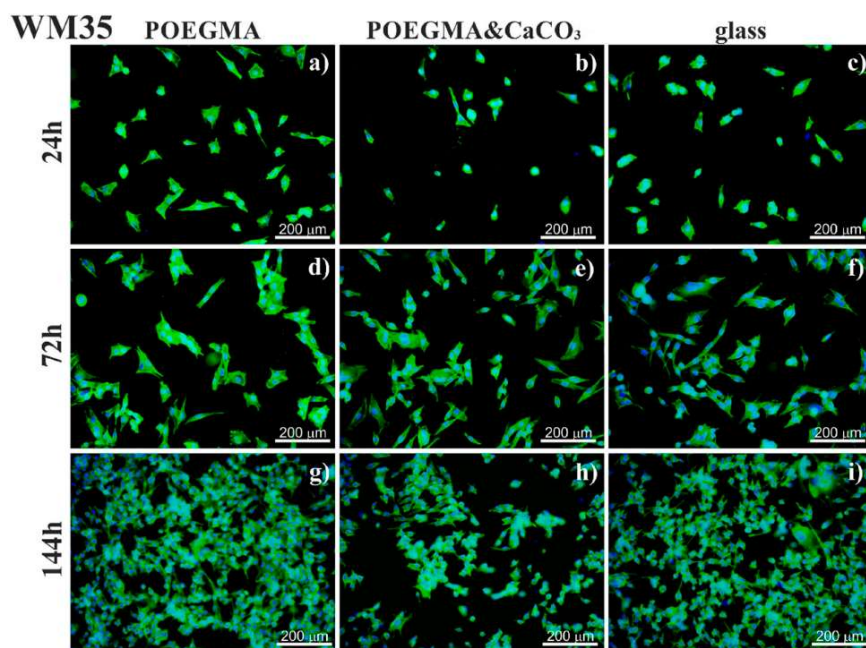


Figure 35. Fluorescence images of cancerous WM35 cells after 24 (a–c), 72 (d–f) and 144 h (g–i) of culture on the glass surface (c,f,i), pOEGMA (a,d,g) and pOEGMA-grafted brush coatings with incorporated CaCO₃ nanoparticles (b,e,h)

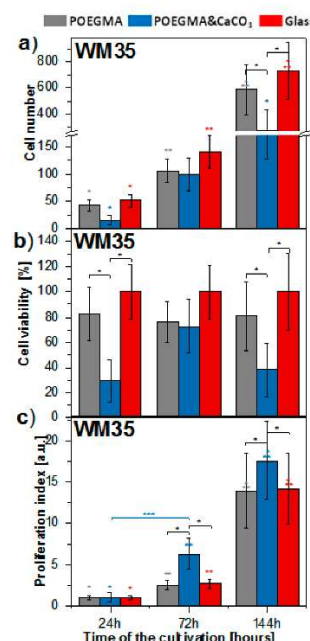


Figure 36. The quantitative analysis of the impact of CaCO₃ nanoparticles embedded in pOEGMA brush on melanoma cells

The results of the quantitative analysis of cell growth are presented in Fig. 36. The presence of CaCO₃ nanoparticles in the coating increased the proliferation index of WM35 melanoma cells compared to the control sample and the pOEGMA brush coating. In turn, the calculated viability of cells cultured on the coating with CaCO₃ nanoparticles after 24 hours of incubation was reduced to 30%, while after 72 hours of incubation it increased to 70%, but still remained lower than for pOEGMA. After 144 hours of incubation, the viability of the cells calculated for cells cultured on pOEGMA with embedded CaCO₃ nanoparticles decreased again down to 40%. However, the proliferation indices calculated for WM35 cells indicate the effective growth of cells on all examined substrates, significantly enhanced for the POEGMA coating with embedded CaCO₃ NPs, therefore, the low viability of WM35 melanoma cells may be related more to the low adhesive properties of the coatings than to their cytotoxicity.

Osteoblastic cells MC3T3-e1. The totally non-adhesive properties of pOEGMA to osteoblastic cells have been reported in the literature [110,111]. In contrast, in this study, the adhesion of the osteoblastic MC3T3-e1 cell line to pOEGMA-based coatings was observed after 24 hours of incubation (Fig. 37). The grafting density and molecular weight have an impact on the adhesive properties of the brush-coatings. Since the literature sources do not provide such information, the results obtained cannot be reliably compared with the data from the literature. However, statistical analysis (Fig. 38) revealed

that the amount of osteoblastic MC3T3-e1 cells decreased with incubation time, most probably due to cell detachment during incubation. The release of cells results in extremely low values of the viability and proliferation index.

MC3T3-E1

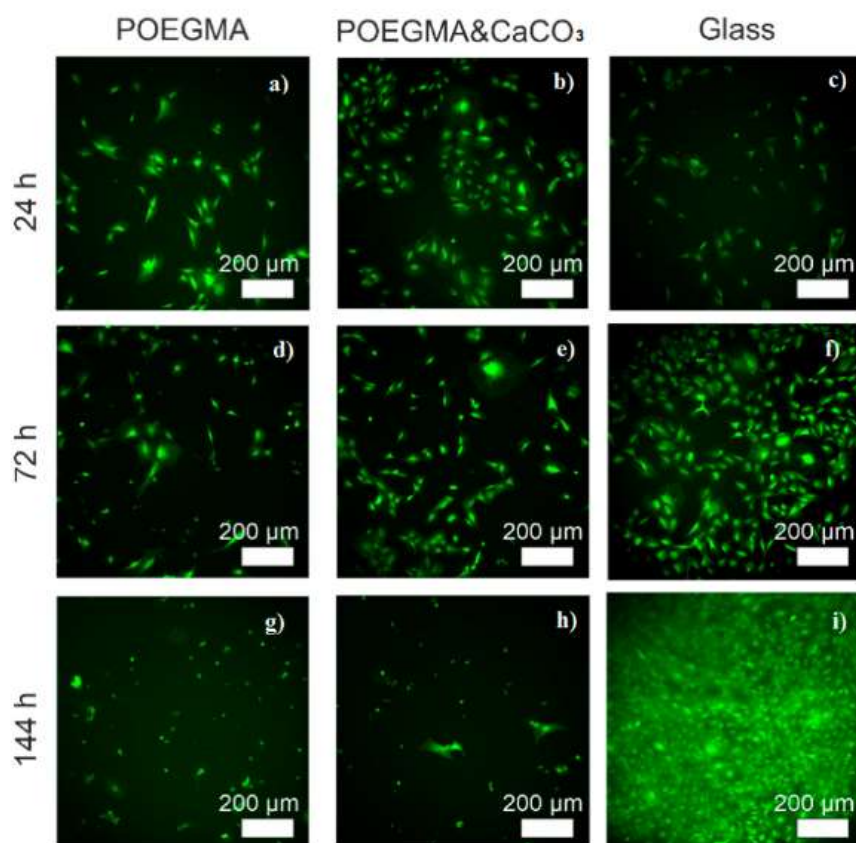


Figure 37. Fluorescence images of osteoblastic cells MC3T3-e1 after 24 (a–c), 72 (d–f) and 144 h (g–i) of culture on the glass surface (c,f,i), pOEGMA (a,d,g) and pOEGMA-grafted brush coatings with incorporated CaCO₃ nanoparticles (b,e,h)

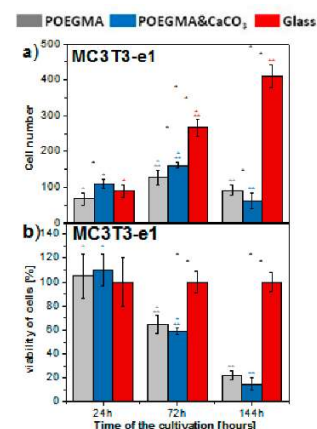


Figure 38. Quantitative analysis of the impact of CaCO₃ nanoparticles embedded in pOEGMA brush on osteoblastic cells MC3T3-e1

Cell behavior on various surfaces is strongly dependent on the physicochemical properties of the surface, namely, hydrophobicity, charge, and roughness (see Section I.2) [32, 38 - 41]. X. She and others studied the adhesion of cells to pOEGMA brush grafted to surfaces with different roughness and showed that cell adhesion is higher on surfaces with higher roughness [32]. According to the results presented in this Section, the incorporation of CaCO₃ nanoparticles increased the roughness of the coating, but a significantly higher adhesion of cells to the surfaces containing the embedded CaCO₃ nanoparticles was not detected. However, X. She et al [32] studied the impact of the roughness of the chemically homogeneous surface. In our case, the surface is not chemically homogeneous since the CaCO₃ nanoparticles are present on the surface, as confirmed by ToF-SIMS analysis and SEM imaging.

The incorporation of CaCO_3 nanoparticles modified the physical and chemical properties of the sample and led to increased hydrophilicity of the coating. Recent advances in the engineering of SPB coatings for cell detachment show that, in many cases, cells release from brushes in an extended hydrophilic coil conformation [31] when the surface is hydrophilic. On the other hand, cells can adhere to the surface in a hydrophilic state via polar interactions that can occur between cells and inorganic nanoparticles. Moreover, in Subsection III.1 it was shown that pOEGMA swells in water, which may impact the roughness of the surface [101]. The results presented in this Section revealed that the influence of CaCO_3 nanoparticles embedded in the pOEGMA brush is strongly cell dependent. However, the determination of the most important parameter, determining the interactions between the examined cells and coatings is not possible.

The literature reports low cytotoxicity of CaCO_3 nanoparticles to cells [113,114], and even improved cell proliferation [115]. On this basis, it is concluded that decreased cell viability on the surface of pOEGMA grafted brush-coating with embedded CaCO_3 nanoparticles may be related rather to the weak adhesion of cells to the coating and their spontaneous detachment during incubation than to the toxic effect of CaCO_3 nanoparticles.

IV.3. Conclusions

The CaCO_3 nanoparticles were successfully fabricated inside of pOEGMA brush via the biomineralized precipitation from solutions of CaCl_2 and Na_2CO_3 . Fabricated pOEGMA brush coatings and pOEGMA brush coatings containing CaCO_3 nanoparticles were studied with ToF-SIMS, XPS, AFM and water contact angles. The LCST of pOEGMA and pOEGMA with embedded CaCO_3 nanoparticles was found at 20°C with water contact angles. The SEM detected squared-like CaCO_3 nanoparticles. The concentration of CaCO_3 nanoparticles, determined with XPS was $1.88 \pm 0.53\%$.

The influence of CaCO_3 nanoparticles on three cell lines: human skin cells (HaCaT), melanoma (WM35), and osteoblasts (MC3T3-e1) was studied. The study revealed that the fabricated nanocomposite is low-fouling against osteoblasts (MC3T3-e1) and noncytotoxic against healthy skin (HaCaT) and melanoma (WM35) cell lines. The embedded CaCO_3 nanoparticles demonstrated a strongly cell-dependent influence on examined cells.

V. Smart polymer brush coatings for thermo-controllable bacteria killing

The bacteria-resistant surfaces known nowadays are divided into two groups: (I) surfaces that repel bacteria and (II) surfaces that contain the antibacterial agent that kills bacteria [21]. The anti-fouling surfaces prevent the adhesion of dangerous bacteria, while the surfaces containing the antibacterial agent kill the bacteria, preventing their further spreading. High attention is paid to smart antibacterial coatings that activate their antibacterial properties under external stimuli, such as light, pH, or temperature, and are capable of a 'kill-release' strategy [48,116].

Smart antibacterial coatings that activate their antibacterial properties with temperature stimuli may find numerous applications for the prevention of the formation of bacterial film on daily-used, frequently touched surfaces such as smartphones, cash machines, windows, and many others.

In Subsection IV.1 it was shown that the pOEGMA brush and pOEGMA with embedded CaCO_3 nanoparticles undergo the LCST-driven transition at 20 °C. Stetsyshyn and others found an interesting temperature switch of wettability also in pVP grafted SPB coatings [1,29]. The antibacterial properties of silver are well known [117]. Furthermore, in work [71] it was shown that pOEGMA, pVP, and p(OEGMA-co-VP) provide the appropriate properties for the fabrication of silver nanoparticles inside the brushes.

On the basis of these results, pOEGMA and pVP were selected for the fabrication of novel smart antibacterial nanocomposites containing the silver nanoparticles embedded into SPB, dedicated for thermo-stimulated bacteria killing. SPB coatings were fabricated following ATRP (see II.1.2). Silver nanoparticles were fabricated inside the SPB coating by reducing AgNO_3 with NaBH_4 as a reducing agent (see II.1.5). The silver ions were adsorbed from 0.005 M aqueous solution of AgNO_3 for 30 minutes and reduced by 0.2 M aqueous solution of NaBH_4 for 12 hours. The composition of the fabricated coatings was studied with ToF-SIMS (see II.2.1) and XPS (see II.2.2). The thermal response of the SPB coatings and the SPB coatings containing silver nanoparticles were studied by measuring the water contact angles at different temperatures (see II.2.8). The morphology of the fabricated nanocomposites was studied using AFM and SEM (see II.2.3 and II.2.4).

The thermo-switchable antibacterial activity of the fabricated nanocomposites was tested against representatives of Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*) (see II.3.5 and II.3.6).

V.1. Characterization of the smart polymer brush coatings for thermo-controllable bacteria killing

The chemical composition of the coatings and the effective incorporation of silver nanoparticles into the fabricated coatings were examined with XPS (Fig. 39a and Fig. 39d), ToF-SIMS (Fig. 39b and

Fig. 39e), and SEM working in EDX mode (Fig. 39c and Fig. 39f). The XPS spectra show two peaks at 367.8 and 373.8 eV, corresponding to the states 3d_{5/2} and 3d_{3/2} of Ag, respectively, confirming the presence of Ag. A more detailed analysis of the XPS spectra implied that the abundance of Ag in pOEGMA equals 0.49% and that in pVP equals 0.32%. The EDX analysis showed the series of AgL peaks, confirming the presence of Ag in the coatings. In turn, in the ToF-SIMS spectra of pOEGMA and pVP (Fig. 39b and Fig. 39e) SPB coatings with embedded Ag nanoparticles two peaks were recorded, at m/z ratio equal to 106.9 and 108.9, corresponding to $^{107}\text{Ag}^+$ and $^{109}\text{Ag}^+$ silver isotopes, respectively, providing additional verification of the successful incorporation of Ag nanoparticles into the coatings.

