Physicochem. Probl. Miner. Process., 55(6), 2019, 1535-1548 | Physicochemical Problems of Mineral Processing

http://www.journalssystem.com/ppmp

ISSN 1643-1049

Received July 29, 2019; reviewed; accepted October 17, 2019

Effect of chitosan, hyaluronic acid and/or titanium dioxide on the physicochemical characteristic of phospholipid film/glass surface

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Abstract: The production of preparations, whose destination action takes place in close proximity to living cells, increases the necessity to carry out studies concerning the determination of the biomaterial surface effect on the cellular response. In achieving this goal, physicochemical characteristic of the surface can be helpful. This can be established based on topography, chemical composition, wettability, and surface energy analysis. In addition, determining the changes of these properties which can occur as a result of surface modification will allow prediction of cell behaviour when contacting with biomaterial. In the study, the Langmuir-Blodgett technique was used. It enabled the transfer of the Langmuir monolayer formed from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) to a solid support. The DPPC film imitated a natural biological membrane capable of interacting with the components of the liquid subphase: chitosan (Ch), hyaluronic acid (HA) and/or titanium dioxide (TiO_2). Depending on the type and strength of interactions of phospholipid molecules with the components of the subphase, the films obtained on the solid support were characterized by specific surface properties. Their characteristics based mainly on values of the work of adhesion in connection with films topography, allowed for statement that it is possible to form semi-interpenetrating Ch network in which HA is entrapped, contributing to the enhanced adhesion of the DPPC film, additionally intensified by TiO_2 inclusion. This type of research permit for better understanding of the interactions at the interface, cell membrane-Ch/HA/TiO_2 and can be important in the creation of a new generation of skin or tissue substitutes.

Keywords: chitosan, titanium dioxide, hyaluronic acid, phospholipid, adhesion, topography

1. Introduction

Biomaterials can be used to facilitate wound healing or as implants that restore the body's functionality. Biocompatibility, biosafety and interactions between the implant surface and cells in the environment play an important role in their design (Thevenot et al., 2008; Ulusan et al., 2019).

In the group of materials with potential application as skin substitutes, there are mainly substances of natural origin, such as various types of proteins, peptides (collagen, fibrin) and polysaccharides. These substances contain numerous groups that allow their modification. They can polymerize to form interpenetrating polymer networks (IPNs) with desirable properties. Such 3D network formed with two or more polymers of micropatterned structures closely mimic the heterogeneous architecture of native tissues (Tiwari and Bahadur, 2019). These physically or chemically cross-linked networks share the properties of each polymer and thus, properties of the biomaterial can be regulated by varying the individual component. The extraordinary simplicity of processing allows the production of preparations in different forms, such as gels, films, fibers, which exhibit a number of additional, specific physicochemical properties that make them suitable for various applications, e.g. biomedical. For example, hydrogels are an ideal "scaffold" for cells that can be tuned to allow long-term cell proliferation and survival (Tiwari and Bahadur, 2019).

DOI: 10.5277/ppmp19081

Scaffolds for the extracellular matrix (ECM) are also created primarily from porous, biocompatible and biodegradable materials that serve to provide the right environment for optimal cell growth and function. Porosity has a direct impact on their functionality during biomedical applications, e.g. in reconstructive surgery or skin substitutes. A good biomaterial that ideally maps the ECM structure must have appropriate physical and chemical properties. First of all, it should be characterized by a suitable texture because the shape of the implant (tissue) affects the physiological reaction of the body. The scaffolding architecture also directly influences the behaviour of the cells. This is due to the fact that the ECM provides signals directly deciding on specific interactions between the cells and the environment. It is also important to adjust the rate of matrix degradation, because if it is too slow, it can cause disturbances in cell migration and proliferation as well as nutrient and oxygen deficiencies in the developing tissue. However, if it is too fast, it may disrupt the mechanical and structural integrity of the implant and as a result lead to incomplete development of the growing tissue. Consequently, both cases lead to inhibition of regeneration process as a result of mismatches in the tissue growth rate and scaffold degradation (Loh and Choong, 2013).

Nanocomposite scaffolds based on polysaccharides have gained wider application in medicine in the field of drug delivery, tissue engineering and wound healing (Mohandas et al., 2018; Dodero et al., 2019). The most popular chitosan (Ch) is a derivative of the chitin and is obtained as a result of its chemical deacetylation. It is a subject of many studies, due to the chemically attractive structure containing numerous hydroxyl and amine groups. The number of $-NH_3^+$ groups is determined by a parameter called the deacetylation degree (*DD*). The *DD* value is also a criterion for distinguishing chitosan from chitin. Namely, substances with $DD<50\%$ are classified as chitin, while its derivative has $DD>50\%$, because this level of free amino groups allows for solubility in acidic aqueous solutions. In addition, it has a number of unique features, such as biocompatibility, biodegradability, non-toxicity – promising for biomedical applications, including skin tissue engineering. It has been reported that chitin and chitosan degradation products can help regenerate the wounded area by proliferating fibroblasts (Mohandas et al., 2018). Application similar to that of Ch is demonstrated by hyaluronic acid (HA), both are polyelectrolytes. HA has hydroxyl and carboxyl groups (Jooybar et al., 2019) which presence allows to produce internal hydrogen bonds and to ease of functionalization (Dodero et al., 2019). High molecular weight HA consists of repeated *N*-acetylglucosamine and glucuronic acid connected with β-1,4 and β-1,3 glycosidic linkages arranged alternately. As a result, it takes the form of a rigid chain in the aqueous environment (Dodero et al., 2019). HA molecules bind strongly to the water molecules and are highly hydrated, thus forming the viscoelastic gel (Tiwari and Bahadur, 2019). The HA-based hydrogels can map the extracellular matrix. The HA presence in the ECM where it is involved in maintaining its elasticity and proper hydration of tissues, attracted our attention. In addition, this unsulfated glycosaminoglycan is present in various body fluids, such as synovial fluid, serum, saliva (Asparuhova et al., 2019). The studies carried out by Asparuhova et al. (2019) proved that the presence of HA allowed to maintain the viability of oral fibroblasts, and also increased their proliferative and migration abilities. Moreover, it was found that it promotes healing of wounds without scarring because it can be an ideal scaffold replacing damaged tissues with stimulating effects on proper cell secretion and differentiation (Jooybar et al., 2019).

