

# PHYSICOCHEMICAL CHARACTERISTICS OF TITANIUM DIOXIDE/CHITOSAN/HYALURONIC ACID BIOMATERIAL IN RELATION TO ANTIBACTERIALITY AND BIOCOMPATIBILITY

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## Abstract

The primary goal of this research was the multi-aspect physicochemical characterisation of a hybrid material based on chitosan, a hyaluronic acid, and TiO<sub>2</sub> in terms of antibacterial and biocompatible properties. To harness their natural potential, chitosan and hyaluronic acid were employed, as well as titanium(IV) oxide to enhance mechanical stability. The physicochemical behaviour and stability of binary and ternary systems were investigated, along with wettability, topography and surface free energy. Biomimetic methods were used to approximate the behaviour and interactions of components within individual dispersions with representative phospholipids of model biological membranes. Understanding these parameters is essential for the proper functioning of the biomaterial within a living organism. Two different types of phospholipids (1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphocholine) were selected for the study of cell membranes. In contrast, the sodium salt of 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) was used as a typical bacterial model. The obtained results confirmed the possibility of potential applications of the spectrum of chitosan/hyaluronic acid-based systems in the cosmetics, medical and pharmaceutical industries.

**Keywords:** chitosan, hyaluronic acid, titanium dioxide, biomimetic methods, antibacterial character, biocompatibility

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## **1. Introduction**

Advancements in implantology and tissue/regenerative medicine have led to more attention being paid to the application-related properties of newly developed composites, thereby increasing the specific performance requirements for such materials. In response to these challenges, natural organic materials have gained popularity, especially biopolymers. An undeniable advantage of biopolymers, particularly polysaccharides, is the similarity of their properties to those of the extracellular matrix. The polysaccharides most commonly used in the medical, pharmaceutical and cosmetic industries include chitosan and hyaluronic acid. Their broad applicability is attributed to excellent biocompatibility, biodegradability and low toxicity. Moreover, they do not elicit allergic reactions; their degradation products are non-cytotoxic and are readily metabolised by host tissues [1–11].

Titanium dioxide (TiO<sub>2</sub>) is an inorganic material widely used in the pharmaceutical and cosmetic industries, commonly employed as an excipient and an active component in pharmaceutical formulations, such as tablets and dispersions. However, its biological effects are strongly correlated with particle size. Nanoparticles may be toxic to the body, but those with larger sizes of several dozen to several hundred nanometres may have beneficial effects. Literature reports indicate that drug delivery systems utilising titanium(IV) oxide particles exhibit potential as anticancer agents. They improve the effectiveness of the drug while lowering the dose, thereby reducing toxicity and other undesirable side effects. Moreover, the addition of TiO<sub>2</sub> to biomaterial matrices can strengthen mechanical properties, which is why they can be suitable for the reconstruction of organs [5–9].

Chitosan can serve as a biomaterial matrix. It is a natural polymer with amine/hydroxyl groups that are responsible for the adsorption capacity and enable easy chemical and enzymatic modification. This is particularly important in the development of controlled-release systems for active substances, e.g., drug delivery matrices. Due to its molecular structure, chitosan inhibits the growth of microorganisms, has mucoadhesive properties and accelerates wound healing by stimulating immune system cells. Moreover, chitosan-based formulations form a protective layer on the skin, protecting it against harmful external factors. Positively charged moieties present in the structure of chitosan make it a cationic polyelectrolyte (pK<sub>a</sub> ≈ 6.3), which promotes the formation of complexes with the anionic glycosaminoglycans, GAGs. One of the GAGs found in the dermis is hyaluronic acid (HA). This combination offers several advantages from a pharmaceutical perspective. Systems with purely cationic surfaces are generally undesirable due to significantly reduced circulation time and/or bioavailability in biological environments. However, on a surface containing only anionic groups, protein adsorption and/or macrophage uptake are limited. Therefore, differently charged surfaces seem optimal, as the charges can be mutually shielded. This enables control over specific properties, such as thickness, roughness, and surface charge, which can be easily adjusted [5–11].