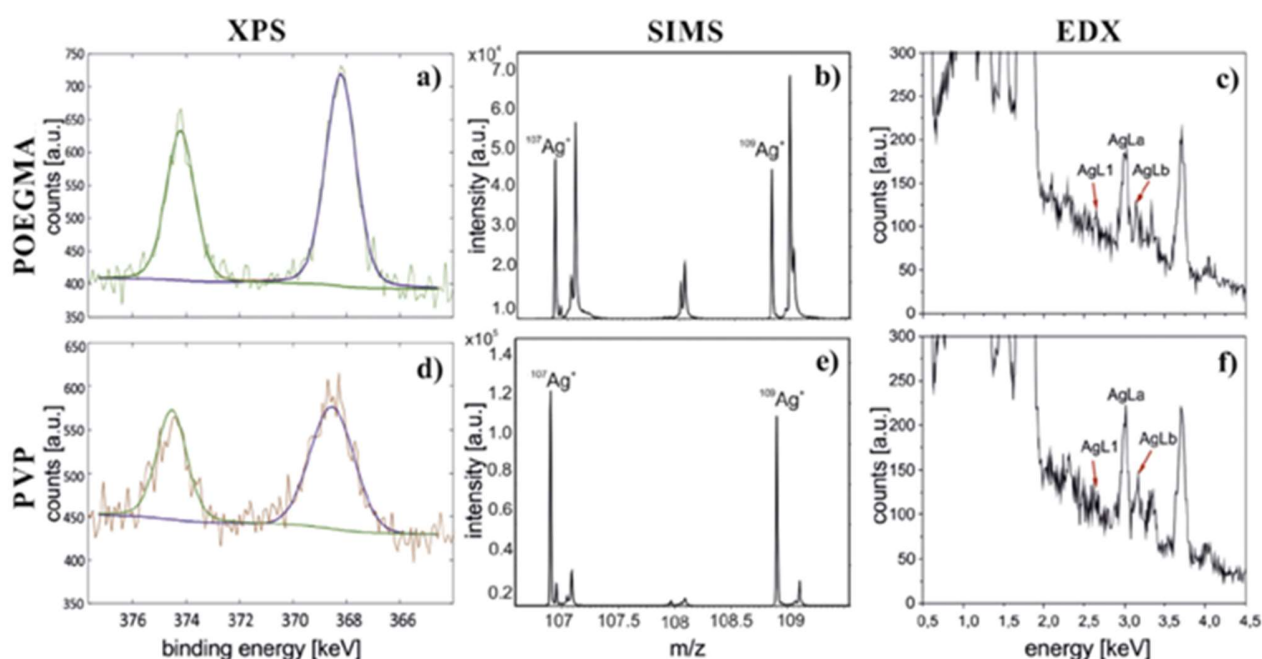


Figure 39. Verification of the presence of Ag in POEGMA (top row) and PVP (bottom row) grafted brush coatings using XPS (a-d), ToF-SIMS (b-e), and EDX (c-f). All spectra were obtained from random points within the field of view of the images.

To study the distribution of the Ag nanoparticles on the surface of SPB coatings, SEM (Fig. 40a and Fig. 40d) and TOF-SIMS mapping (Fig. 40b, Fig. 40c, Fig. 40e and Fig. 40f) were applied. The TOF-SIMS maps recorded for $\text{C}_4\text{H}_7\text{O}^+$ and $\text{C}_7\text{H}_8\text{N}^+$ ions corresponding to pOEGMA and pVP, respectively, reveal an effective coverage of the surface by polymers (Fig. 40b and Fig. 40e). The imaging of $^{107}\text{Ag}^+$ ions reveals sparse regions with higher intensity of ions (Fig. 40c), which may be related with Ag agglomerates, which were also observed in the SEM analysis (Fig. 40a). SEM image recorded for pVP-based coating shows the homogeneous distribution of Ag nanoparticles (Fig. 40d) in the polymer matrix. This arrangement is confirmed using TOF-SIMS results (Fig. 40e and 40f), which present a

uniform distribution of $C_7H_8N^+$ ions (Fig. 40e) typical for pVP and Ag^+ ions (Fig. 40f) characteristic for nanoparticles.

The detailed analysis of high-resolution SEM and AFM working in topography and phase contrast modes depicts rod-like and spherical nanoparticles on the surface of pOEGMA and pVP surfaces, respectively. The size of the rod-shaped nanoparticles on the surface of pOEGMA is approximately $40 \pm 7 \times 80 \pm 14$ nm. In turn, the size of the spherical silver nanoparticles on the surface of the pVP is approximately 14 ± 6 nm (Fig. 41).

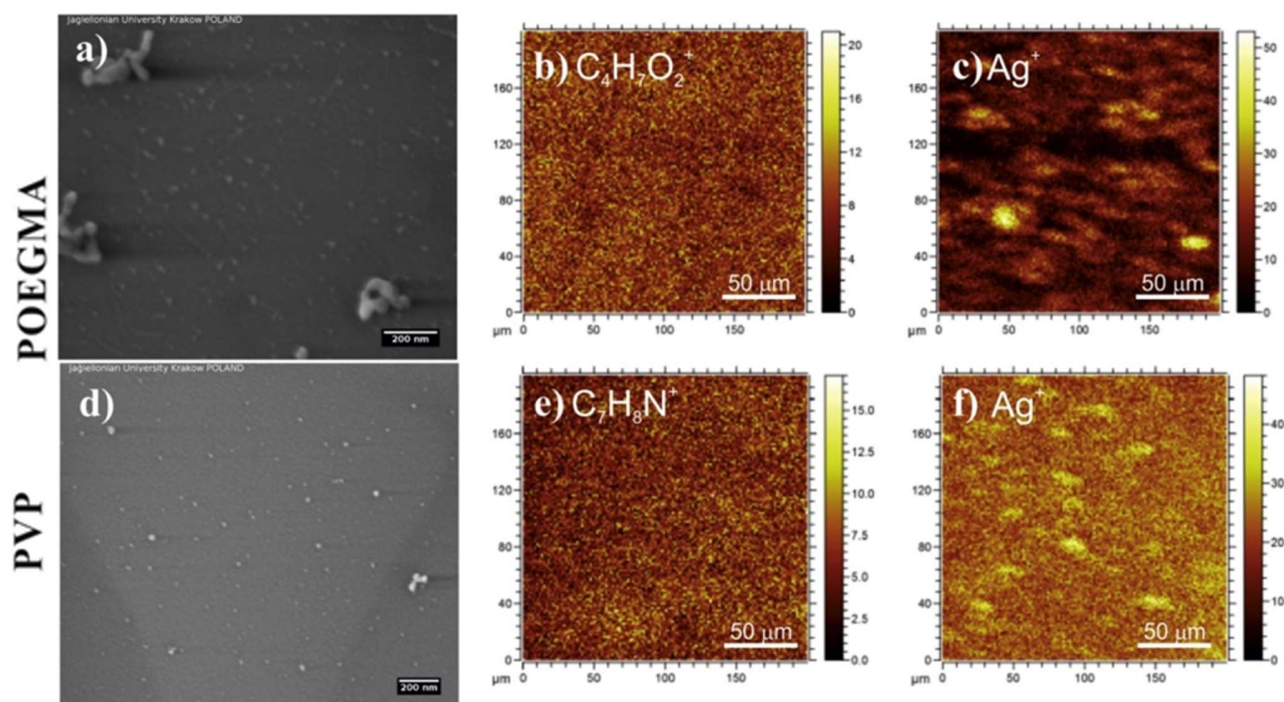


Figure 40. The homogeneity of the pOEGMA (upper row) and pVP (bottom row) coatings with Ag nanoparticles confirmed using SEM (a-d) and ToF-SIMS imaging of polymer matrix (b-e) and Ag (c-f).

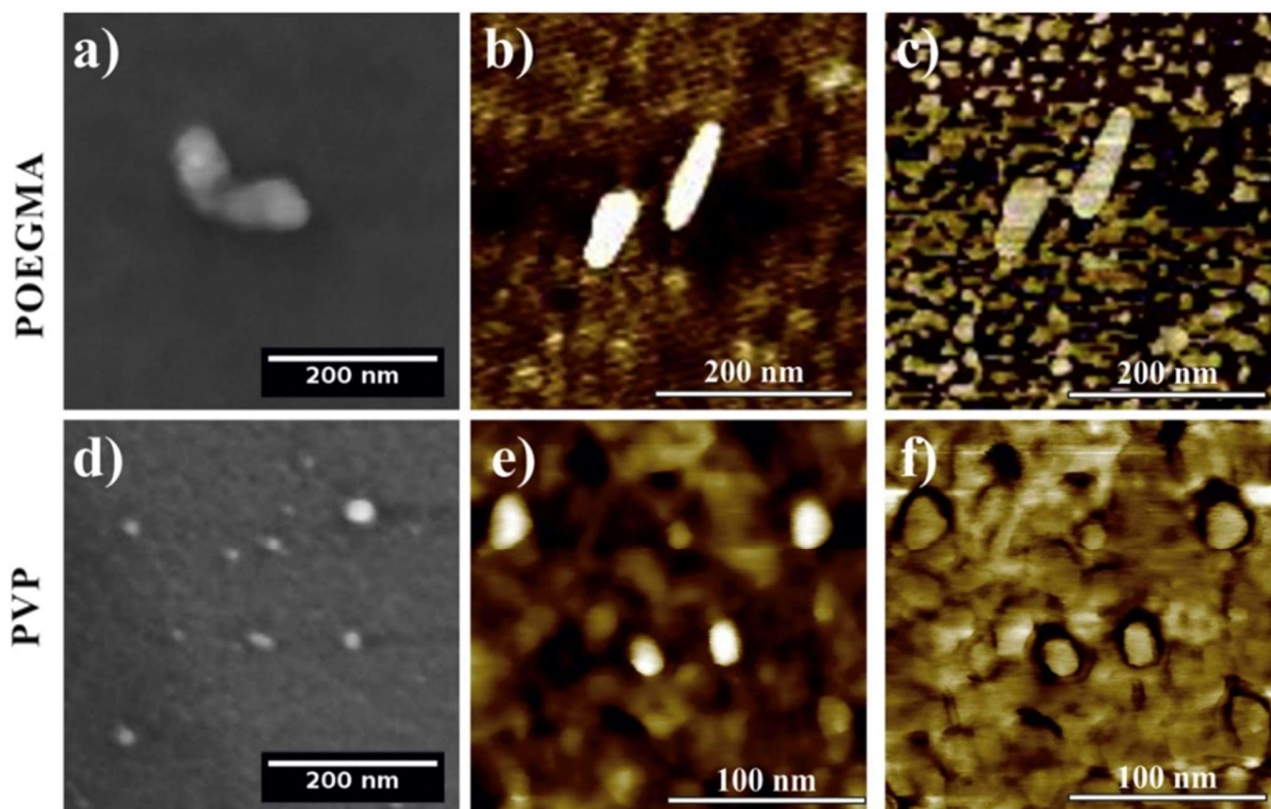


Figure 41. Silver nanoparticles in pOEGMA (upper row) and pVP (bottom row) grafted brushes recorded using SEM (a, d) and AFM working in topography (b, e) and phase contrast (c, f) modes.

To study the thermal response of the SPB coatings, measurements of the water contact angles for temperatures ranging from 5 to 40 °C were employed. The thermal dependence of the water contact angle of pOEGMA and pVP with and without Ag nanoparticles are akin to a sigmoid with the deflection point at 20 °C for pOEGMA-based coatings and at 12 °C for pVP-based coatings (Fig. 42). Temperature-stimulated changes in water contact angles are typical for both pOEGMA [1,101], and pVP [1,28,29], implying that the presence of Ag nanoparticles does not weaken the thermo-responsive properties of the coatings. However, coatings with embedded Ag nanoparticles are significantly more hydrophilic, compared to the ‘pure’ ones.

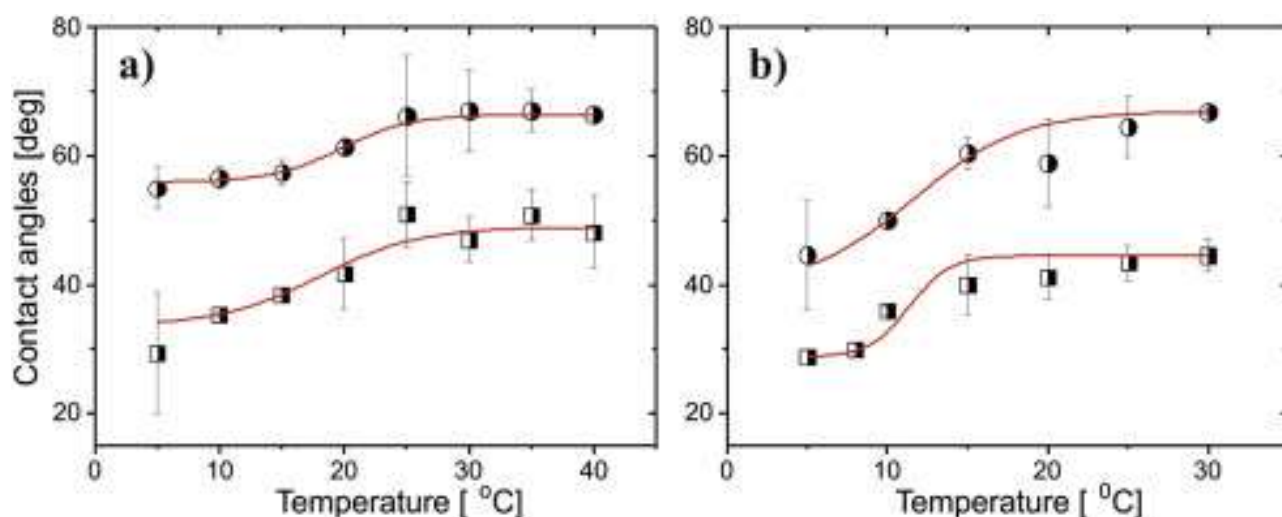


Figure 42. Temperature dependences of water contact angles determined for ‘pure’ SPB coatings (circles) and the SPB coatings with embedded Ag nanoparticles (squares) measured for pOEGMA (a) and pVP-based (b) coatings.