Despite many positive features of HA that decide about its use in regenerative medicine, some limitations have been noticed such as: rapid degradation *in vivo*, low stability and difficulty in cross-linking (Ratanavaraporn et al., 2018). The problem is also the doubtful mechanical strength of the constructed scaffolds, necessary to create favorable conditions for supporting the cells growth. As in the case of chitosan, rheological and mechanical properties of HA can be successfully improved by combining with other substances, e.g. polymers, other bioactive molecules or nanoparticles (Labie et al., 2019; Li et al., 2019). This is possible due to the presence of places generated by the above-mentioned functional groups contained in the polymer structure where potential coupling is created (Tiwari and Bahadur, 2019).

With a presumption that polysaccharides promote cell proliferation; the other component contributes to the mechanical superiority. In this case the solution may be the reinforcement in the form of small solid particles or nanoparticles introduced into the matrix. The chitosan matrix containing nanometallic components has an enormous potential in the field of wound dressings due to its antibacterial properties (Mohandas et al., 2018). On one hand, the nanoparticles enhance the mechanical properties of the matrix, but on the other hand, combining them with the appropriate biodegradable material results in a reduction of their cytotoxicity, and consequently, possible effects on the health and environment (Behera et al., 2017; Qu et al., 2019).

For our studies titanium dioxide (TiO_2) was chosen because the application of 'particles reinforcement' into the chitosan scaffold improves the mechanical properties, water absorption and oxygen permeability, cell growth and their proliferation (Montaser et al., 2019) as well as bactericidal activity (Behera et al., 2017; Mohandas et al., 2018; Qu et al., 2019). In addition, an improvement in biodegradation properties was noted after the inclusion of TiO_2 (Mohandas et al., 2018; Montaser et al., 2019). Therefore, the combination of Ch/TiO_2 can be successfully used in tissue engineering as well as in dressings that accelerate wound healing (Peng et al., 2008). Such clinical applications include also hyaluronic acid (HA) (Ratanavaraporn et al., 2018; Wang et al., 2019; Laffleur et al., 2019; Labie et al., 2019).

On the other hand, during the preparation of materials whose potential applications are related to contact with living cells, there is a need to carry out research to determine the effect of biomaterial surface on the cellular response. The response from the environment very often depends strictly on the surface topography of the biomaterial. The use of the Langmuir-Blodgett technique for the transfer of the DPPC Langmuir monolayer stretched on the aqueous subphase containing chitosan (Ch), hyaluronic acid (HA) and/or titanium dioxide (TiO_2) to a solid support permits to obtain materials suitable for such analysis. More insightful characteristic can allow, among others, to determine the type and strength of phospholipid interactions with subphase components (Blodgett and Langmuir, 1937). Thus, this technique can be helpful to obtain the expected response from the biological environment to presence of a biomaterial.

In the literature a lot of papers about scaffolds made of different natural substances are available, e.g. protein, peptides (collagen, fibrin), polysaccharide and mixed system, combination of Ch and HA (Ahsan et al., 2018; Sheikholeslam et al., 2018; Croisier et al., 2014), Ch and TiO_2 (Behera et al., 2017; Kumar, 2018; Archana et al., 2013; Peng et al., 2008). But mixed system Ch/HA/TiO_2 proposed by us remains unexplored and, in our opinion, it is very interesting from the medical point of view. The detailed aim of our research was topographic analysis of the surface in connection with work of adhesion for the prepared films containing Ch, HA and/or TiO_2 (and Langmuir monolayers formed on them) transferred from the solution to a solid support to characterize their potential interactions with the cell membrane.

2. Materials and methods

2.1. Materials

To prepare the Langmuir monolayer, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (>99% from Sigma) dissolved in the chloroform (98.5% from Avantor Performance Materials Poland S.A.) was used. For the DPPC subphase preparation the following substances were used: acetic acid (99.9% from Avantor Performance Materials Poland S.A.), chitosan (with a molecular weight of 100.000-300.000 and deacetylation degree of *DD*=82% from Acrōs Organics, ACRS34905), titanium dioxide (P-25 from Evonik), hyaluronic acid (commercial 1% solution of high-molecular HA). The ethyl alcohol (96% from Avantor Performance Materials Poland S.A.) and the deionized water (purified by the Milli-Q Plus 185 system from Millipore (USA) – conductivity 18.2 MΩ·cm) were used as a cleaning agents for the optical glass plates (36 mmx24 mmx1 mm, As Polonia, Poland).

2.2. Preparation of the subphase

Phospholipid DPPC, one of the main components of biological membranes, was used to prepare a model membrane. 1 mg/cm^3 solution was used for the test – it was prepared by dissolving an appropriate sample of lyophilized powder in the chloroform. As a subphase for DPPC: acetic acid 0.1% (AA), chitosan (Ch) in acetic acid 0.1 mg/cm^3, titanium dioxide (TiO_2) in acetic acid 1.2 mg/cm^3, hyaluronic acid (HA) in acetic acid (v/v 0.5 cm^3/dm^3) and the mixtures AA/Ch/TiO_2, AA/Ch/HA, AA/HA/TiO_2, AA/Ch/HA/TiO_2, were used. The ratio of the components was determined based on the previous studies (Wiącek et al., 2018a), so that a stable connection between chitosan and titanium dioxide was formed. All components were applied as received, only TiO_2 was purified according to the procedure described in our previous papers (Gozdecka and Wiącek, 2017; Wiącek et al., 2018a).

2.3. Preparation of the solid support

The glass plates were pre-cleaned and degreased by dipping in deionized water and in ethyl alcohol. In addition, they were exposed to low temperature plasma of air containing N_2, N_2^+, O_2, NO, and OH; for 1 min in the Pico system; Diener Electronic (Germany). The plasma treatment caused introduction of the additional functional groups onto the glass surface (-OH, >N-H, >N-OH) (Xu et al., 2018). Their presence made the surface more polar (hydrophilic) with increased amount of groups capable for effective bonding of the applied film components to the support surface by the Lewis acid-base interactions (Scheme 1, subsection 4.1.). Detailed modifications were described in the previous papers (Ładniak et al., 2019b; Ładniak et al., 2019c).