In medicine, cosmetology and pharmacy, hyaluronic acid is often found in the form of sodium or potassium salt, i.e., a polyanion that is negatively charged in a wide pH range (pK<sub>a</sub> ≈ 3). It also plays a significant role in many biological processes, such as inhibiting cancer progression and metastasis formation. Due to its ability to interact electrostatically, it can create a nanocomposite hydrogels, effectively used in drug delivery systems (targeted anticancer therapy). Even if the gel form ensures easy degradation, its mechanical resistance is insufficient to fully utilise its valuable properties under various environmental conditions. One way to improve gel's functionality may be to create hybrid organic-inorganic materials with controlled mechanical properties. The high molecular

weight polysaccharide-based networks bind low molecular weight ions and act as an osmotic buffer. Such networks can sterically exclude various molecules depending on their size, which is essential in protecting tissues against the entry of bacteria, fungi, and viruses [11–14].

The main goal of the research was a detailed analysis of a newly developed biomaterial composed of an inorganic (TiO<sub>2</sub>) and organic substances (polysaccharides, chitosan and hyaluronic acid). The impact of individual ingredients, as well as their two-/ three-component dispersions, on the properties of model cell membranes in terms of biocompatibility and antibacterial properties was determined. It was assumed that the combination of inorganic TiO<sub>2</sub> and organic biopolymers would create a hybrid material of specific physicochemical properties, constituting mutual reinforcement/synergy of the unique properties of individual components. The characteristics of the resulting materials will be discussed in relation to biocompatibility and antibacterial properties.

## 2. Materials and Methods

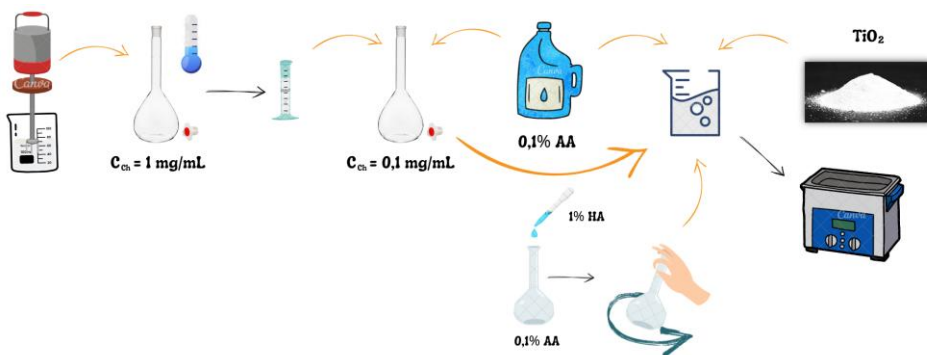
### 2.1. Materials

#### 2.1.1. Model Membranes

The three most characteristic phospholipids (purity  $\geq 99\%$ , Sigma-Aldrich) of various chemical natures were selected as main components of biological membranes: (a) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) for the human membrane model, and (b) 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (DPPG) for the bacterial membrane model. Fresh 1 mg/mL solution of phospholipids DPPC/DOPC in chloroform (purity  $\geq 98.5\%$ , Avantor Performance Materials) was also prepared and stored for a maximum of 2 days at  $-20^{\circ}\text{C}$ . A chloroform/methanol/water mixture (1:2:1.8 v/v/v) was applied to isolate lipids from the bacterial mass of *E. coli* or *S. aureus*. Chloroform (98.5%):methanol (99.8%) 4:1 mixture was used for DPPG dissolution. *E. coli* isolated lipids were dissolved in a chloroform:methanol mixture (9:1), while *S. aureus* extracted lipids were dissolved in pure chloroform.

#### 2.1.2. Dispersions/Subphases

Titanium dioxide (TiO<sub>2</sub> P-25 Evonik, 10 - 30 nm mean size, fine-grained), chitosan (Ch, Acros Organics, ACRS34905, 100,000 - 300,000 g/mol, degree of deacetylation  $82.28 \pm 0.93$ ) and hyaluronic acid (HA, 1.60 - 1.80 MDa, 1% commercial solution) were used to prepare the tested subphases. The 0.1% acetic acid (AA) solution was prepared from concentrated 99.5% - 99.9% acid (Sigma-Aldrich). The substances were dispersed in 0.1% AA in various combinations. The equal final components content for each mixture was ensured: 0.1 mg/mL of Ch, 0.5 mL/L of HA, 1.2 mg/mL of TiO<sub>2</sub>. The subphases' pH was equal to 3.4 - 3.8. During mixture homogenisation, distilled water from the Milli-Q Plus 185 system (Millipore, USA) and a homogeniser SilentCrusher M (Heidolph, Germany) were applied. The general scheme of preparation of TiO<sub>2</sub>/chitosan (Ch) and/or hyaluronic acid (HA) dispersion is presented in Figure 1.