V.2.Characterization of the antibacterial properties

The antibacterial activity of the fabricated pOEGMA and pVP coatings with embedded Ag nanoparticles was tested against representative strains of Gram positive bacteria (*Staphylococcus aureus*, ATCC 25923) and Gram negative bacteria (*Escherichia coli*, ATCC 25922). First, the coatings were incubated in suspension of bacterial strains for 24 hours at 4 °C and 37 °C. Then, the bacterial cells were inoculated on standard agar, cultured and counted (see II.3.5).

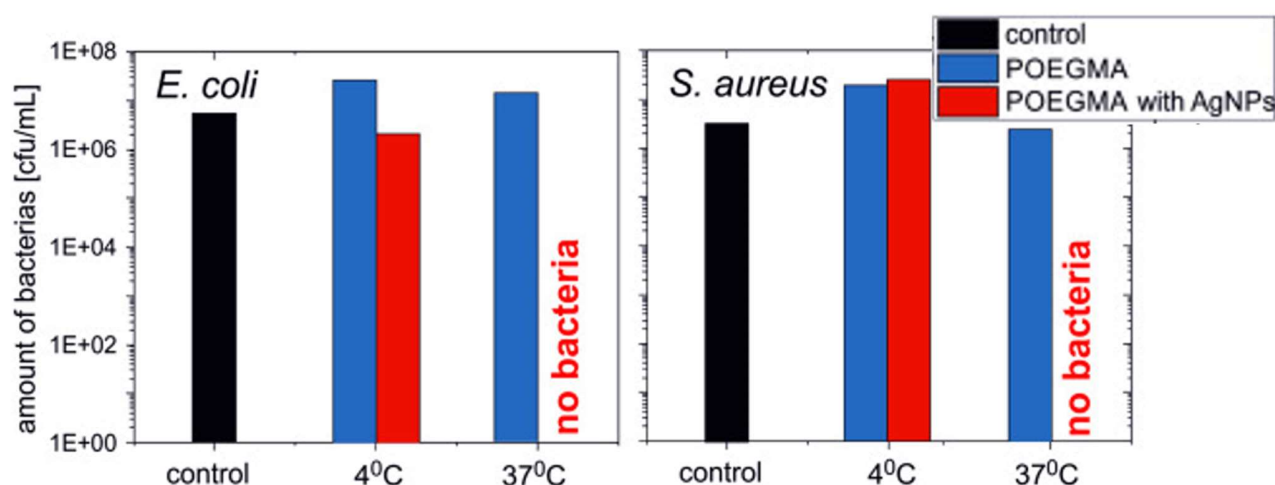


Figure 43. Amount of *E. coli* and *S. aureus* bacteria obtained from the solution after incubation with pOEGMA SPB coatings (blue) with silver nanoparticles (red) and on the control sample (black).

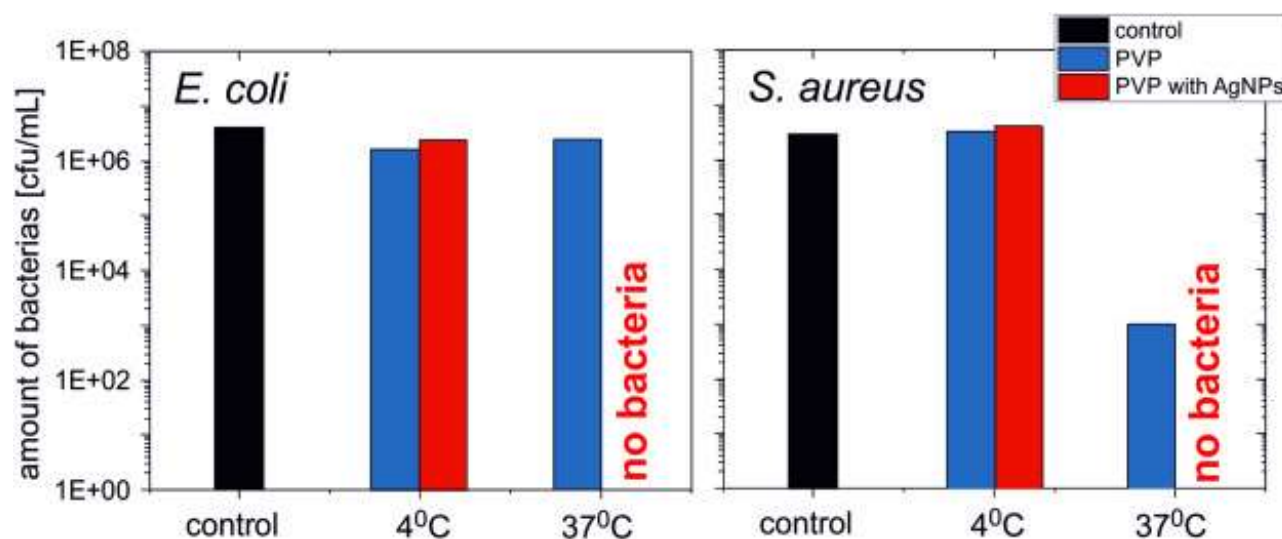


Figure 44. Amount of *E. coli* and *S. aureus* bacteria obtained from the solution after incubation with pVP SPB coatings (blue) with silver nanoparticles (red) and on the control sample (black).

The quantitative analysis, presented in Figures 43 and 44 revealed that for bacterial suspensions initially incubated with the coatings at 4 °C, the amounts of both bacterial strains were large and similar for all examined substrates. In turn, for bacterial suspensions initially incubated with the coatings at 37 °C, bacteria cells were found only for the samples cultured from the suspension incubated with the coatings which did not contain any silver nanoparticles. After incubation at 37°C with pOEGMA and pVP containing silver nanoparticles, no bacteria were observed (see Fig. 43 and Fig. 44).

The results presented in Fig. 43 and Fig. 44 confirm that pOEGMA and pVP brushes with embedded silver nanoparticles have very strong antibacterial properties at 37 °C, as no bacteria able to proliferate were found in the solutions incubated with them (non-contact killing, see I.3). To check whether these coatings are effective against bacteria also in direct contact (contact killing, see I.3), the bacteria remained on the incubated coatings were stained with the LIVE/DEAD fluorescence kit, enabling differentiation between live and dead bacteria, and pictured by the fluorescent microscope (see II.3.6). The results revealed that on the pOEGMA and pVP coatings bacteria were alive after incubation at 4 °C and 37°C. In turn, for pOEGMA and pVP brush containing silver nanoparticles all bacteria were dead after incubation at 37 °C, while at 4 °C the bacteria were alive (Fig. 45), confirming the thermo-switchable antibacterial properties of the coatings with embedded silver nanoparticles.

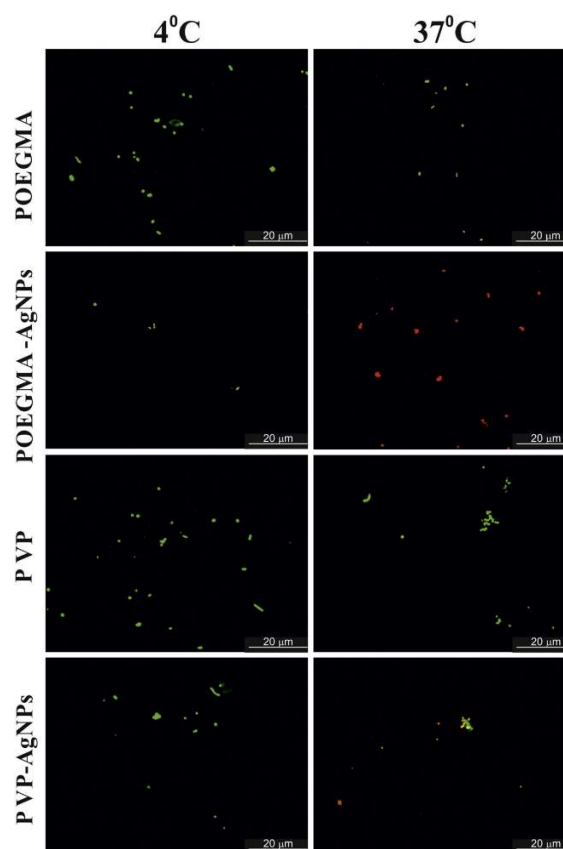


Figure 45. Fluorescent microscopy pictures of alive (green) and dead (red) *S. aureus* bacteria on the surface of pOEGMA and pVP with and without silver nanoparticles after incubation for 24 hours at 4°C and 37°C

V.3.Conclusions

Novel thermo-responsive nanocomposites based on silver nanoparticles embedded in pOEGMA and pVP SPB coatings grafted to the glass surface were successfully fabricated. The temperature transitions of wettability of the pOEGMA and pVP with embedded silver nanoparticles were determined by the water contact angle. It was shown that the incorporation of Ag nanoparticles does not block the thermoresponsiveness of the nanocomposites, which exhibit temperature transitions at 20°C and 12°C, respectively. The ToF-SIMS measurements proved the successful fabrication of the nanocomposites. The incorporation of Ag nanoparticles into the coatings was verified with XPS and ToF-SIMS. The results of the XPS study revealed that the abundance of silver was equal to 0.49% in pOEGMA and 0.32% in pVP. The detailed analysis of nanoparticles, performed with SEM as well as AFM topography and phase-contrast imaging revealed the elongated, rod-like shape of silver nanoparticles for POEGMA grafted brush coatings, in contrast to the pVP coating, where the nanoparticles have rather a spheroid shape.

The antibacterial activity of the fabricated nanocomposites was studied against *E. coli* (representative of Gram-negative bacteria) and *S. aureus* (representative of Gram-positive bacteria) at 4 and 37 °C (below and above the LCST of the grafted polymer brushes, respectively). The study revealed that below the temperature transitions nanocomposites are not toxic against both bacteria strains whereas they exhibit strong antibacterial activity against both strains above transitions temperature.

The fabricated novel thermo-switchable nanocomposites are promising materials for applications in food packaging, medical laboratories, and in air-conditioning systems where the bacteria can proliferate and cause danger to human health. Moreover, the metallic nanoparticles probably are strongly bonded in a polymer matrix, and the coating should prevent the release of whole nanoparticles from the coating and thereby their accumulation in human organs. The cytotoxicity and the release of the embedded nanoparticles in the polymeric matrix are studied in the next Section.

VI. “Smart” non-cytotoxic antibacterial POEGMA based nanocomposite

In Section V, the thermo-switchable antibacterial activity of the smart nanocomposites based on pOEGMA and pVP with embedded silver nanoparticles was reported. Currently, it is well known that pOEGMA brushes exhibit low cytotoxicity for various types of cells and are widely used in biomedicine [96,97,118]. Therefore, pOEGMA-based nanocomposites with embedded silver nanoparticles are promising materials for prevention of bacteria spreading between the humans. However, the applications of the surfaces containing the metallic nanoparticles are restricted because of the possible risk that these nanoparticles cause. Metallic nanoparticles can be released from the surface and accumulate in human organs such as the liver, spleen, lungs, and kidney [119–121]. Furthermore, silver nanoparticles released from the coating have potential genotoxicity activated by various mechanisms, in particular, the production of excess reactive oxygen species, resulting in oxidative stress, reduced glutathione levels, elevated lipid peroxidation, inflammation, DNA damage, altered cell cycle and proliferation capacity, and apoptosis and necrosis [122–125].

The silver nanoparticles embedded into the polymer brush matrix are bound to the brush and should not be able to be released. To confirm this hypothesis, the release of silver nanoparticles in an aqueous environment was studied. To study the potential cytotoxicity of the fabricated nanocomposites, two cell lines were cultured on them: human skin HaCaT cells and WM35 melanoma cells.

The optimal conditions for the fabrication of a smart noncytotoxic nanocomposite were determined experimentally. The pOEGMA brush-coatings of different thicknesses were fabricated following the ATRP method (see II.1.2) by varying the time of polymerization of the brush. Then, the silver nanoparticles were embedded into the brush for different concentrations and time of silver adsorption, following the procedure described in Subsection II.1.5. Finally, the morphology and thermo-responsiveness of the fabricated nanocomposites were studied with AFM and water contact angles, respectively.

VI.1. Characterization of “smart” antibacterial and non-cytotoxic coating

The pOEGMA brushes were grafted onto the glass plates following ATRP. To characterize the growth of the pOEGMA brush coatings, they were examined with ellipsometry (see II.2.7). In Fig. 46 the increase of brush thickness with increasing polymerization time is presented, visualizing the kinetics of the polymerization. After 16 hours of polymerization, the coatings were fabricated with a thickness of 60 nm, and the polymerization was not terminated, as visible in the further increase of the thickness of the SPB coating.

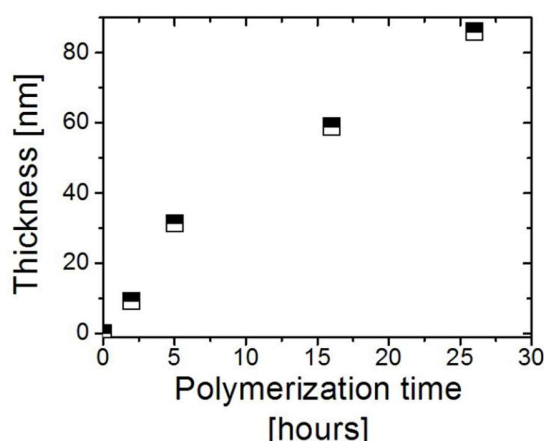


Figure 46. Thickness of grafted pOEGMA brush coatings versus polymerization time

To study the dependence of the thermal response of the pOEGMA brush-coating on the polymerization time, the water contact angles at various temperatures were studied for pOEGMA coatings fabricated with different polymerization times (Fig. 47a). The LCST occurs in coatings prepared for polymerization times longer than 5 hours of polymerization and does not vanish with increasing polymerization time.