2.4. Langmuir-Blodgett technique

The Langmuir-Blodgett trough was filled with the subphase solution. The glass plate was immersed in a vertical position in the subphase, after which a DPPC film was applied on its surface. The monolayer was compressed at a rate of 10 mm/min, up to a pressure of 35 mN/m – at which it was transferred to a solid support, by ascending a glass plate at a speed of 5 mm/min. This value of pressure was chosen similar to that in the earlier papers (Ładniak et al., 2019a; Ładniak et al., 2019b; Ładniak et al., 2019c) because it corresponds to the pressure of the biological membranes. The reference samples were made analogously but without the DPPC monolayer use. The Wilhelmy plate method was used to measure the surface pressure. Such prepared plates with the film-coated of subphases with different compositions, with or without the DPPC monolayer, were placed in a vacuum oven until the next day.

2.5. Work of adhesion

The work of adhesion for individual systems was determined based on the pre-made wettability measurements (made by considering the contact angle values of test liquids). The three probe liquids with known, well-defined surface tension values and its individual components were used, i.e. water (by Milli-Q), formamide and diiodomethane (analytical grade reagents from Sigma). Measurements of advancing contact angles (θ_a is the contact angle found at the advancing edge of a liquid drop) (Chibowski and Jurak, 2013; Yuan and Lee, 2013) were made at room temperature in an air atmosphere. The interpretation of the obtained results and details of the experiment are described in (Ładniak et al., 2019b; Ładniak et al., 2019c). Then the work of adhesion W_A of a liquid to the solid surface was calculated using the following formula (Wiącek et al., 2017; Wiącek et al., 2018b):

$$W_A = \gamma_L(1 + \cos\theta_a) \tag{1}$$

where γ_L is the surface free energy of liquid and θ_a is the advancing contact angle of the probe liquid.

2.6. Topography

Activated plates with the deposited film were tested using an optical profilometer (Contour GT by Bruker (Germany)). This technique enables quick and non-contact mapping of surface in three dimensions at different magnification. The surface roughness analysis was conducted based on the obtained values of the following parameters: Ra – the arithmetic average deviation of the profile from the mean line, Rq – the mean square deviation of the profile from the mean line and Rt – the distance of the highest profile point to the lowest one on the average line.

3. Results

3.1. Work of adhesion

Based on the surface characterization by measuring the wettability of film surfaces deposited on the plasma-activated glass plates, it was possible to describe the process of adhesion from the values of work of adhesion. This physical quantity allows to determine a magnitude of affinity of the given surfaces for each type of liquid. Two polar liquids but differing in chemical nature, i.e. water (W) and formamide (F) as well as a non-polar one – diiodomethane (D) were used in the research.

3.1.1. Glass plate before and after plasma treatment

Glass plates before and after plasma modification, as used in these measurements, were described in details in our previous papers (Ładniak et al., 2019b; Ładniak et al., 2019c). As a result of the plasma treatment, the surface of the glass plates became more polar. The contact angles values of the probe liquids used for the test decreased, i.e. they wetted the activated surface to a much greater extent (Ładniak et al., 2019b; Ładniak et al., 2019c). Thus, a much higher level of adhesion was obtained.

The increase in the value of the adhesion work after plasma treatment is caused by the introduction of additional functional groups, i.e. -OH, >N-H, >N-OH (Xu et al., 2018), on the glass surface which allows to create strong bonds with the contacting substances (Wiącek et al., 2018b). This indicates that the surface becomes more attractive and binds better the substances from the closest environment. Thus, more force is needed to separate such a layer from the support surface.

3.1.2. Adhesion process on the subphase film surface

In case of the reference systems (without DPPC monolayers), the lowest work of adhesion values in the range of 86.8–99.7 mJ/m^2, were obtained for diiodomethane, and the highest in the range of 131.7–143.7 mJ/m^2 for water (Table 1). The work of adhesion with regard to formamide took intermediate values, however, in this case the smallest differences between the individual prepared films were obtained and the W_A was on average about 114.2 mJ/m^2.

The analysis of the all W_A values for D showed that only two samples deviated from the others, i.e. AA/TiO$_2$ and AA/HA/TiO$_2$. These cases showed greater affinity for this non-polar liquid, consequently slightly higher W_A (99.7 and 96.5 mJ/m^2) values were obtained (Table 1). For the other compositions, the work of adhesion remained at a similar level of 89.9 mJ/m^2. However, in the case of surface analysis using water, a similar level of value (140.1 mJ/m^2) was obtained for the majority of films. Only the use of AA/Ch and AA/Ch/HA subphases resulted in a decrease of this value to 132.3 and 131.7 mJ/m^2, respectively. A slightly higher than mentioned, but still lower than the average value was obtained for the film containing all components, i.e. AA/Ch/HA/TiO$_2$.

3.1.3. Adhesion process on the subphase film surface with the DPPC monolayer

The presence of the DPPC monolayer contributed to the reduction of adhesion performance (adhesion loss is observable) for all tested subphases of each probe liquid used with the largest changes occurring for formamide (Table 1). Only for the AA/HA/TiO$_2$ film there were no significant changes, i.e. the adhesion values obtained on the subphase film and on the phospholipid monolayer transferred from this system, were almost identical (within the limits of the measurement error).

After the DPPC film transfer, slightly larger (than in the case of AA/HA/TiO$_2$) differences in the values of W_A parameter but also distinguishable from the other subphases, were noted for AA/TiO$_2$. Here the adhesion decreased for D from 99.7 to 83.3 mJ/m^2, for F from 115.2 to 107.9 mJ/m^2 and for W from 138.4 to 114.5 mJ/m^2. Interestingly, in this case one of the lowest changes in adhesion work values for formamide was observed. The difference between the value in the subphase film and on the DPPC monolayer transferred from the subphase film surface was only 7.3 mJ/m^2.

It is worth noting that the adhesion at a similar level, i.e. within the limits of measurement error, was noted on the DPPC monolayer transferred from the subphases AA/Ch/TiO$_2$ and AA/Ch/HA/TiO$_2$. At the same time, the large values obtained for AA/HA/TiO$_2$/DPPC can be very informative regarding the interactions occurring between the individual components of the subphase, i.e. Ch–TiO$_2$, HA–TiO$_2$, Ch–HA–TiO$_2$.