**Figure 1.** Scheme of preparation of TiO<sub>2</sub>/chitosan/hyaluronic acid-based dispersion.

### 2.1.3. Substances Used in Wettability Measurements

Wettability measurements were conducted using two polar liquids (water and formamide) and one apolar liquid (diiodomethane). The surface tension of the probe liquids needed for the calculation of the apparent surface free energy (SFE) was taken from van Oss and Good [15]. Wettability parameters are essential in relation to the growth process of microorganisms and, indirectly, biocompatibility and antibacterial character.

## 2.2. Methods

The most important factors affecting interaction between the cell and the material include: chemical composition, topography, wettability and surface free energy. Knowledge of these parameters provides the basis for skilful control of the properties of the surface layer, while, in turn, the given surface properties of the biomaterial allow achieving the expected response from the biological environment, and using it for the directional behaviour of cells on the surface of the biomaterial.

### 2.2.1 Biocompatibility. Model Biological Membranes

The term ‘biocompatibility’ complements the concept of biomaterial, thereby imposing on it several physicochemical properties in relation to the functions it performs in specific applications. Biocompatibility is “*the ability of a material to perform appropriately while producing a positive host response that is closely tailored and specific*” [1–4]. Biomaterials must possess appropriate mechanical and strength properties. Above all others, biomaterials must be compatible with the surrounding tissues, human blood and its components, i.e., must exhibit high hemo- and biocompatibility, low or zero thrombogenicity and carcinogenicity, adequate host reaction, appropriate osteoconduction, osteoinduction and mineralisation [5–11].

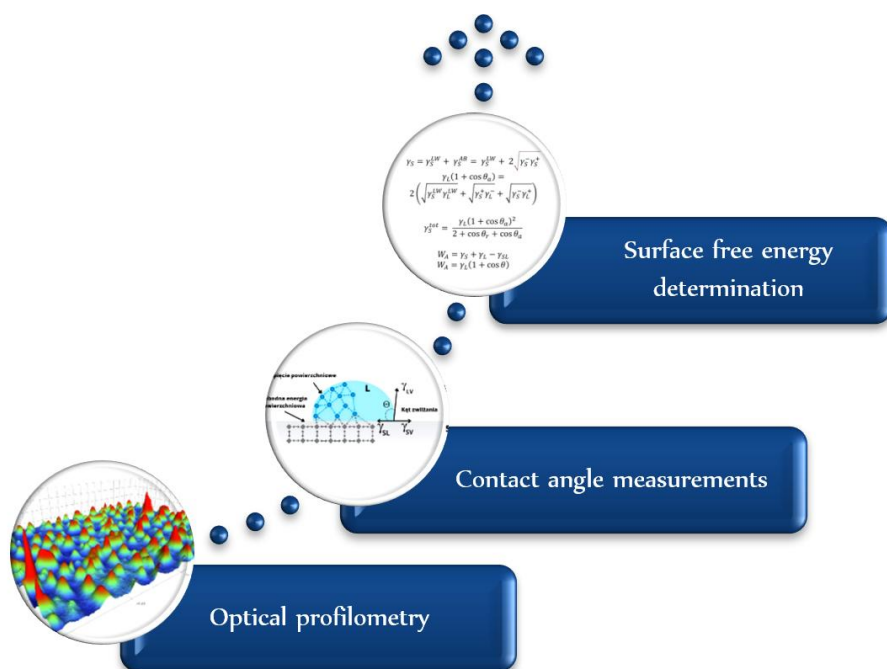
The host response is defined as “*the response of a living organism to the presence of a material that may or may not be neutral and non-toxic, taking into account the defence mechanisms of the entire immune system or its individual components*”. Therefore, to fully understand which parameters characterising the biomaterial are most important in creating formulations intended for contact with living tissue, it is necessary to take into account their impact on the properties of biological membranes, i.e., the outermost elements of cells with which the foreign body comes into contact [11–14, 16, 17]. According to the literature [5–11], the most essential physicochemical and biological factors influencing biocompatibility are surface modification and/or degradation, surface composition, e.g., charge, functional groups; surface micro- and macrostructure, hydrophilic-hydrophobic