To optimize the conditions for the embedding of the silver nanoparticles in pOEGMA coatings, the silver ions were adsorbed from the 5 mM aqueous solution of AgNO_3 for 15 minutes and 30 minutes, then from 3 mM aqueous solutions of AgNO_3 for 15 and 30 minutes followed by reduction of silver by NaBH_4 as described in Subsection II.1.5. After that, the thermal dependence of the water contact angles was measured (Fig. 47). In Fig. 47b, the thermal response of the pOEGMA brush-coating after 5 hours of polymerization with embedded silver nanoparticles from the 5 mM aqueous solution of AgNO_3 for 15 and 30 minutes of adsorption is presented. These results show that the thermal response of the pOEGMA coating after 5 hours of polymerization is totally blocked by the silver nanoparticles at both concentrations and adsorbing times. From Fig. 47c it can be seen that the thermal response of pOEGMA occurs when the polymerization time of pOEGMA increases up to 16 hours and AgNO_3 is adsorbed from 5mM aqueous solution of AgNO_3 for 15 minutes; after 30 minutes of adsorbing, the thermal response of pOEGMA (16 hours of polymerization) is blocked. In Fig. 47d the thermal response of pOEGMA (16 hours of polymerization) with embedded nanoparticles from a 3mM solution of AgNO_3 for 15 and 30 minutes is presented and it can be seen that the thermal transitions of pOEGMA are detectable in both cases.

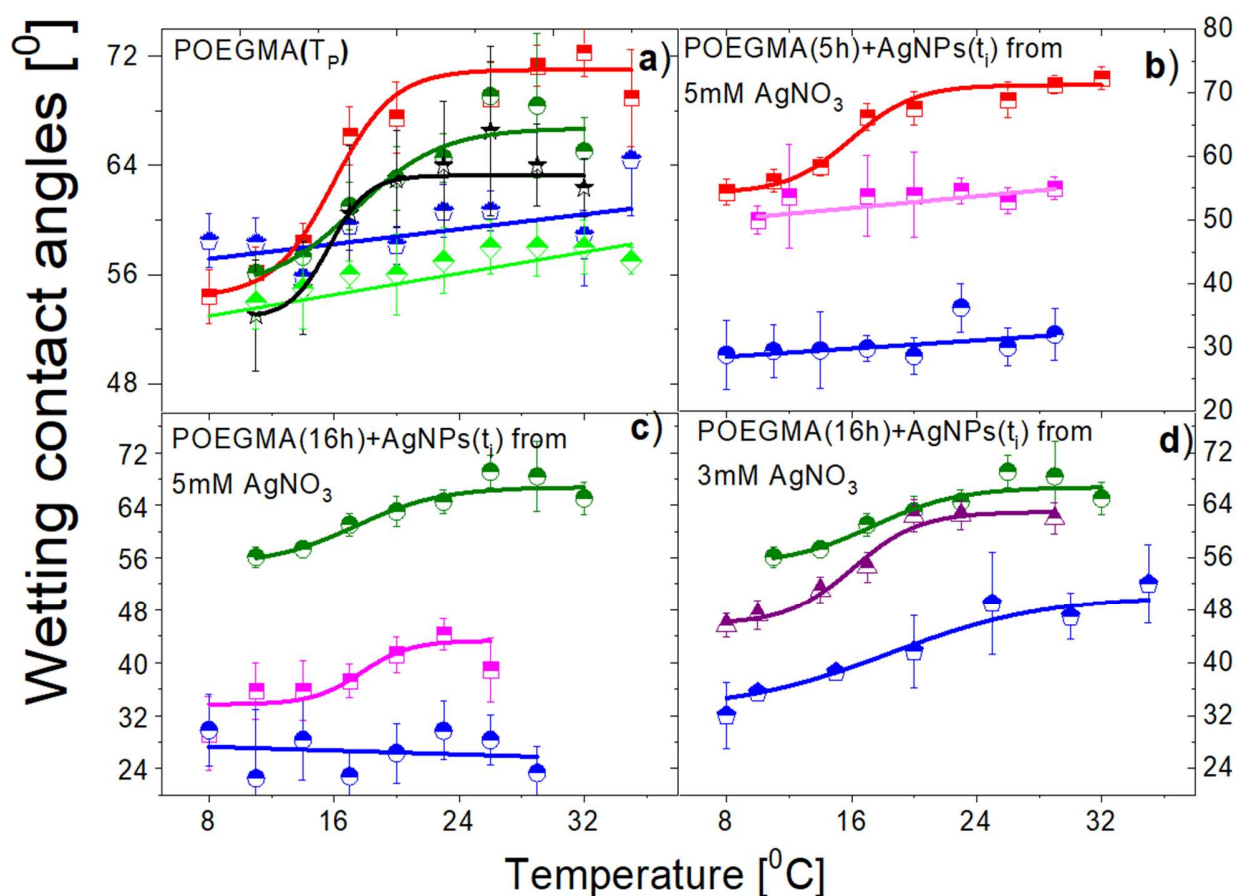


Figure 47. Temperature dependences of water contact angles determined for grafted pOEGMA brush coatings fabricated with different polymerization times T_P , without (a) and with (b–d) silver nanoparticles embedded in brushes after exposure to 5 mM (b and c) and 3 mM AgNO_3 solutions (d) for different immersion times t_i , followed by reduction in the 0.2 M NaBH_4 solution for 12 h. The conditions of synthesis of silver nanoparticles (b–d) are optimized for the pOEGMA brush coatings obtained after 5 h (b) and 16 h (c and d) of polymerization. The data corresponding to different polymerization times ($T_P = 0$ h), green open diamonds; 2 h, blue open triangles; 5 h, red open squares; 16 h, olive open circles; 26 h, black open pentagons and non-zero silver nanoparticles synthesis times ($t_i = 15$ min, violet solid squares; 30 min, blue solid circles) are marked by open and solid symbols, respectively.

The morphology of the fabricated coatings was studied with AFM (Fig. 48). The pOEGMA coating has a relatively smooth surface with small patch structures and does not show any phase contrast (Fig. 48, left side). In turn, the morphology of pOEGMA brush-coating with embedded silver nanoparticles after 15 minutes of adsorbing the AgNO_3 ions is quite rough, showing high phase contrast. With increasing immersion time in AgNO_3 , the surface of pOEGMA with embedded nanoparticles

becomes smooth. and the phase contrast decreases, suggesting that the nanoparticles start to form a strongly metalized surface.

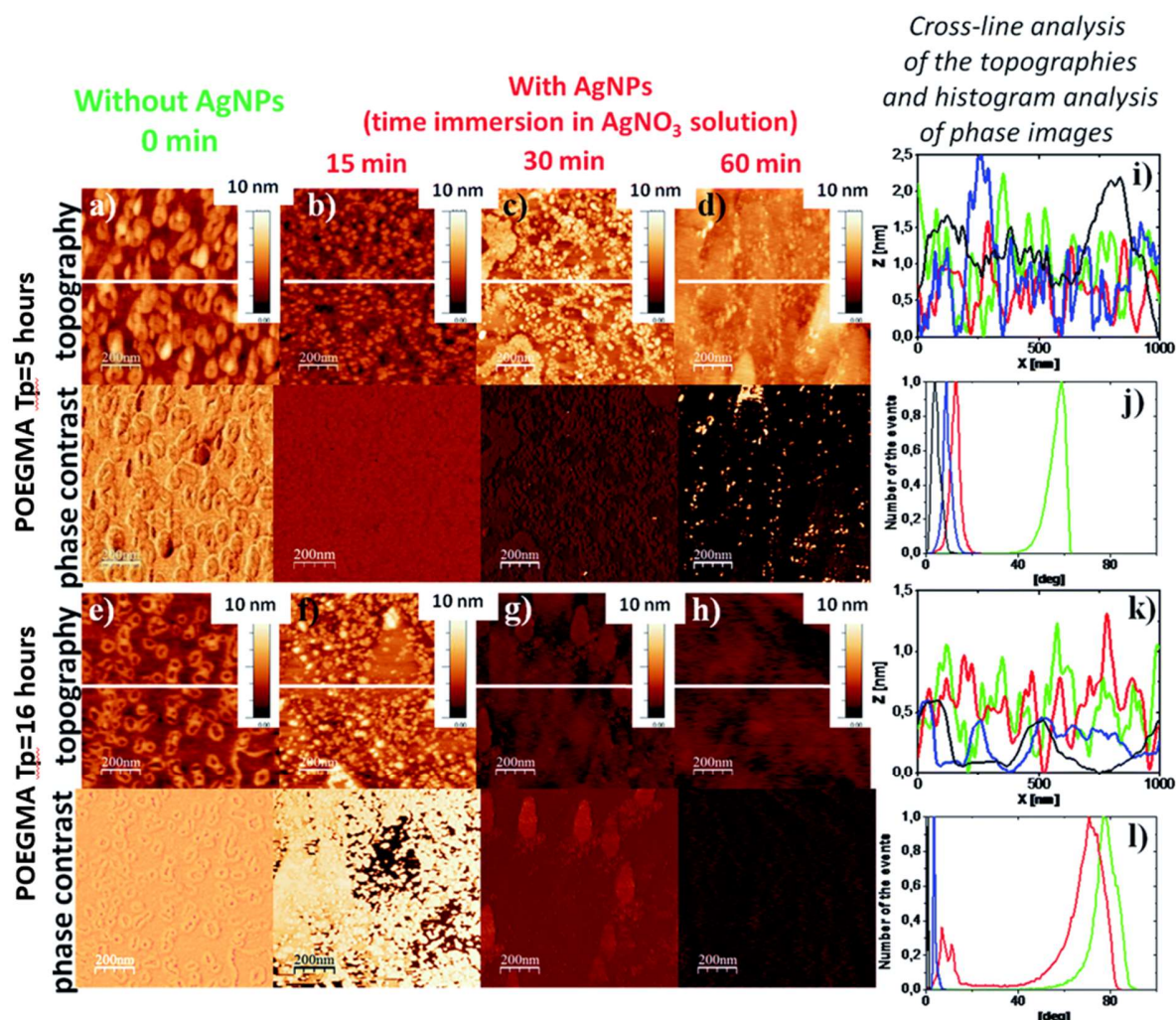


Figure 48. AFM images (topography and phase contrast, recorded in a dry state) of the grafted pOEGMA brush coatings fabricated with different polymerization times ($T_P = 5$ h, a–d; $T_P = 16$ h, e–h) prior to (a and e) and after incorporation of silver nanoparticles (b–d and f–h) – resulting from immersion for different times in 5 mM AgNO_3 solution, followed by reduction in 0.2 M NaBH_4 solution for 12 h. Cross-line analysis of topographies (i and k) and histogram analysis of phase images (j and l) recorded for pOEGMA coatings fabricated with different polymerization times ($T_P = 5$ h, i and j; $T_P = 16$ h, k and l) without (green) and with incorporated silver nanoparticles after 15 min (red), 30 min (blue) and 60 min (black) of silver immobilization.

Based on the analysis of the fabricated coatings, the most appropriate conditions for the fabrication of pOEGMA brush-coatings were concluded, that are, the optimal polymerization time for

the fabrication of the “smart” pOEGMA antibacterial coating is approximately 16 hours, and the highest possible amount of silver nanoparticles that does not block the thermal response is reached after 15 minutes of immersion in a 5 mM aqueous solution of AgNO_3 .

VI.2. Release of silver from the pOEGMA coating in aqueous media

Surfaces containing heavy metal nanoparticles have limited applications in biomedicine, as the nanoparticles could release the surface and accumulate in the body, leading to serious side effects [122–125]. It is assumed that nanoparticles incorporated into the polymer brush are strongly bound to the macromolecule and are not capable of releasing the brush followed by their accumulation in the human body [20]. To confirm this hypothesis the release of silver from the brush in water was studied employing XPS.

To do this, the concentration of silver in “as prepared” samples was first measured with XPS and then the measurements were repeated after the immersion of the samples for a certain time in water. The ratio of silver concentration (Ag) determined after immersion in water for a certain time to the initial concentration (Ag_0) of silver in the 'as prepared' samples is shown in Fig. 49. The release of silver nanoparticles has a durable character and the abundance of silver does not drop below 35% of the initial concentration even for very long incubation times (almost two months), thus suggesting good anchoring of the nanoparticles by the polymer brush.

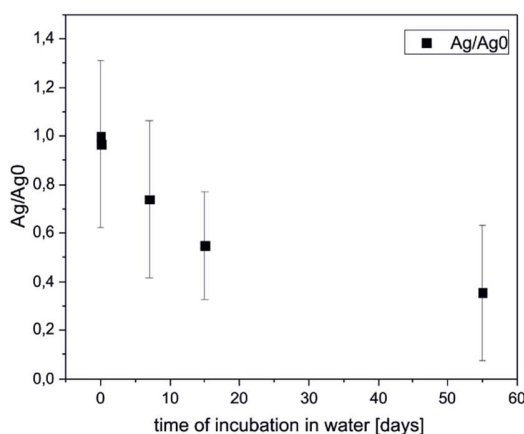


Figure 49. Silver release from pOEGMA grafted brush

VI.3. Effect of silver nanoparticles embedded in pOEGMA on human cells

To study the influence of silver nanoparticles embedded in the brush on cell viability, human skin cells HaCaT and melanoma WM35 cells were cultured on the 'pure' grafted brush coating of pOEGMA, as well as the pOEGMA brush with embedded silver nanoparticles for 24, 48 and 144 hours

(see Subsection II.3.3). The fluorescent images of the HaCaT and WM-35 labeled cells recorded after each culture time are presented in Figs. 50 and 51 respectively, giving insight into the proliferation of cells. HaCaT cells form the confluent cellular sheet on pOEGMA and pOEGMA with embedded nanoparticles after 144 and 72 hours, respectively. These data suggest that the HaCaT cells grow faster on the pOEGMA with embedded nanoparticles than on the “pure” pOEGMA. In turn, the images recorded for the melanoma cells indicate that they grow slower than the HaCaT cells.