Slight changes in the test liquid adhesion to the DPPC film surface after the addition of the further component to the subphase were also noted when analyzing the AA/HA/DPPC and more complex AA/Ch/HA/DPPC systems. W_A values obtained in the above-mentioned cases remained within the measurement error and for D were equal to approximately 73.1 mJ/m^2, for F – 77.4 mJ/m^2, and for W – 119.6 mJ/m^2. While in the absence of a phospholipid monolayer in these subphases there were

Table 1. Work of adhesion values of activated glass plates surface with adsorbed different subphases, with or without the DPPC monolayer, calculated based on the contact angles of three probe liquids: diiodomethane (D), formamide (F) and water (W)

Subphase	**Work of adhesion values (mJ/m^2)**					
	without DPPC monolayer			**with DPPC monolayer**		
	D	**F**	**W**	**D**	**F**	**W**
H_2O	88.7±0.9	115.3±0.3	138.1±1.1	73.8±1.3	69.6±8.4	112.6±3.7
AA	86.8±1.2	112.7±0.9	139.1±1.6	73.1±1.3	85.4±6.6	114.3±3.5
AA/Ch	89.8±1.3	112.5±0.6	132.3±1.7	79.8±4.7	85.7±2.4	128.1±1.9
AA/HA	89.1±0.3	115.2±0.2	140.4±2.0	73.3±0.9	75.8±1.1	118.6±2.6
AA/TiO_2	99.7±0.5	115.2±0.3	138.4±3.5	83.3±2.5	107.9±1.5	114.5±1.5
AA/Ch/HA	90.2±1.1	112.5±0.4	131.7±2.0	72.8±0.8	79.0±1.8	120.7±3.1
AA/Ch/TiO_2	92.1±1.2	115.0±0.3	140.6±1.6	76.9±0.7	95.5±3.3	124.7±0.7
AA/HA/TiO_2	96.5±0.5	116.0±0.0	143.7±0.2	94.7±1.8	115.4±0.5	139.4±3.5
AA/Ch/HA/TiO_2	92.6±1.2	113.2±0.5	135.2±0.7	76.3±0.6	93.4±2.5	127.1±1.1

slight differences in the levels of formamide (115.2 mJ/m^2, 112.5 mJ/m^2, respectively) and water (140.4 mJ/m^2; 131.7 mJ/m^2, respectively) adhesion. However, the diiodomethane adhesion (taking into account the measurement standard deviation) had a relatively constant level in both cases.

In general, the presence of the DPPC monolayer contributed to the reduction of the hydrophilicity of the tested surfaces compared to the reference systems, i.e. the nature of the film subphase surface deposited on the modified glass plates. This is due to the specific arrangement of phospholipid molecules in the monolayer with polar heads towards the subphase film (inwards), and non-polar hydrocarbon chains towards the air (outwards) strictly dependent on the subphase composition. From these considerations, it can be stated that owing to the precise selection of the subphase composition, it is possible to obtain a film with the desired properties (less or more hydrophilic).

3.2. Surface topography

The optical profilometer images of the air plasma activated glass plates covered with the films of AA, AA/Ch, AA/HA, AA/TiO_2, AA/Ch/HA, AA/Ch/TiO_2, AA/HA/TiO_2, AA/Ch/HA/TiO_2, with or without the DPPC monolayer, are presented in Fig. 1. The corresponding profiles pointed out by the sheet are also shown. The images illustrate the topographical organization of the surfaces on the micrometer scale with the nanometer resolution in height.

As follows from the topography images some protrusions are visible on all surfaces. The presence of titanium dioxide particles in the prepared films is additionally manifested in the form of characteristic peaks with height of about 6.8 nm – AA/TiO_2, 4.0 nm – AA/Ch/TiO_2, 12.5 nm – AA/HA/TiO_2, 2.4 nm – AA/Ch/HA/TiO_2. Even distribution of protrusions over the entire surface indicates that the films are uniformly transferred onto activated glass plates by the LB technique. The heights in the z axis, visible on the profiles vary within 1.1–13.5 nm. Deposition of the DPPC monolayer induces the formation of distinct valleys with about 1.2–9.7 nm depth in the film structure of AA/DPPC, AA/Ch/DPPC, AA/HA/DPPC, AA/Ch/HA/DPPC, which contributes to an increase in heights of irregularities of all tested surfaces. In the case of TiO_2-containing films (AA/TiO_2/DPPC, AA/Ch/TiO_2/DPPC, AA/Ch/HA/TiO_2/DPPC) the topography is slightly different. The differences between the highest and the lowest points are in the range of 3.7 to 15.8 nm or 4.7 to 10.9 nm, for the films with or without DPPC, respectively. Only the AA/HA/TiO_2/DPPC film shows completely dissimilar surface morphology than in the absence of DPPC. The surface is heavily folded and the peak-to-valley difference reaches up to 70 nm as profile indicated.

The surface nano-roughness (<1 μm) affects early biological response. It is important for protein adsorption, cell adhesion, and thus affects the rate of cell differentiation, migration and proliferation. Therefore, the roughness parameters are discussed in detail in the next paragraph.