character, wettability and surface free energy, topography (roughness, stiffness), crystallinity, cell adhesion, cell proliferation and more specific cellular uptake. Therefore, topography and wettability analysis, as well as biomimetic methods (model biological membranes based on the Langmuir and Langmuir-Blodgett techniques) [18–21] were used in current research. Model biological membranes (human or bacterial) can help determine the mechanisms of interactions between membrane components and proteins, drugs, or human and bacterial cells. Some of the most commonly used models are Langmuir monolayers, liposomes or bilayers deposited on a solid substrate. All models utilise phospholipids, the main components of human/bacterial membranes. The types of phospholipids and their proportions are selected based on the composition of their natural counterparts.

### 2.2.2. Optical Profilometry and XPS Analysis

Plates (mica or air plasma-activated glass) with deposited films of individual, two- or three-component dispersions were analysed using an optical profilometer (Contour GT by Bruker (Germany), Figure 2). The evaluated topography parameters include the arithmetic average deviation (Ra), the mean square deviation (Rq) of the profile from the mean line, and the distance between the highest and lowest points on the average line.

The Prevac system with the monochromatic hemispheric analyser (R4000; VG Scienta, Rogów, Poland) was used for XPS spectra of TiO<sub>2</sub>, Ch/TiO<sub>2</sub>, HA/TiO<sub>2</sub> and Ch/HA/TiO<sub>2</sub>. XPS analyses were conducted under the UHV conditions in the analytic chamber under 5 · 10<sup>-9</sup> mbar vacuum. The Casa XPS program was applied to quantitatively analyse the examined samples.



**Figure 2.** Typical methods of biomaterials' surface characterisation.

### 2.2.3. Contact Angle Measurements

The contact angle measurement (Figure 2) was conducted by a GBX apparatus (France) in a thermostatic chamber at 20°C. The obtained contact angles (two polar liquids, water and formamide, and one apolar, diiodomethane) reflected the real energetic state of the film surface. Surface tension and its components for all probe liquids used in wettability measurements are summarised in Table 1 [15]. The WinDrop++ program was used for the analysis of the registered drop image and the contact angles on the left and right sides of the drop.

**Table 1.** Surface tension and its components of the probe liquids.

Probe liquid	Surface energy [mN/m]				
	$\gamma_L$	$\gamma_L^{LW}$	$\gamma_L^-$	$\gamma_L^+$	$\gamma_L^{AB}$
Water	72.8	21.8	25.5	25.5	51.0
Formamide	58.0	39.0	2.28	39.6	19.0
Diiodomethane	50.8	50.8	0.0	0.0	0.0

Note.  $\gamma_L$ , surface tension of a liquid;  $\gamma_L^{LW}$ , the apolar (Lifshitz-van der Waals) component;  $\gamma_L^{AB}$ , the polar (Lewis) acid-base component;  $\gamma_S^-$ , electron-donor component;  $\gamma_S^+$ , electron-acceptor component.

### 2.2.4. Surface Free Energy Calculations

The contact angle measurements were used to determine surface free energy using the Lifshitz-van der Waals/acid-base (LWAB) and contact angle hysteresis (CAH) methods. The LWAB approach assumes that the total surface free energy ( $\gamma_S$ ) is a sum of two components: the apolar (Lifshitz-van der Waals) component ( $\gamma_S^{LW}$ ), related to long-range interactions, and the polar (Lewis) acid-base ( $\gamma_S^{AB}$ ) components:

$$\gamma_S = \gamma_S^{LW} + \gamma_S^{AB}. \quad (1)$$

As the acid-base component consists of electron-donor ( $\gamma_S^-$ ) and electron-acceptor ( $\gamma_S^+$ ) parameters, the final free surface energy expression becomes:

$$\gamma_S = \gamma_S^{LW} + \sqrt{2 (\gamma_S^- \gamma_S^+)}. \quad (2)$$

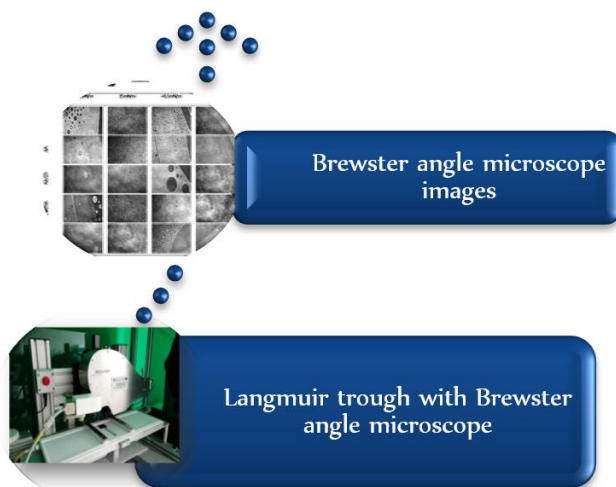
In the CAH (contact angle hysteresis) model, estimation of the surface free energy ( $\gamma_S$ ) is based on the advancing ( $\theta_a$ ) and receding ( $\theta_r$ ) contact angles of a liquid of known surface tensions ( $\gamma_L$ ):

$$\gamma_S = \frac{\gamma_L (1 + \cos \theta_a)^2}{(2 + \cos \theta_r + \cos \theta_a)}. \quad (3)$$

A drop of the test liquid with a 6  $\mu$ L volume was placed, and the advancing contact angle was measured. After the liquid drop volume decreased of 1/3 the receding contact angle was measured. Hysteresis refers to the difference between the advancing and receding contact angles. The total surface free energy ( $\gamma_S^{\text{tot}}$ ) values can be expressed as the arithmetic mean of surface free energy values ( $\gamma_S$ ) calculated separately from the contact angle hysteresis of water ( $\gamma_S^W$ ), formamide ( $\gamma_S^F$ ) and diiodomethane ( $\gamma_S^{\text{DM}}$ ).

### 2.2.5. Langmuir Monolayers by Brewster Angle Microscope

Langmuir monolayers at the water-air interface are the most commonly used membrane model, which is obtained by means of the Langmuir trough [7–10]. To check the biocompatibility of the dispersions, their effect on model biological membranes was analysed. Surface pressure-mean molecular area ( $\pi$ - $A$ ) compression isotherms were analysed with a Langmuir-Blodgett standard trough (KSV NIMA, Figure 3). First, a chloroformic solution of the tested phospholipid was used to create a monolayer. Next, the one-hour stability of the compressed monolayers was analysed, followed by decompression tests. Compression of the monolayers to 35 mN/m occurred through the movement of barriers towards the trough centre (total area 780 cm<sup>2</sup>) at a constant, pre-set speed of 10 mm/min.



**Figure 3.** Scheme of the apparatus used for Langmuir monolayer investigation and typical Brewster angle microscope images.

A Brewster angle microscope (BAM, Accurion GmbH, Germany) was used to visualise nanofilm layers. Owing to the single-layer optical model, the thickness of the phospholipid film on the AA, AA/Ch, AA/HA, and AA/Ch/HA subphases was evaluated.

### 2.2.6. Stability of TiO<sub>2</sub>/Polysaccharide System

Particle Sizer (Malvern) and Zeta Pals-BiMass Zetameter (Brookhaven Instruments Corporation) operating at pH 3 - 7 and 20 ± 0.1°C were used to evaluate the average size (based on the Stokes-Einstein equation), polydispersity index (PDI) and electrophoretic mobility (zeta potential from the Smoluchowski equation) of the chitosan/hyaluronic acid/TiO<sub>2</sub> systems in acetic acid solution. The best proportions between chitosan, hyaluronic acid, and TiO<sub>2</sub> were selected to guarantee the relative stability of the tested system, which prompted in-depth research [5–7].