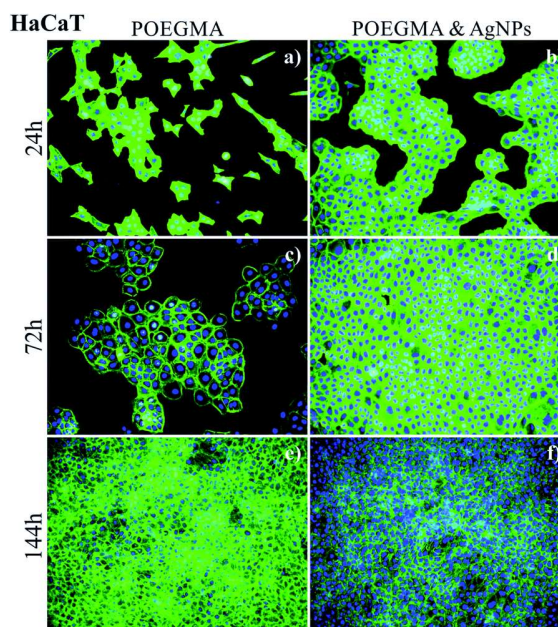


Figure 50. Growth of HaCaT cells on 'pure' pOEGMA and pOEGMA with embedded nanoparticles

However, the amount of cells on the substrate depends on two factors: (I) the number of adhered cells, and (II) their ability to proliferate. The proliferation index is the ratio of the amount of cells at a given time to the amount of adhered cells (initial amount of cells on the substrate); hence, it gives the information about the ability of the cells to proliferate independently of their initial adhesion to the substrate.

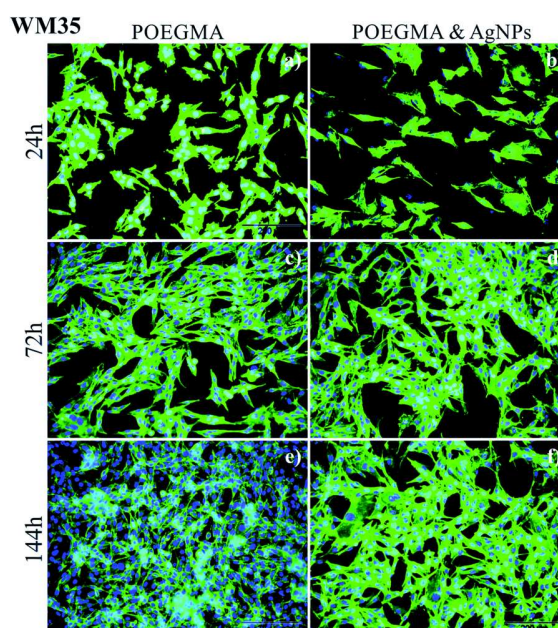


Figure 51. Growth of WM35 cells on 'pure' pOEGMA and pOEGMA with embedded nanoparticles

To determine the ability of cells to proliferate, they were counted using ImageJ software and statistical analysis was performed, followed by the calculation of proliferation indices (Fig. 52).

The proliferation indices of WM-35 cells on 'pure' pOEGMA and pOEGMA with embedded silver nanoparticles are almost comparable for 144 hours of culture. In case of HaCaT cells, the proliferation indices of the cells decrease slightly after 144 hours of culturing on the pOEGMA coating with embedded silver nanoparticles and increase significantly for the coating without them. This effect may suggest the release of silver ions from the coating, which has a slightly cytotoxic effect for long incubation times. This hypothesis is strengthened by the analysis of the amount of cells, which for both cell lines (WM35 and HaCaT) after 144 hours of culturing on pOEGMA without the silver nanoparticles starts to exceed the amount of cells on pOEGMA with silver nanoparticles.

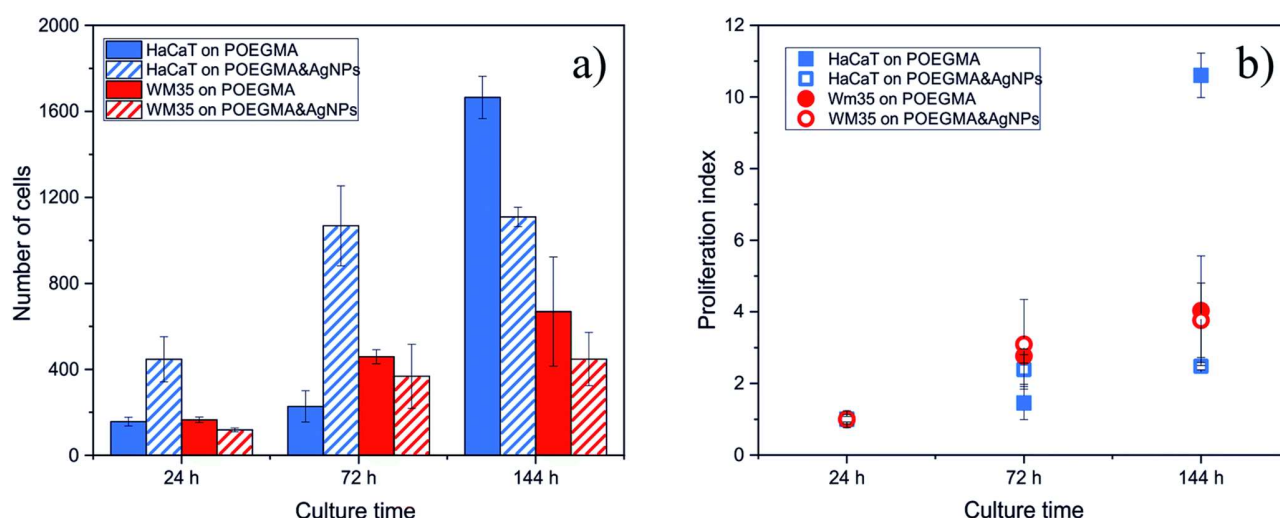


Figure 52. Impact of silver nanoparticles embedded in pOEGMA grafted brush coatings on the amount (a) and proliferation index (b) of HaCaT and WM35 cells.

The results obtained suggest that silver nanoparticles embedded in pOEGMA grafted brush affect different cell lines differently, similarly to the results reported for CaCO_3 nanoparticles in Section IV. For short exposure times, the silver nanoparticles do not show cytotoxic effect toward HaCaT cells (normal human skin cells). In turn, the number of cancer cells decreases slightly, but this effect is not conclusive enough to postulate an anticancer effect of the silver nanoparticles embedded in the brush. Furthermore, partial detachment of cells during incubation, which affects the proliferation index of the cells, cannot be excluded.

VI.4. Conclusions

The nanocomposite based on pOEGMA coating with embedded silver nanoparticles was successfully fabricated and studied with AFM, XPS, ellipsometry, and water contact angles. The temperature responsiveness of the fabricated nanocomposite turned out to be strongly dependent on the thickness of the polymer brush coating and the amount of embedded silver nanoparticles. The appropriate conditions for the fabrication of smart temperature-responsive pOEGMA with embedded silver nanoparticles were established.

The release of nanoparticles from the coating was studied with XPS in a water environment and showed that the release of nanoparticles has a prolonged character and the abundance of silver in the coating does not drop below 35% of the initial concentration after 55 days of immersion. The prolonged character of decreasing the amount of silver in the coating suggests that the full silver nanoparticles do not release the pOEGMA brush. The decrease in the amount of the silver is rather due to the release of the ions in aqueous media.

Silver nanoparticles embedded in the pOEGMA coating do not have cytotoxic effect on human skin HaCaT cells for a short exposure time (<72 hours) and may have slight but detectable influence on melanoma WM35 cells. The non-toxicity towards the human skin cells promises the human-oriented applications of fabricated coating, namely for preventing the bacteria proliferation on daily-used frequently touched surfaces such as smartphones, windows, and other glasses.

The fabricated coatings have at least three advantages. (I) Temperature-dependent antibacterial properties have great potential for applications, from medical laboratories to food packaging, where the uncontrolled growth of microorganisms at elevated temperatures is a natural but undesired process. (II) Heavy metal nanoparticles embedded in the polymer brush do not have a cytotoxic effect against normal (non-cancer) HaCaT cells for short exposure times. (III) The release of silver in an aqueous medium is a durable process, in which only silver ions are released from the brush.

VII. The calorimetric study of the interaction between proteins and smart polymer grafted brushes based on pOEGMA and pVP

When the nanoparticle is injected into the blood, it starts to interact with proteins immediately and the protein corona is formed [56,126,127]. The structure of the protein corona may be divided into two layers: soft protein corona and hard protein corona. The layer of hard protein corona is in strong contact with the nanoparticle being non-capable to release the nanoparticle. In turn, the proteins of the soft protein corona interact weaker with the nanoparticle and can be replaced with other proteins [128–130].

The representative measure of the 'hardness' of the protein corona is the free energy of adsorption derived from the binding affinity. The ITC is the remarkable technique that allows the determination of the binding affinity, enthalpy and amount of the binding sites of the protein-nanoparticle interaction. The Gibbs energy and the entropy of the interaction are then determined from the enthalpy and binding affinity. The structure of the protein corona is strongly dependent on the physicochemical properties of the nanoparticles and proteins, such as wettability, surface charge, size, and shape [55].

In this Section, pOEGMA, pVP, and three different p(OEGMA-co-VP) copolymers with different fractions of VP and OEGMA were grafted to nanosized SiO₂ nanoparticles employing ATRP (see II.1.3). The weight fraction of the polymer brushes and the chemical composition of the fabricated polymer grafted brushes were determined with TGA (see II.2.6) and XPS (see II.2.2), respectively. The size, shape, and structure of the prepared polymer grafted brushes were determined from TEM pictures (see II.2.4). The thermal trends of ξ -potential of the fabricated polymer grafted brushes were recorded with DLS (see II.2.5). The interactions between the fabricated polymer grafted brushes and the most abundant proteins in human blood (human serum albumin (HSA), immunoglobulin (IgG) and fibrinogen (Fbr)), protein mixture corresponding to 5 times diluted human plasma, and human plasma were studied with ITC below and above the transition temperature of the fabricated polymer brushes (see II.3.7). According to the results described in Sections IV and V, and in the work [28] it was assumed that the temperature transitions of the fabricated polymers are between 15-25°C.

VII.1. Fabrication of pOEGMA, pVP, and p(OEGMA-co-VP) brushes grafted to nanosized SiO₂ nanoparticles

The silica nanoparticles having an average hydrodynamic diameter of 72±17 nm were synthesized by hydrolysis of tetraethylorthosilicate (TEOS) according to the Stöber method (see II.1.3). The pOEGMA, pVP and p(OEGMA-co-VP) were grafted to fabricated SiO₂ nanoparticles following ATRP (see II.1.3). The procedure required three steps: (I) amination of the nanoparticle surface with

APTES, (II) grafting of the ATRP initiator to amino groups, and (III) ATRP of the monomers. (see Subsection II.1.3

VII.2. Characterization of the polymer-brush coated nanoparticles

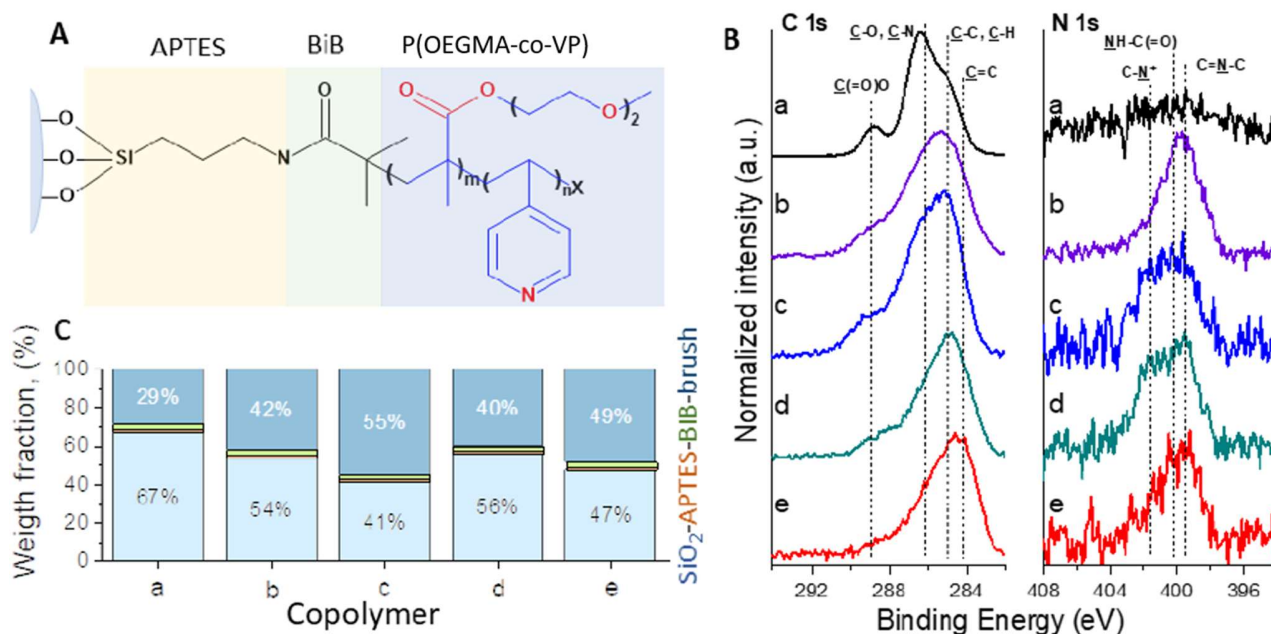


Figure 53. The composition of grafted polymer brushes. (A) p(OEGMA-co-VP) brush structure; (B) high resolution C1s and N1s core level XPS spectra of pOEGMA@SiO₂ (a), p(OEGMA-co-VP 3:1)@SiO₂ (b), p(OEGMA-co-VP 1:1)@SiO₂ (c), p(OEGMA-co-VP 1:3)@SiO₂ (d) and p(VP)@SiO₂ (e); (C) composition of SiO₂ nanoparticles with grafted polymer brushes from thermogravimetric analysis: lower (light blue) - SiO₂ core, yellow - APTES; green - BiB, upper (dark blue) - grafted brush

The chemical composition of the fabricated polymer brush coated SiO₂ nanoparticles was studied with XPS (see Fig. 53B). The fabricated nanoparticles contain hydrophilic and hydrophilic moieties marked by red and blue in Fig. 53A. When comparing the high resolution XPS spectra of homopolymers (pOEGMA and pVP) and copolymers (p(OEGMA-co-VP)), a gradual decrease in the contribution of OEGMA in copolymer p(OEGMA-co-VP) was observed mainly by decreasing the broad ethylene glycol C-O band at 286.2 eV and the ester C(=O)-O band at 289.0 eV (see Fig. 53B) [131,132]. The expected fractions of OEGMA and VP were theoretically calculated using the Mayo-Lewis equation and are in good agreement with the concentrations of VP determined from XPS (see Table 4).