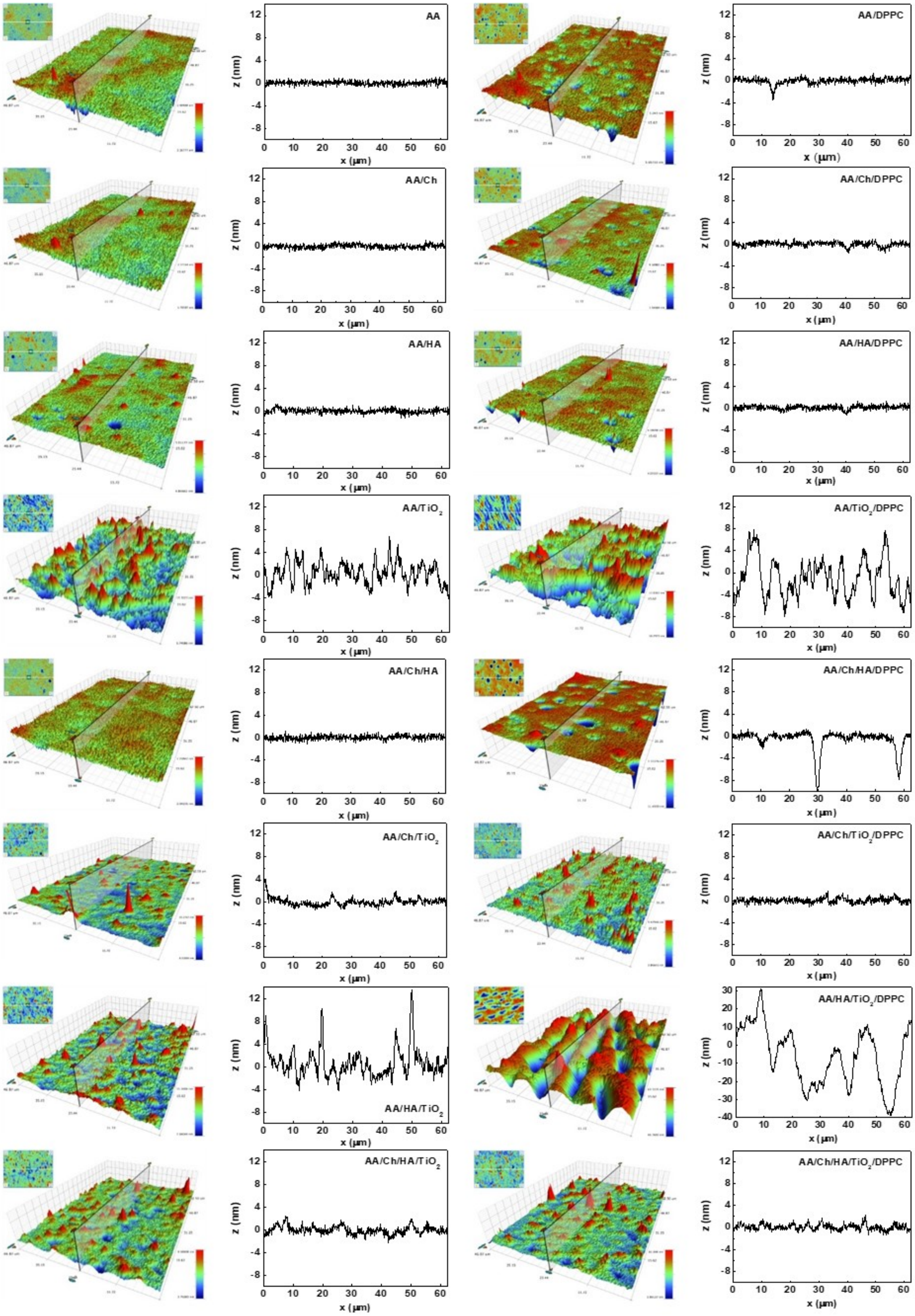


Fig. 1. Profilometric 2D and 3D images with the cross-section (profiles) along the sheet for the air plasma activated glass plates covered with the films of AA, AA/Ch, AA/HA, AA/TiO_2, AA/Ch/HA, AA/Ch/TiO_2, AA/HA/TiO_2, AA/Ch/HA/TiO_2, with or without the DPPC monolayer (the image size 47 µm x 62 µm)

3.2.1. Analysis of the Ra and Rq parameters

The determined roughness parameters, average roughness (Ra), root-mean-square (Rq) average roughness, and peak-to-valley difference (Rt), are included in Table 2. The topographic analysis of the subphases film surface deposited on the activated glass support showed that the Ra and Rq parameters assumed similar values for not containing TiO$_2$ particles systems, i.e. around 0.3 and 0.4 nm, respectively (Table 2). However, which was predictable due to the TiO$_2$ particle size, its introduction to the subphase caused an increase in these values, small for the systems containing also chitosan, i.e. AA/Ch/TiO$_2$ and AA/Ch/HA/TiO$_2$ – Ra≈0.5 nm, Rq≈0.7 nm in both cases, and significant for the titanium dioxide itself (AA/TiO$_2$) as well as in combination with HA (AA/HA/TiO$_2$) – Ra≈1.5 nm, Rq≈2.0 nm also for both systems.

Table 2. Values of Ra, Rq and Rt parameters of activated glass surfaces covered by the subphase film, with or without the DPPC monolayer

Subphase	**without DPPC monolayer**			**with DPPC monolayer**		
	Ra **(nm)**	Rq **(nm)**	Rt **(nm)**	Ra **(nm)**	Rq **(nm)**	Rt **(nm)**
AA	0.30±0.01	0.37±0.01	5.40±1.49	0.39±0.02	0.53±0.02	9.20±0.42
AA/Ch	0.28±0.01	0.35±0.02	4.10±1.56	0.33±0.01	0.44±0.04	13.00±3.22
AA/HA	0.31±0.03	0.40±0.05	7.90±1.72	0.31±0.01	0.41±0.07	8.30±2.04
AA/TiO$_2$	1.53±0.04	1.98±0.01	19.00±3.34	3.38±0.10	4.08±0.14	29.00±2.53
AA/Ch/HA	0.31±0.01	0.40±0.02	4.70±0.50	0.56±0.00	1.05±0.04	15.00±4.50
AA/Ch/TiO$_2$	0.45±0.01	0.68±0.01	20.00±1.19	0.38±0.03	0.50±0.06	8.30±0.78
AA/HA/TiO$_2$	1.50±0.21	2.02±0.32	38.00±4.24	13.67±1.22	16.72±1.46	108.00±5.56
AA/Ch/HA/TiO$_2$	0.53±0.08	0.72±0.13	13.00±3.81	0.51±0.01	0.72±0.04	14.00±1.81

The deposition of a lipid monolayer caused a minimum increase in the values of these parameters (Table 2) for AA, AA/Ch and AA/HA (for the other systems the changes were significant). The addition of both biopolymers to the subphase solutions (i.e. AA/Ch/HA) resulted in a 2-fold increase compared to the systems without a DPPC monolayer. The biggest changes were observed for the systems containing TiO$_2$. In the case of AA/TiO$_2$/DPPC there was a 2-fold increase (in reference to AA/TiO$_2$) while after the additional introduction of HA there was a drastic increase in roughness (AA/HA/TiO$_2$: Ra=1.5 nm, Rq=2.0 nm; AA/HA/TiO$_2$/DPPC: Ra=13.7 nm, Rq=16.7 nm).