The effect of biopolymer (polysaccharide, 0.1 mg/mL Ch or 0.5 mL/L of 1% HA) on the TiO<sub>2</sub> system stability and interfacial layer properties was also studied by the static multiple light scattering method using the Turbiscan analyser, enabling examination of the concentrated dispersions without dilution.

The stability of the systems can be determined based on TSI (Turbiscan Stability Index) values. To calculate the TSI, the light intensity is mathematically compared over

the entire height of the cell, based on the difference between scans, according to the following formula [6–9]:

$$TSI = \sum_i \frac{\Sigma_h(\text{scan}_i - \text{scan}_{i-1})}{H} \quad (4)$$

For every scan in the history of samples, a new point is added to its destabilisation kinetics, representing the TSI value for this ageing time. TSI values enable the determination of polysaccharide efficiency on dispersion stabilisation.

### *2.2.7. Antibacterial Activity*

Two methods have been applied to test the antibacterial activity of the biomaterial. The first method relies on the Langmuir monolayer technique and Brewster angle microscopy. The researchers used the synthetic phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (in the form of sodium salt) and lipids isolated from *E. coli* and *S. aureus* bacteria as models of bacterial membranes. The other method assumes the determination of antibacterial activity with microbiological techniques. The basic method consisted of incubating the tested dispersions with suspensions of individual bacteria at 37°C on an agar medium for 24 hours. The bactericidal effect was determined by counting colony-forming units (CFU). Moreover, with a carefully selected set of dyes, the fluorescence method was used to distinguish live bacterial cells from dead ones. The dyes differed in their spectral characteristics and ability to penetrate bacterial cells. The details of antibacterial activity testing were described by us elsewhere [16].