Table 4. Composition of the polymer brushes

Sample	[OEGMA]	[VP]	$f_{[OEGMA]}$	$f_{[VP]}$	Calculated molar fraction in copolymer		Experimental molar fraction (XPS)	
					F_{DEGM_A}	F_{VP}	F_{OEGMA}	F_{VP}
P(OEGMA)@SiO ₂	2.17	0	1	0	1	0	1	0
p(OEGMA-co-VP 3:1)@SiO ₂	1.62	0.52	0.76	0.24	0.56	0.44	0.63	0.37
p(OEGMA-co-VP 1:1)@SiO ₂	1.12	1.13	0.50	0.50	0.37	0.63	0.59	0.41
p(OEGMA-co-VP 1:3)@SiO ₂	0.63	1.75	0.26	0.77	0.22	0.78	0.37	0.63
p(VP)@ SiO ₂	0	2.52	0	1	0	1	0	1

Moreover, the TGA analysis performed revealed that the weight fraction of the polymer brushes varies from 29% to 56% of the total weight of the sample (see Fig. 53c).

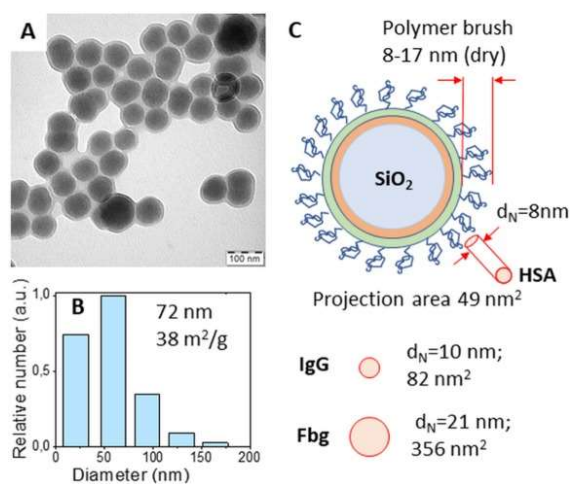


Figure 54. The morphology of SiO₂ nanoparticles with grafted polymer brushes. (a) TEM micrograph; (b) number weighted size distribution of SiO₂ cores (TEM); (c) schematic comparison of the dry polymer shell thickness on SiO₂ nanoparticles with hydrodynamic diameters of proteins and the area of their projection on a flat surface.

TEM analysis (see Section II.2.4) revealed that fabricated SiO₂ nanoparticles have a spherical shape and an approximate size of 72 nm (Fig. 54a,b). The nanoparticles are surrounded with a bright hollow, that is, the polymer-brush coating having the approximate thickness of 7- 17 nm (see Subsection II.2.6 and Fig. 54a). The thickness of the polymer brush is comparable to the diameter of the proteins (Fig. 54C).

Both, the polymer brush coated SiO₂ nanoparticles and the proteins demonstrate negative surface charges in milliQ water and phosphate buffer (pH 7.4) at temperatures 10 - 40 °C in terms of ζ -potential (see Subsection II.2.5 and Fig. 55). The ζ -potential of pOEGMA and p(OEGMA-co-VP) in water is akin to sigmoid with a deflection point around of 22°C in water due to LCST. In turn, ζ -potential of pVP increased around 20 °C due to transition mimicking LCST [28].

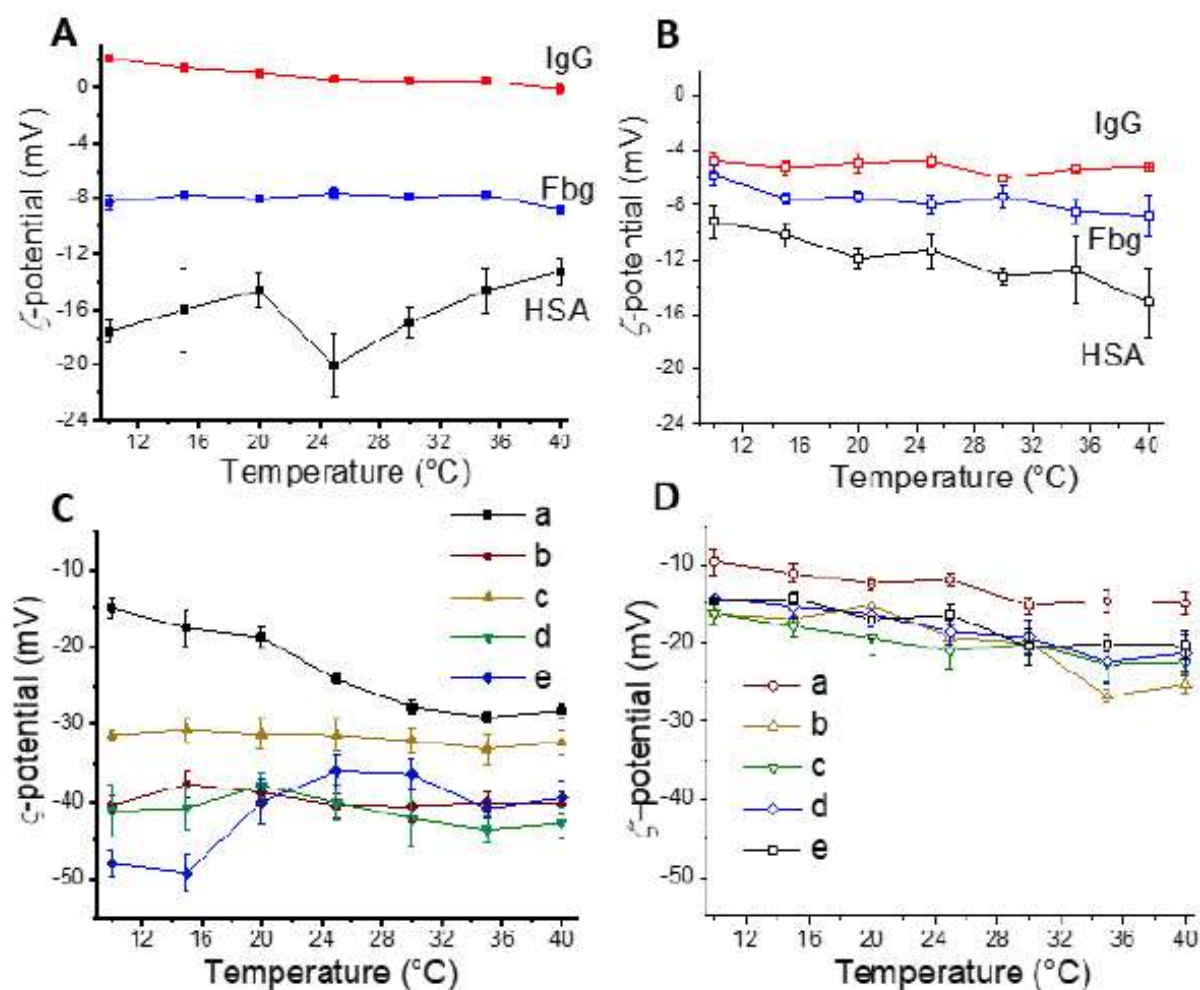


Figure 55. Evolution with temperature of ζ -potential of proteins HSA, IgG, and Fbg (A,B) and nanoparticles p(OEGMA)@SiO₂ (a), p(OEGMA-co-VP 3:1)@SiO₂ (b), p(OEGMA-co-VP 1:1)@SiO₂ (c), p(OEGMA-co-VP 1:3)@SiO₂ (d) and p(VP)@SiO₂ (e) (C,D), determined in MilliQ water (A,C) and PB (B,D)

VII.3. The interaction between proteins and a smart polymer brush grafted to SiO₂ nanoparticles

In Fig. 56 two representative calorimetric titrations are depicted as heat flux ($\mu\text{J}\cdot\text{s}^{-1}$) vs time (min): the endothermic interaction of p(OEGMA-co-VP 3:1)@SiO₂ with fibrinogen (Fbg) at 10 °C (A) and the exothermic interaction of p(OEGMA-co-VP 1:3)@SiO₂ with HSA at 37 °C (B).

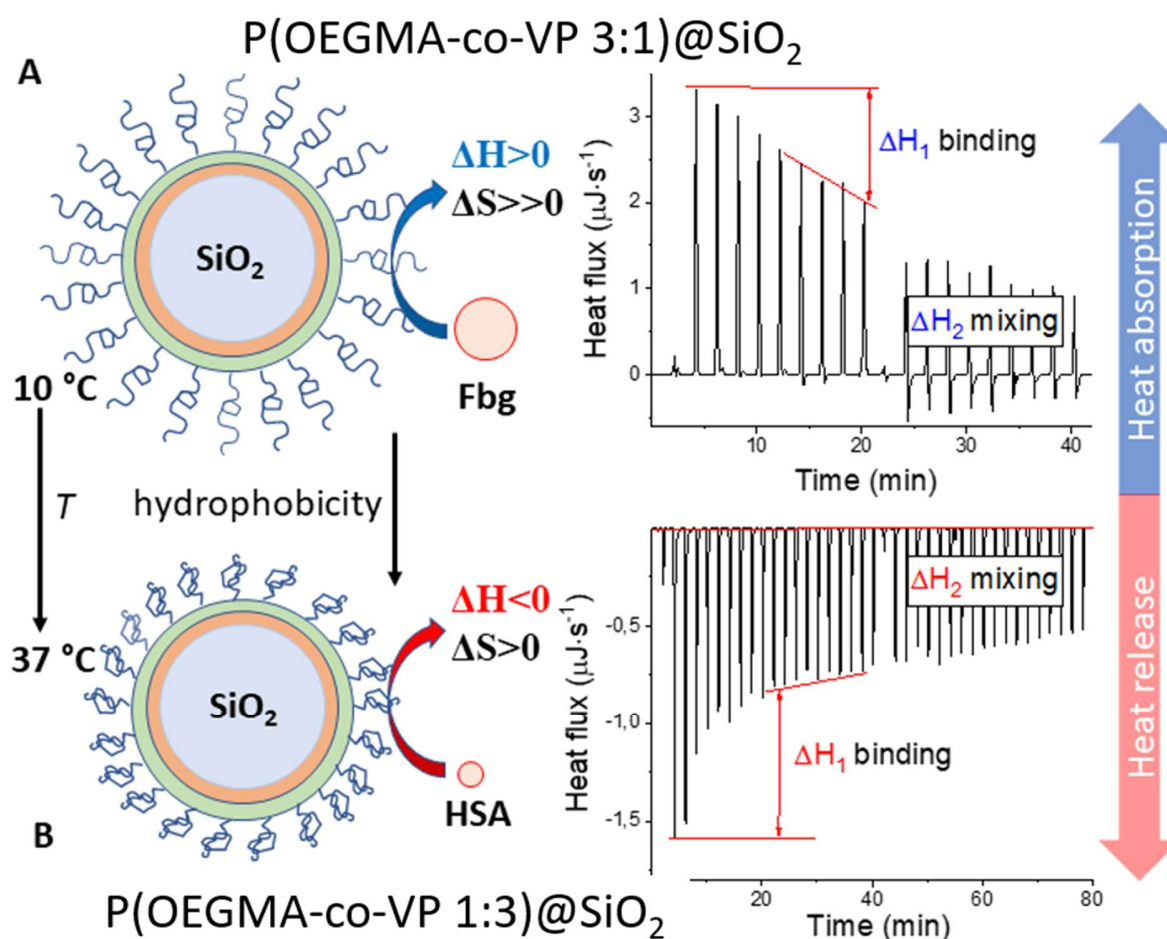


Figure 56. Representative ITC curves of the titration of proteins to nanoparticles with grafted thermoresponsive brushes performed at different temperatures: (A) Fbg adsorbing to p(OEGMA-co-VP 3:1)@SiO₂ at 10 °C and (B) HSA adsorbing to p(OEGMA-co-VP 1:3)@SiO₂ at 37 °C. Endothermic and exothermic ITC curves reveal entropy-driven ($T\Delta S > 0$, $\Delta H > 0$) and enthalpy-driven ($\Delta H < 0$) interactions attributed to polar and hydrophobic associations, respectively.