Worth noticing changes occurred for the systems containing chitosan molecules. The transfer of the DPPC monolayer from the AA/Ch/TiO$_2$ subphase resulted in a decrease in the Ra and Rq parameters (0.4 and 0.5 nm) compared to the reference system (0.5 and 0.7 nm, respectively). It is surprising that after the further addition of hyaluronic acid into such system, there was a slight increase in the roughness of the transferred monolayer (AA/Ch/HA/TiO$_2$/DPPC: Ra=0.5 nm, Rq=0.7 nm), compared to the system without HA (Table 2). However, with regard to the systems without lipid molecules (i.e. AA/Ch/HA/TiO$_2$), the film topography remained unchanged (Table 2). This suggests that the presence of a DPPC monolayer in the case of a system containing all components (the most complicated, multicomponent subphase) did not cause any changes on the surface. This can point out to the preferential and regular (uniform) distribution of phospholipid molecules for different subphases.

3.2.2. Analysis of the Rt parameter

The total surface roughness characterized by the Rt parameter assumed usually higher values than the Ra and Rq parameters (Table 2). In addition, it could be observed that the subphase film on the activated glass support surface, in the absence of the DPPC monolayer, exhibited significant discrepancies for the individual systems (Table 2). The lowest value was obtained for the glass plates surface covered by the AA/Ch subphase (Rt=4.1 nm), and the highest for AA/HA/TiO$_2$ (Rt=38.3 nm). The support pulled out of the system containing both biopolymers (AA/Ch/HA) showed an intermediate roughness value (Rt=4.7 nm) of its surface compared to the samples covered with the single components (AA/Ch: above mentioned, AA/HA: Rt=7.9 nm).

Larger values were obtained for the glass plates with the adsorbed subphases containing TiO_2, and the additional inclusion of Ch or HA to that system increased the irregularities occurring on the surface. Interestingly, the support with the subphase film containing all components, i.e. AA/Ch/HA/TiO_2, has a medium roughness parameter of 13 nm, eliminating the effect of TiO_2 on its increased value.

Deposition of the DPPC monolayer on the glass plate resulted in an increase in its total roughness, but to varying degrees for individual subphases (Table 2). Slight differences were found for AA/HA/DPPC and AA/Ch/HA/TiO_2/DPPC compared to the subphase film roughness transferred to the support without the monolayer. In the other systems the presence of lipid film caused more significant changes, the values were higher from 3.8 nm in the case of the plate surface with adsorbed AA to even 69.8 nm for the sample with AA/HA/TiO_2. However, the most frequently the difference between the support covered by subphase film, with or without the DPPC monolayer, was around 10 nm. Only for the plate with the AA/Ch/TiO_2 system the presence of a monolayer caused a drop in its roughness from 20.2 nm to 8.3 nm (difference equals 11.9 nm).

4. Discussion

As was mentioned, scaffolds are created primarily from biocompatible and biodegradable materials. A suitable texture (as a rule to the adequate porosity) has a direct impact on optimal cell growth and their functionality because the shape is one of the main parameters that affect the physiological reaction of the body. Extracellular matrix, ECM provides signals directly deciding on specific interactions between the cells and the environment. In addition, the rate of matrix degradation is also important because too slow or too fast degradation lead to inhibition of tissue regeneration as a result of mismatches in the tissue growth rate and scaffold degradation (Loh and Choong, 2013). We hope that the substances used by us (i.e. chitosan, hyaluronic acid and titanium dioxide) in a suitable combination may be able to meet the requirements of the ECM substitute.

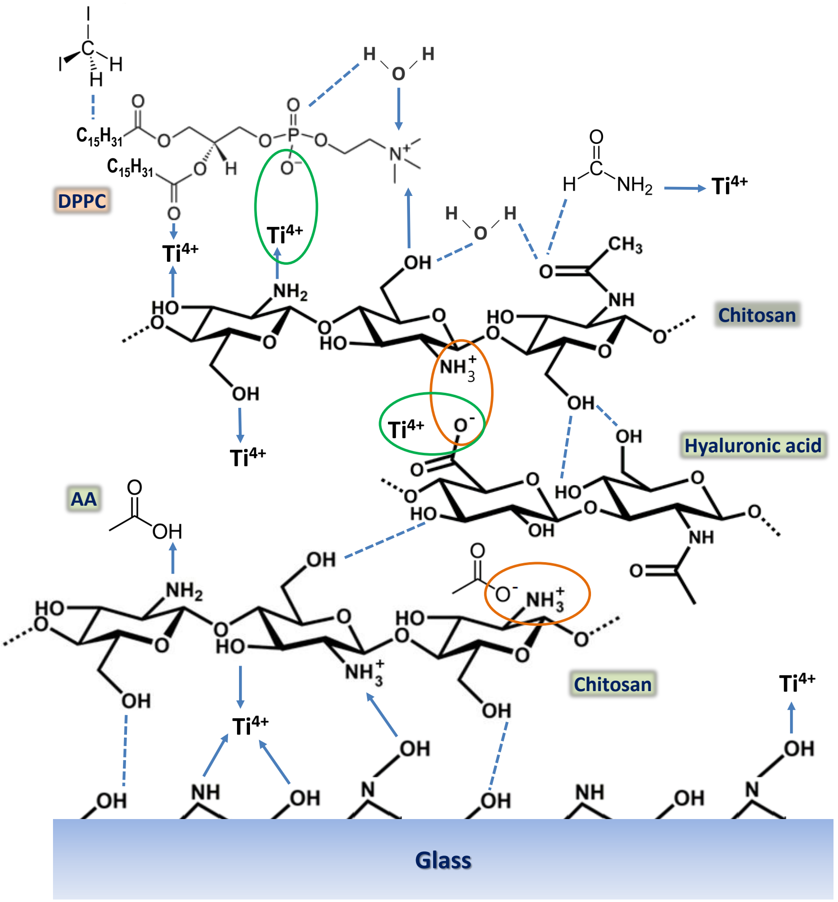
4.1. Interactions of DPPC with Ch, HA and/or TiO_2 and probe liquids

To explain the changes in the surface roughness, adhesion between the activated glass and the molecules and/or particles of subphase components should be taken into consideration. The increase of mutual interactions between components causes the formation of more tightly packed monolayers characterized by the decreasing values of the roughness parameters. Consequently, the condensed films are less permeable for the probe liquids used for the contact angle measurements, and finally, the smallest work of adhesion values were found.