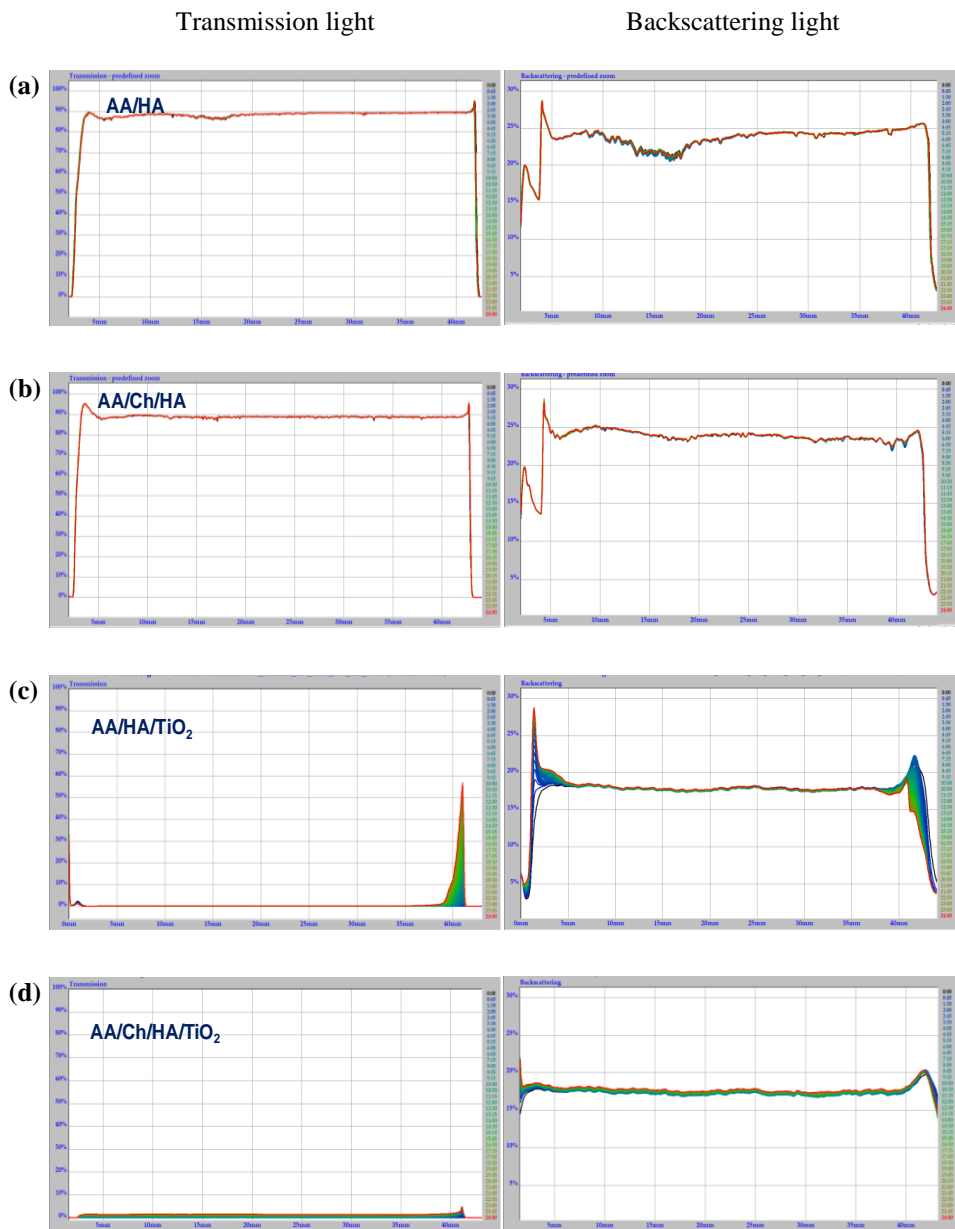
## **3. Results and Discussion**

### **3.1. Physicochemical Characteristic of TiO<sub>2</sub>/Polysaccharide System**

The physicochemical characterisation concerned the determination of the stability of TiO<sub>2</sub>/polysaccharide systems. It was noticed that titanium dioxide dispersions are very sensitive to pH changes; therefore, correcting this parameter makes it possible to obtain a system with improved stability, which was additionally strengthened after adding hyaluronic acid. Chitosan can also enhance the photocatalytic properties of TiO<sub>2</sub>, which is important in the production of protective agents against solar radiation or dressing preparations. This combination of these ingredients, first, makes it possible to use smaller amounts of TiO<sub>2</sub>, second, affects the functional properties of the preparation itself. Using chitosan as a thickening agent is also technologically advantageous [5–7].

The degree of swelling and resistance of various chemicals to solvents often used in pharmacy and medicine (water, ethanol and glycerol) was also analysed. TiO<sub>2</sub> particles slightly inhibit the chitosan's swelling properties and increase its stability. This behaviour benefits the drug delivery process because it results in slower and, thus, more effective drug release. Chitosan, which creates a thin polymer layer on the surface of TiO<sub>2</sub> particles, increases the repulsive forces between TiO<sub>2</sub> particles and prevents aggregation. This process was confirmed by three times lower TSI values than those obtained for the TiO<sub>2</sub> base dispersion without the polysaccharide [6–9].

The stability tests were established from the changes of the system structure and, particularly, the changes of the interfacial layer. Turbiscan analyser holds the electroluminescence diode, which emits the collimated light beam of 880 nm wavelength passing through the studied systems and two synchronised detectors (the transmission and backscattering). The obtained data are presented in Figure 4 as being dependent on the intensities of transmission or backscattering as a function of time.



**Figure 4.** Changes of transmission and backscattering light as a function of baseline vs. cuvette bottom for the tested systems: (a) AA/HA, (b) AA/Ch/HA, (c) AA/HA/TiO<sub>2</sub>, (d) AA/Ch/HA/TiO<sub>2</sub> vs. time (0 – 24 h). *Note.* Y-left-axis refers to 0 - 100% transmission or 0 - 30% backscattering, Y-right-axis represents time from 0h at the top, to 24 h at the bottom.

Figure 4 indicates that the use of hyaluronic acid did not significantly change the physicochemical properties of the TiO<sub>2</sub>/Ch system. At the same time, HA constitutes an additional factor that strengthens dispersion stability and biocompatibility. Three-component systems based on chitosan, hyaluronic acid and titanium(IV) oxide have properties strictly controlled by the pH of the environment. All dispersions are homogeneous and stable at a pH range that corresponds to the target pH in the human body (i.e., 3.4 - 4.8). Moreover, the positive charge favours interactions with negatively charged cell membranes [7–10].