Titration with solutions of different proteins that had similar mass concentrations yielded comparable heat flux amplitudes, which were lower than most reported elsewhere [133–135]. The heat flux trends sharply to the baseline at the beginning of titration, pointing to the binding event between the protein and the nanoparticle. After the binding is complete, still significant heat flux slowly trending to

baseline is observed. A similar effect in the work of Gal et al. [133] resulted in poor fitting of ITC isotherms with a 'one binding site' model, which requires the heat flux to disappear to a baseline after binding is complete. The successful attempt to fit similar ITC isotherms with the “two independent binding sites” model was realized by de Castro et al [136]. In the present work, the isotherms were fitted with the model of “two independent binding sites”.

The enthalpy changes, obtained from the ITC experiments, were in the range 10^0 – 10^1 kJ·mol⁻¹ (except Fbg with molecular weight of 338 kDa and ΔH in the range of 10^2 kJ·mol⁻¹), in the same range as most of the enthalpy changes reported elsewhere, obtained by the ITC [54] and from the van 't Hoff analysis of K_a versus T dependencies for the adsorption of proteins on nanoparticles [133,136]. The interaction between proteins and particles occurs against Coulomb forces as their ξ -potentials are negative (except the negligible positive charge of IgG in water, Fig. 55). In the absence of Coulomb attractive forces, proteins and particles will interact either by hydrophobic or polar association. Hydrophobic interaction is often discussed as entropy driven [137], according to the hypothesis that the water is structured near the hydrophobic surfaces and the release of this structured water to the bulk after the association of the hydrophobic surfaces contributes to the gain in entropy. However, neutron scattering studies did not reveal any difference for water on the hydrophobic surface and in bulk [61]. But the water near the hydrophobic surface possesses hydrogen bonding capacity and can recombine after the association of the hydrophobic surfaces with the release of ~ 21 kJ·mol⁻¹. Therefore, the hydrophobic interaction is observed in ITC as an exothermic (enthalpic) process with an arbitrary value of ΔS . The polar surfaces are already hydrated and their association is accompanied by the release of water from the hydration shell, which contributes to entropy gain ($\Delta S > 0$). But the amount of new hydrogen bonds is comparable to the number of broken hydrogen bonds and the ΔH for the polar association is often low, either positive or negative. The case with $\Delta H < 0$ is indistinguishable from the hydrophobic association, and the case of $\Delta H \approx 0$ is not measurable with ITC. Only the endothermic process accompanied by an entropy gain ($\Delta H > 0$, $\Delta S > 0$) can be attributed to the polar (entropic) association.

The interaction of HSA with polymer brushes on the surface of SiO₂ nanoparticles, was endothermic for pOEGMA at both 10 and 37 °C ($\Delta H > 0$ and $\Delta S > 0$, Fig. 57a and B, Table 5). The HSA is adsorbed on pOEGMA most probably by entropy-driven hydrogen bonding as the adsorption is stronger below LCST, with K_a 5×10^5 M⁻¹ for 10 °C, where the pOEGMA is in the extended coil state, than above LCST with K_a 5×10^4 M⁻¹. The interaction of HSA with p(OEGMA-co-VP) and pVP is exothermic and is also accompanied by the entropy gain at both temperatures. At 10 °C, K_a is as low as 10^5 M⁻¹, but at 37 °C K_a increases from 10^6 to 10^7 M⁻¹ with an increased fraction of VP, so it can be concluded that HSA is bound by hydrophobic interactions with pVP fragments. In consequence, neither

pOEGMA nor pVP possess the non-fouling property toward HSA, as it is adsorbed to both by a different mechanism. However, the adsorption on pVP containing copolymers at 10 °C (below LCST for both polymers) is weaker in terms of K_a .

IgG at 10 °C is exothermically interacting with $K_a 6.5 \times 10^6 \text{ M}^{-1}$ only with the pVP homopolymer (Fig. 57c, Table 5). At 37 °C the interaction of IgG with p(OEGMA-co-VP) and pVP homopolymer brushes takes place in the same manner ($\Delta H < 0$, $\Delta S > 0$) as for HSA, with K_a ranging from 7.9×10^5 to $2.5 \times 10^6 \text{ M}^{-1}$ for copolymers and $4.2 \times 10^7 \text{ M}^{-1}$ for pVP homopolymer. The IgG is binding by a hydrophobic exothermic mechanism to the pVP and its copolymers with OEGMA above LCST. The pOEGMA homopolymer is, in turn, repellent for IgG at 10 and 37 °C even despite its low positive ξ - potential.

The interaction of Fbg with polymer brushes was endothermic, except pVP homopolymer at 10 °C, and demonstrated little dependence on temperature (Fig. 57, e and f). The surface coated with the pOEGMA homopolymer is non-fouling with Fbg below and above LCST, but p(OEGMA-co-VP) readily adsorbs Fbg by the entropy-driven process. Similarly, for the other proteins studied, the affinity of Fbg in terms of K_a is stronger than for brushes with higher content of VP (Table 5).

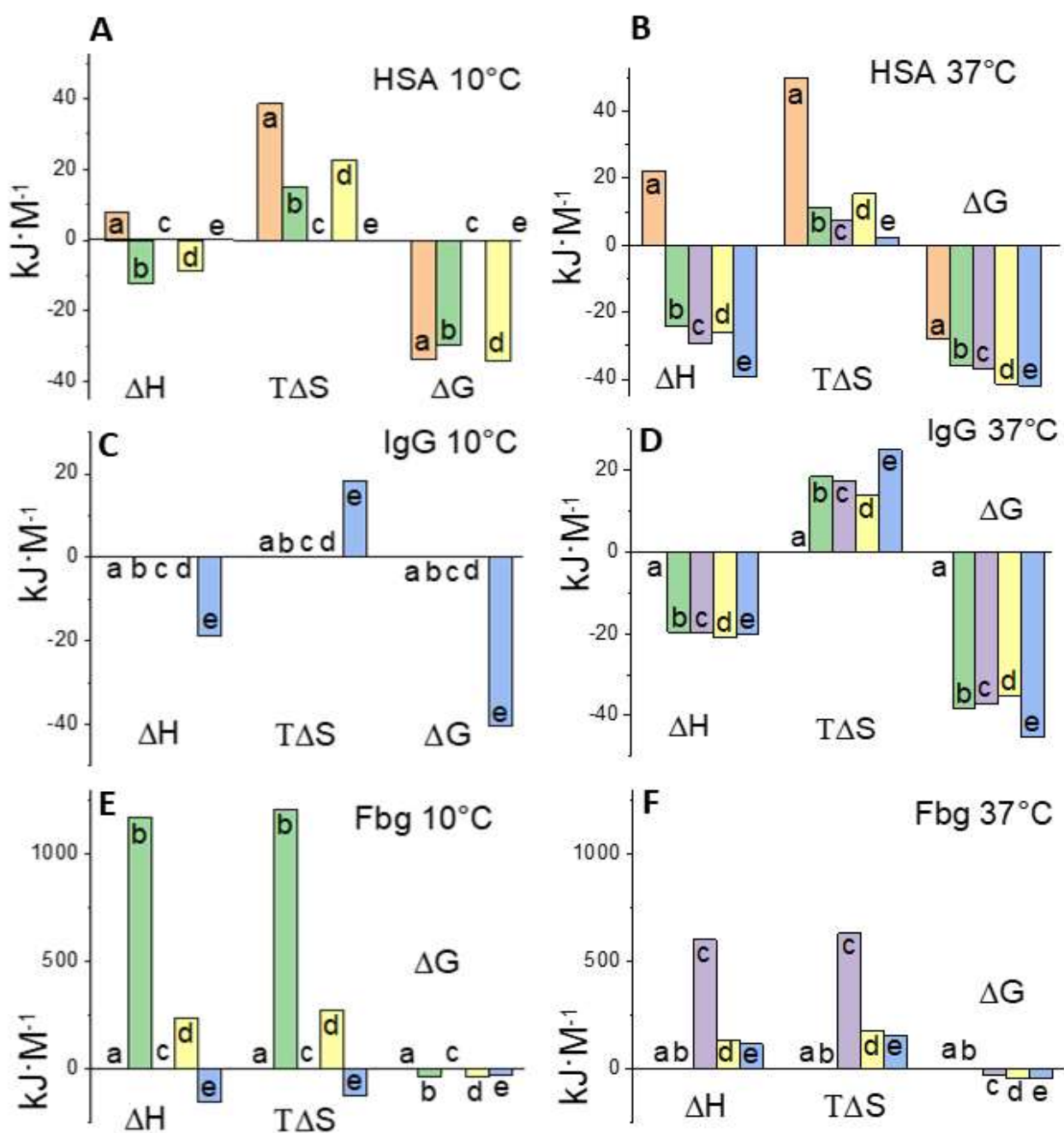


Figure 57. Thermodynamic parameters determined from ITC for HSA (A, B), IgG (C, D) and Fbg (E, F) adsorbing at 10°C (left) and 37°C (right) to p(OEGMA)@SiO₂ (a), p(OEGMA-co-VP 3:1)@SiO₂ (b), p(OEGMA-co-VP 1:1)@SiO₂ (c), p(OEGMA-co-VP 1:3)@SiO₂ (d), pVP@SiO₂ (e) nanoparticles. The bars mark the values of enthalpy (ΔH), entropy ($T\Delta S$) and binding free energy (ΔG)

Table 5. The parameters of interaction between the proteins and the polymer brush coated SiO₂ nanoparticles

Temperature	10 ⁰ C					37 ⁰ C			
Protein	Polymer	K _a , M ⁻¹	ΔH, M ⁻¹	ΔS cal* M ⁻¹ *K ⁻¹	[Binding sites], mM	K _a M ⁻¹	ΔH, M ⁻¹	ΔS cal* M ⁻¹ *K ⁻¹	[Binding sites], mM
HSA	pOEGMA	4.8E5	1.9E3	32.6	0.011	5.0E4	5.3E3	38.6	0.080
	p(OEGMA-co-VP) 3:1	1.0E5	-2.9E3	12.7	0.072	1.06E6	-5.8E3	8.83	0.020
	p(OEGMA-co-VP) 1:1	0	0	0	0	1.65E6	-7.0E4	5.76	0.015
	p(OEGMA-co-VP) 1:3	5.8E5	-2.0E3	19.2	0.017	9.57E6	-6.2E3	11.9	0.011
	pVP	0	0	0	0	1.1E7	-9.4E3	1.88	0.080
IgG	pOEGMA	0	0	0	0	0	0	0	0
	p(OEGMA-co-VP) 3:1	0	0	0	0	2.5E6	-4.7E3	14.2	0.006
	p(OEGMA-co-VP) 1:1	0	0	0	0	1.8E6	-4.7E3	13.4	0.006
	p(OEGMA-co-VP) 1:3	0	0	0	0	7.9E5	-5.0E3	10.8	0.008
	pVP	6.49	-18.7	64.4	0.003	4.2E7	-4.8E3	19.3	0.003
Fbg	pOEGMA	0	0	0	0	0	0	0	0
	p(OEGMA-co-VP) 3:1	3.4E6	2.8E5	1.2E3	0.003	0	0	0	0
	p(OEGMA-co-VP) 1:1	0	0	0	0	5.0E4	1.4E5	486	0.003
	p(OEGMA-co-VP) 1:3	5.3E6	5.7E4	230	0.003	3.93E6	3.3E4	135	0.003
	pVP	5.4E5	-3.7	-104	0.003	5.2E6	2.8E4	120	0.003

For three proteins studied, the adsorption mechanisms were changing from entropic to enthalpic with increasing VP content (Lewis base) and increasing surface hydrophobicity, promoted by the elevation of the temperature above LCST for thermoresponsive grafted brushes. The smaller globular

proteins HSA and IgG demonstrated affinity for diverse surfaces, while the larger glycoprotein Fbg was mostly inert. Seemingly, for efficient adsorption of Fbg by entropic polar association, the amphiphilic surface of p(OEGMA-co-VP) is preferable over sole pOEGMA or pVP. The concentrations of the binding sites, derived from the fits, were in the same range with those estimated for HSA and Fbg and lower than those predicted for IgG.