The decrease in adhesion after transfer of the DPPC monolayer to the activated glass plate surface from the different subphases (Table 1) suggests a specific orientation of the DPPC molecules on the support with their hydrocarbon chains directed outwards and their polar heads with carbonyl, phosphate oxygen atoms and tri(methyl) ammonium groups directed inwards the film. The polar moieties are capable of interacting with those of Ch, HA and TiO_2 by the electrostatic and/or hydrogen bond forces while a polar fragments interact by the Lifshitz-van der Waals (*LW*) forces (Scheme 1). As a consequence, the polar groups engaged in the interactions with the film component are shielded from contact with water and formamide making the work of adhesion, regarding to these liquids, lower. A significant decrease in the water adhesion process indicates a reduction in the character of the hydrophilic surface. The small change in the W_A value in the case of D, suggests that there was a significant share of the Lifshitz-van der Waals interactions by means of which D interacts and which may occur between the liquid and the nonpolar hydrocarbon chains of the DPPC molecule.

4.2. Characteristics of the HA and TiO_2-containing films

Small changes in the adhesion values were observed after the DPPC film transfer from the AA/TiO_2 and AA/HA/TiO_2 subphases to the solid (Table 1). This indicates the penetration of each component into the monolayer structure so that they (HA and/or TiO_2) can be available for droplets of probe liquids during the contact angle measurements (Ładniak et al., 2019c), and/or can cause DPPC molecules partial reorganization (Wiącek, 2007a; Wiącek, 2007b) in such a way, that they create similar conditions for possible impacts as in the case of absence of DPPC monolayer. HA molecules contain multiple functional groups such as hydroxyl, carboxyl and *N*-acetyl as well as the hydrophobic patches. All are responsible for the production of intermolecular interactions with the Langmuir monolayers (Herzog et al., 2019). At the pH studied (3.1–3.6) HA molecules possess both undissociated and negatively charged dissociated carboxylic groups (pKa 3-4 (Mero and Campisi, 2014)). Along with –OH groups they can be sites for attachment of other molecules. Hence they presumably interact with TiO_2 particles and contribute to the interactions with $-PO_4^-$ and $-N(CH_3)^+$ groups of the DPPC molecules via electrostatic and/or Lewis acid-base forces. The other groups, not engaged in the mutual interactions within the film components, interact with the probe liquids by H-bonds (O-H⋯O or C-H⋯O) as well as by *LW* forces.



Scheme 1. Possible types of interactions and sites of the network creating groups of the studied films

Our previous studies proved that the DPPC monolayers transfer from the AA/TiO_2 and AA/HA/TiO_2 subphases were the most packed (simultaneously, the DPPC molecule takes up the same area as in other systems) (Ładniak et al., 2019a), which confirms the existence of strong interactions between the subphase components and the phospholipid film. The presence of TiO_2 particles in the DPPC monolayer also proves the roughness parameters values obtained during the optical profilometer analysis because for the systems containing titanium dioxide, the *Rt* values were close to particle size received in the earlier study, namely, the smallest one had a 10–30 nm. (Wiącek et al., 2018a).

In the case of the plate surface with the AA/HA/TiO_2 system, the heights of the roughness (Table 2) suggest that these subphase components do not form a durable connection and only spatially interact with each other creating a kind of network. The formation of an irregular structure by HA and TiO_2 can explain the significant increase (3-fold) of roughness parameter – *Rt* of glass covered by the AA/HA/TiO_2 system after embedding of the DPPC monolayer (Table 2).

4.3. Characteristics of the Ch and TiO_2-containing films

The above considerations were not confirmed in the case of AA/Ch/TiO_2 (Table 2) because the DPPC monolayer transfer from this system to the solid resulted in the reduction in its roughness (compared to the surface of glass plates with the subphase without the lipid film). As it was previously suggested (Gozdecka and Wiącek, 2017; Gozdecka and Wiącek, 2018; Wiącek et al., 2018a; Ładniak et al., 2019a; Ładniak et al., 2019b; Ładniak et al., 2019c) chitosan most likely creates with TiO_2 a stable formation (Scheme 1), by the spatial effect of hydrogen bonds between Ch and TiO_2 (Behera et al., 2017) or by electrostatic effects (Kumar, 2018). As follows from the literature data the structure of chitosan in the presence of titanium dioxide becomes more rigid and mechanically resistant while maintaining adequate flexibility, being an ideal substrate for cell growth (Peng et al., 2008; Behera et al., 2017; Kumar, 2018; Qu et al., 2019).

The analysis of roughness of glass plates surfaces covered with AA/Ch/TiO_2 and AA/Ch/TiO_2/DPPC also suggests the formation of a durable connection – the Rt values (Table 2.) decreased in the presence of the monolayer which indicates the creation of the conditions promoting its adhesion by subphase components.

4.4. Characteristic of the Ch, HA and TiO_2-containing films

Small changes in the values of adhesion work for the probe liquids between AA/Ch/HA/TiO_2 and AA/Ch/HA/TiO_2/DPPC cases (difference: D=16.3 mJ/m^2, F=19.8 mJ/m^2, W=8.1 mJ/m^2) and no changes in the surface roughness before and after transfer of the DPPC monolayer, suggest that the combination of these substances (i.e., subphase components: Ch, HA, TiO_2) enables obtaining energetically and topographically different surfaces although favorable for biological membranes.

The chemical structure of Ch and HA indicates the complementarity of their functional groups (Scheme 1). The positive amino groups ($-NH_3^+$) in Ch allow forming the ionic complexes with negative carboxylate groups ($-COO^-$) of anionic polymer HA (Kaderli et al., 2015; Park et al., 2013). By dissolving HA in a crosslinkable Ch solution it is possible to form the chitosan network in which high molecular weight HA is entrapped, producing a semi-interpenetrating network. To promote the interactions between carboxylate and ammonium moieties the polymer chains acquire an extended arrangement where uncharged acetylglucosamine units play a role of separators between the carboxylate groups (Tiwari and Bahadur, 2019). Polyelectrolyte complex formation contributes to the enhanced mechanical strength of the composite hydrogels (Park et al., 2013) which is additionally increased by the incorporation of TiO_2 into the Ch/HA network. Cross-sectional SEM images of the Ch hydrogels presented porous microstructures. In contrast, Ch/HA composite hydrogels showed tighter networks with a smaller pore diameter, indicating a higher crosslinking density (Park et al., 2013). These composites have high potential for use as a support of cells and bioactive molecules used for the cartilage treatment. It is most likely that higher crosslinking density will reduce access of the enzyme to the tight network structure of the Ch/HA/TiO_2 system, and subsequently will slow degradation.

Literature data give many examples of natural binary hybrid biocompatible and biodegradable. Relative to pure component such hybrid usually improves substrate adhesion, the proliferative activity and differentiation of different kind of cell cultured on a two-dimensional substrate. Patchornik and co-workers (2012) studies demonstrate that use of Ch/HA hybrid delays osteoarthritis progression and improves knee function. SEM micrographs indicate the marked internal structure of hybrid gel. These authors commented that the larger hyaluronate molecules appear to chaperon and organize the smaller chitosan molecules. Chitosan is an attractive biomaterial for tissue repair because of its structural similarity to glycosaminoglycans (GAG) found in cartilage, its anti-inflammatory effects and ability to promote cartilage growth. In addition, chitosan is enzymatically degraded *in vivo* by lysozyme naturally presented in human cartilage. It is possible that the hyaluronate acts to mitigate the inflammatory effect observed when chitosan is degraded, thus explaining the better weight-bearing and histological features. Other studies (Tiwari and Bahadur, 2019) give description of HA with collagen or alginate system. HA a major component of synovial fluid and extracellular matrix is found in articular cartilage and has been proven to support cell proliferation. The incorporation of HA into collagen or alginate system increases the production of cartilaginous matrix by encapsulated chondrocytes (Park et al., 2013). Besides, HA regulates the viscoelasticity of biofluids, controls tissue hydration, and altogether, acts as scaffold for the organization of ECM.

Another important aspect is to characterize the degradation behaviour of chitosan/HA hydrogels because tissue engineering scaffolds are required to degrade for subsequent tissue regeneration or release of encapsulated bioactive molecules. Chitosan alone is completely resorbed *in vivo* and enzymatically degraded by lysozyme through hydrolysis of acetylated residues. The addition of HA slowed the rate of gel mass loss and the Ch/HA composite hydrogel weight remaining was 50% at 42 days. It is most likely that higher crosslinking density reduced access of the enzyme to the tight network structure of the Ch/HA composite hydrogel, and subsequently slowed degradation. Moreover, relative to pure chitosan hydrogels, there was a significant increase in cell proliferation in the systems containing HA.

5. Conclusions and perspectives

Ongoing research on HA, Ch and their various modifications testifies unarguable share in the advancement of tissue engineering technologies. High hydrophilicity and enzymatic vulnerability of native HA or Ch makes them inferior relative to commercially available synthetic polymers. This can be overcome through mechanical enforcement employing covalent and non-covalent modifications. In addition to improved stability and intrinsic properties, chemical modifications have shown to dictate the biological fate of both. Understanding of Ch/HA interactions, before and after TiO_2 addition, at molecular level (in relation to biological membrane) could enable the preparation of product without affecting the groups involved in biological interactions.

The use of the Langmuir-Blodgett technique leads to obtain the materials that can be further analyzed to understand the interactions of the phospholipid with the components of the subphase and to predict the response from the biological environment. This was done based on calculations of the adhesion work in relation to the surface topography. In addition, some correlations between the changes in surface roughness and work of adhesion values of probe liquids on the activated glass plates surface covered with different subphases, with or without the DPPC monolayer, were found.

For the mixtures, the type and composition of subphase determine strictly the properties of the transferred DPPC film. The presence of the DPPC molecules contributes to lowering the adhesion of the probe liquids in all studied systems, but to a different extent. The data obtained during the topographic analysis proved to be helpful in understanding the changes occurring in the adhesion process. The surface characteristics of monolayers based on the work of adhesion in combination with their topography allows for better insight into the interactions at the interface of the cell membrane – Ch/TiO_2/HA. In turn, knowledge of type and strength of these interactions can be useful in the process of creating a new generation of skin or tissue substitutes. In addition, the combination of these substances can provide a biocompatible system with wound-healing properties and probably adequate in terms of strength.

Based on our present and previous results it was concluded that topography and degree of surface hydrophilicity are the most essential parameters for more profound characteristics of biomaterial utility. Topography usually determines interactions of the biomaterials with the biological environment, firstly the water molecules, then ions and proteins adsorbed, and finally different kinds of surrounding cells. At the same time, bacteria can compete with the cells for surface colonization. It is hard to find one set of the rules to choose which of presented material is the most suitable for the practical applications. That is why full characteristics of materials and their biological response (blood wettability, protein adsorption, cell adhesion, bacterial adhesion and biofilm formation) should be considered separately for particular cases. On the other hand, the material utility strictly depends on its destination. By changing composition of our films, it will be possible to obtain the biomaterial characterized by adequate efficacy.

Biocompatibility is determined by many factors and the correct proportions of HA, Ch and TiO_2 must be maintained. Besides characteristic and applicability of used systems is dependent on type of cell, place of application and individual traits: age, sex, activity. Accurate details of the study bring us closer to answering this question. The most promising will be the system representing the highest bacteriological activity. Nowadays for the obtained surfaces such studies are conducting with *Escherichia coli* and *Staphylococcus aureus*. We hope that the incorporation of HA in the chitosan network will significantly increase cell proliferation and cartilaginous ECM production, and TiO_2 will increase mechanical stability of the system and bacteriological effect. As final effect, these composites will have high potential for use as a medical device in the broad sense of the word.

Acknowledgments

This research was funded by Ministry of Science and Higher Education, grant no. BS-M-03-002-18-D-01.

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