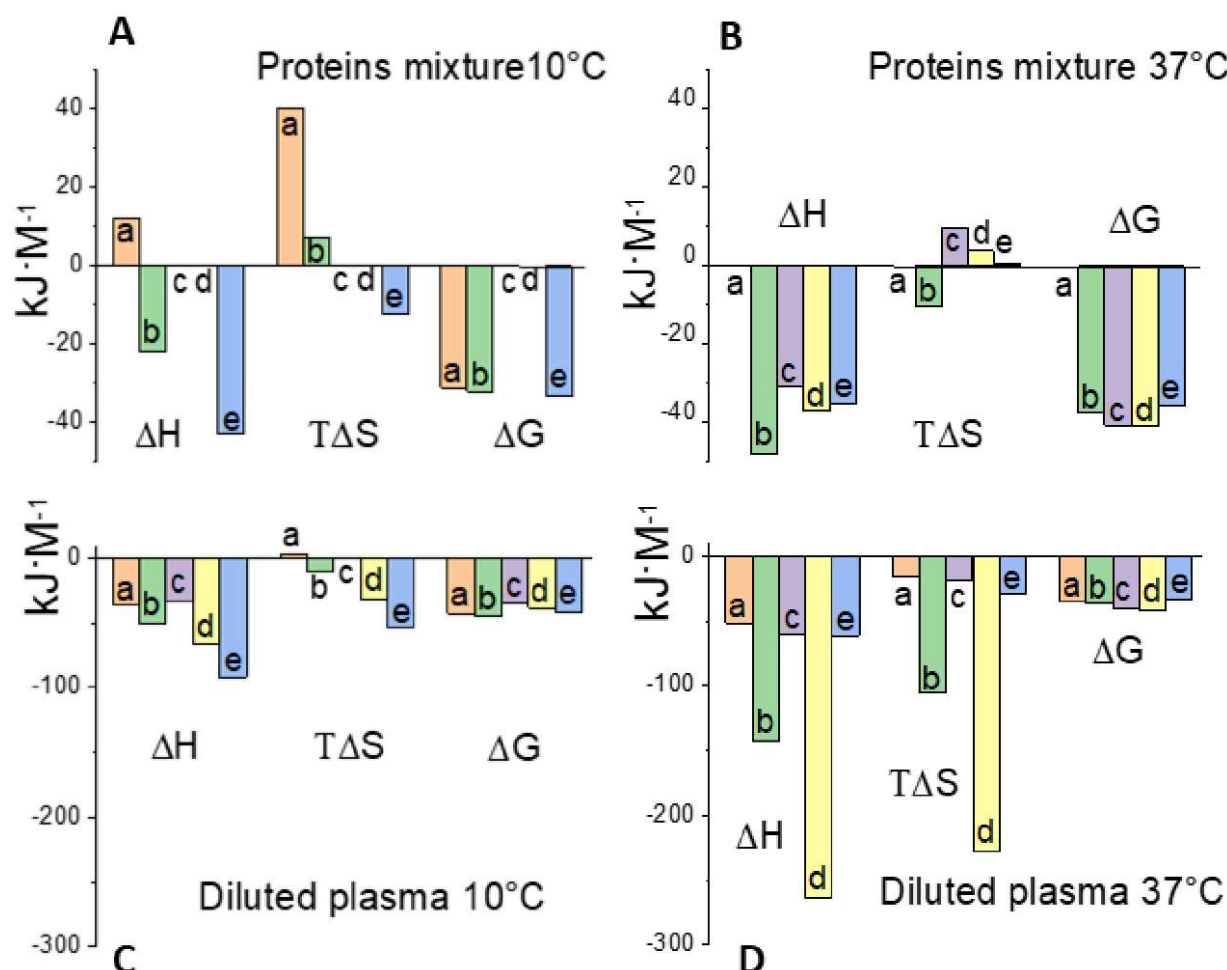


Figure 57. Thermodynamic parameters determined from ITC for proteins mixture (A, B) and diluted plasma (C, D) adsorbing at 10°C (left) and 37°C (right) to p(DEGMA)@SiO₂ (a), p(DEGMA-co-VP 3:1)@SiO₂ (b), p(OEGMA-co-VP 1:1)@SiO₂ (c), p(OEGMA-co-VP 1:3)@SiO₂ (d), pVP@ SiO₂ (e) nanoparticles. Groups of bars mark the values of enthalpy (ΔH), entropy (TΔS) and binding free energy (ΔG).

The mixture of three proteins corresponding to five times diluted blood plasma (HSA – 0.135 mM; IgG 16 μM, Fbg – 2 μM) was titrated in the SiO₂ nanoparticles with grafted polymer brushes. The titration isotherms were normalized to the sum of protein concentrations (0.153 mM). Under competitive

adsorption conditions, the use of total protein concentration is an approximation, which generates the systematic uncertainty in all thermodynamic parameters. The sign of adsorption enthalpy is then the only parameter, which is not affected by the uncertainty in concentrations, the amplitudes of ΔH , the values of K_a and the derived ΔG shall be compared only within the series of similar titrations. Adsorption of the protein mixture on the grafted brushes demonstrated 'HSA-like' behavior – exothermic binding to VP containing polymer brushes and endothermic binding to pOEGMA below LCST (Fig. 57, a and b, Table 6). The K_a increased at a higher VP fraction. Therefore, it can be reasonably suggested that, under given competitive conditions, the adsorption of HSA by hydrophobic forces dominates, because of its higher concentration in the mixture.

Table 6. Thermodynamic parameters of the titration of protein mixture

Sample	°C	[Binding sites], mM	$K_a, \times 10^6$, M^{-1}	ΔG , $kJ \cdot M^{-1}$	ΔH , $kJ \cdot M^{-1}$	ΔS^2 , $J \cdot M^{-1} \cdot K^{-1}$
pOEGMA	10	0.015	0.17	-28.3	11.8	141.8
	37	0	0	0	0	0
P(OEGMA-co-VP) 3:1	10	0.028	0.25	-29.3	-21.9	26.1
	37	0.003	1.97	-37.4	-47.7	-33.2
P(OEGMA-co-VP) 1:1	10	0	0	0	0	0
	37	0.003	6.56	-40.5	-31.0	30.7
P(OEGMA-co-VP) 1:3	10	0	0	0	0	0
	37	0.003	6.98	-40.6	-36.9	12.0
pVP	10	0.002	0.40	-33.3	-42.7	-42.7
	37	0.015	1.01	-35.7	-35.1	1.7

Human blood plasma is stabilized against coagulation with sodium citrate, which shifts the pH to 8.1. The ITC with undiluted plasma yields massive heat flux from pH mismatch, which cannot be compensated and subtracted from blank titrations of plasma to buffer. Therefore, plasma was diluted to 20% vol/vol with 200 mM phosphate buffer (PB). For the ITC of SiO_2 particles with grafted brushes, the plasma concentration was assumed the same as for the protein mixture (0.153 mM) and similar restrictions for the thermodynamic parameters obtained were applied. The initial concentration of the

² The values of ΔS were not used in discussion, as affected by uncertainties in concentrations

binding sites was accepted the same as that for HSA (0.074 mM) as the majority of the blood proteins are of comparable size and molecular weight. The ITC of the grafted polymer brushes showed the exothermic interaction for all polymer brush samples (Fig. 57. c and d) below and above their LCST. The amplitudes of heat flux are two to four times higher than those for ITC with protein mixture, most probably due to the higher fraction of interacting proteins in plasma.

Table 7. Thermodynamic parameters of the titration of diluted plasma (20% vol/vol) to SiO₂ nanoparticles with grafted polymer brushes

Sample	°C	[Binding sites], mM	$K_a \times 10^6$, $\cdot M^{-1}$	ΔG , $kJ \cdot M^{-1}$	ΔH , $kJ \cdot M^{-1}$	ΔS , $J \cdot M^{-1} \cdot K^{-1}$
POEGMA	10	0.003	15.3	-38.9	-36.7	7.9
	37	0.015	0.74	-34.9	-51.5	-5356
P(OEGMA-co-VP) 3:1	10	0.003	29.5	-4.7	-50.2	-34.3
	37	0.016	1.61	-37.1	-143.1	-341.8
P(OEGMA-co-VP)1:1	10	0.003	0.82	-32.1	-32.9	-2.9
	37	0.015	7.76	-40.8	-60.3	-62.8
P(OEGMA-co-VP)1:3	10	0.003	2.93	-34.5	-66.9	-114.6
	37	0.015	9.93	-35.7	-264.0	-736.4
PVP	10	0.002	13.9	-38.8	-92.1	-188.9
	37	0.015	0.36	-33.0	-61.5	-92.0

Under competitive adsorption conditions, hypothetically, the hydrophobic (exothermic) interactions contributed the most to the heat flux. The polymer brushes, non-fouling with individual model proteins are readily adsorbing the unknown proteins from blood plasma. The significant observable difference between plasma and artificial protein mixture is that the affinity of proteins in plasma to the polymer brushes is higher at 10 °C in comparison with 37 °C (Table 7 and Table 6). The explanation of this phenomenon from sole ITC data is not feasible. Also, the concentrations of binding sites that satisfied the 1:1 stoichiometry were found to be lower in one order, than suggested below LCST but lower only 4 times above LCST. Despite uncertainties in concentrations of titrant and analyte, the

ITC gives the evidence that below the LCST, only a small amount of plasma proteins with high affinity are adsorbed, whereas above the LCST the amount of adsorbed proteins increases.

VII.4. Conclusions

Two homopolymers pOEGMA and pVP, and three p(OEGMA-co-VP) copolymers with different content of pVP were successfully grafted to nanosized SiO₂ nanoparticles and studied with XPS, TEM and DLS. XPS confirmed the expected chemical composition of the brush coatings, TEM showed expected nanosized spherical core-shell nanoparticles, and DLS in ξ -sizer regime detected the negative ξ -potential of the coatings.

Amphiphilic non-ionic polymer brushes and blood proteins both demonstrate the negative charge in phosphate buffer and interact against Coulomb forces by hydrophobic or hydrophilic association. The type of interaction can be distinguished by ITC from the sign of enthalpy change: $\Delta H < 0$ for hydrophobic and $\Delta H > 0$ for hydrophilic association, respectively. For the individual proteins HSA, IgG, and Fbg, the presence of VP fragments in polymer brushes favored adsorption below and above T_c. The OEGMA fragments, in turn, reduced (HSA) or entirely blocked (IgG and Fbg) adsorption. OEGMA containing polymers demonstrated LCST-dependent affinity to proteins in terms of K_a, and the p(OEGMA-co-VP) grafted brushes possess switchable high/low fouling properties towards studied model proteins. The proteins from blood plasma are adsorbing on the polymer brushes which showed non-fouling properties toward three individual proteins.

Summary

This thesis is focused on the development and characterization of the novel smart polymer brush coatings for biomedical applications, namely, for temperature-controlled bacteria killing, temperature-controlled protein adsorption, and cell culturing as well as the basic studies of polymer-protein interactions. In a frame of this work, the following results were reached:

1. It was shown that p(OEGMA-co-VP) brush provides appropriate conditions for the culture of cow granulosa cells and the development of oocyte-cumulus complexes.
2. The CaCO_3 nanoparticles were successfully embedded into pOEGMA brush and their influence on human cells was studied. It was shown that the influence of CaCO_3 nanoparticles embedded in the pOEGMA brush is strongly cell-dependent.
3. Smart antibacterial nanocomposites built on pOEGMA and pVP brushes containing embedded silver nanoparticles capable of the 'kill-release' strategy were successfully fabricated. The antibacterial activity of the nanocomposite was tested against the representatives of Gram-positive and Gram-negative bacteria. The fabricated nanocomposites may be employed in broad spectra of applications, starting from medical laboratories through food packaging and finishing in air conditioning.
4. The most appropriate conditions for the fabrication of pOEGMA brush-coatings with embedded silver nanoparticles were concluded, that are, the optimal polymerization time for the fabrication of the "smart" pOEGMA antibacterial coating is approximately 16 hours, and the highest possible amount of silver nanoparticles that does not block the thermal response is reached after 15 minutes of immersion in a 5 mM aqueous solution of AgNO_3 followed by 12 hours of reducing by NaBH_4 .
5. It was shown that the release of silver nanoparticles embedded in the pOEGMA brush has a prolonged character in the water environment, and, hence, they are strongly bound being non-cytotoxic against human skin HaCaT cell line for short exposure time. The non-cytotoxic effect of the nanocomposite makes it attractive for bacteria killing on daily frequently touched surfaces.
6. The smart pOEGMA, pVP, and p(OEGMA-co-VP) brushes were successfully grafted to nanosized SiO_2 nanoparticles. Interactions between grafted smart polymer brushes and the most abundant proteins in human blood were studied with isothermal titration calorimetry, and it was shown that the proteins interact with the polymer brushes against the Coulomb forces via hydrophobic enthalpy- and polar entropy-driven associations. Below LCST, pOEGMA and p(OEGMA-co-VP) were antifouling against human immune globulin; above LCST pOEGMA and p(OEGMA-co-VP) brushes interacted with immune globulin via hydrophobic enthalpy-

driven associations. Human serum albumin interacted with pOEGMA, pVP, and p(OEGMA-co-VP) below and above LCST. The interaction between human serum albumin and grafted brushes was stronger above LCST than below in terms of binding affinity. Human fibrinogen interacted with brushes via associations driven by polar entropy. The LCST of the brushes demonstrated only a weak influence on the affinity of human fibrinogen to the brushes. The interaction between the polymer brushes and human serum shown the 'HSA-like' interaction. The interaction between fabricated smart polymer brushes grafted to SiO₂ nanoparticles with human plasma was realized with quite higher enthalpy than the interaction between the same polymer brushes and mixture proteins corresponding to human plasma. It was assumed that in human plasma are other objects that interact with the brushes.

List of abbreviations

AFM - atomic force microscopy

Amytal - amytal-5-ethyl-5-isoamyl-barbituric acid

APTES - (3-aminopropyl) triethoxysilane

ATP - adenosine 5'-triphosphate

ATRP - atom transfer radical polymerization

BDDNWs - boron-doped diamond nanowires

BSE - backscattered electrons

DLS - dynamic light scattering

DSC - differential scanning calorimetry

EDTA - ethylenediaminetetraacetic acid tetrasodium salt dihydrate

EDX - energy dispersive x-ray analysis

Fbg - fibrinogen

FN - fibronectin

HSA – human serum albumin

IBB - 2-isobutyryl bromide

IgG – immunoglobulin

ITC - isothermal titration calorimetry

LC - liquid crystal

LCST - lower critical solution temperature

Na₂EDTA - disodium ethylenediaminetetraacetic

NMP - nitroxide mediated polymerization

NMR - nuclear magnetic resonance spectrometry

OEGMA - oligo(ethylene glycol) methyl ether methacrylate

PB - phosphate buffer

PBMA - poly(butyl methacrylate)

PCB - poly(carboxybetaine)

PCL - polycaprolactone

PDS - poly(dioxanone)

PGA - poly(glycolic acid)

PHB - polyhydroxybutyrate

PHBV - poly(hydroxybutyratevalerate)

PHV - poly(hydroxyvalerate)

PLA - poly(lactic acid)

PLGA - poly(lactide-co-glycolic acid)

PNIPAM - poly(N-isopropylacrylamide)

POEGMA - poly(oligo(ethylene glycol) methyl ether methacrylate)

PPB - poly(phosphataine)

PSB - poly(sulfobetaine)

PU - polyurethanes

PVP - poly(4-vinyl pyridine)

RAFT - reversible addition-fragmentation chain-transfer polymerization

RMS - root mean square

SE - secondary electrons

SEM - scanning electron microscopy

SPB- smart polymer brush

SPR - surface plasmon's resonance

TEM - transmission electron microscopy

TEOS - triethylorthosilicate

T_g - glass-rubber transition temperature

TGA - thermogravimetric analysis

THF - tetrahydrofuran

ToF-SIMS - time-of-flight-secondary ions spectrometry

VN - vitronectin

VP - 4-vinyl pyridine

XPS - x-ray photoelectron spectrometry

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