Changes in the system stability were monitored for 24 h. Single scans were collected every 30 - 45 minutes. The collected data were used to evaluate TSI values (Table 2).

**Table 2.** Turbiscan stability index (TSI) for the tested polysaccharide/TiO<sub>2</sub> systems.

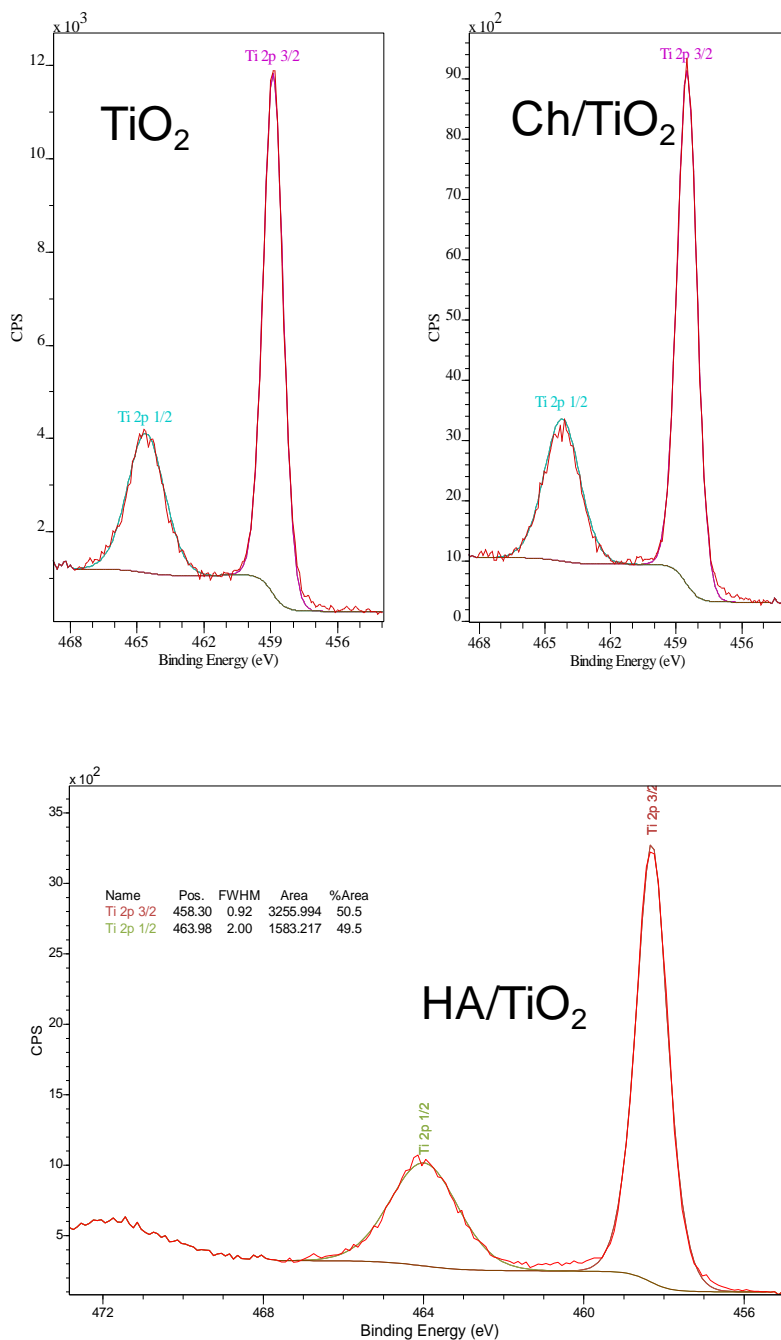
System	Turbiscan stability index (TSI)	
	after 2 h	after 24 h
AA/HA	0.3	1.5
AA/Ch/HA	0.1	1.3
AA/HA/TiO <sub>2</sub>	0.7	5.2
AA/Ch/HA/TiO <sub>2</sub>	1.9	17.3

Generally, the TSI values change within the 0 - 100 range, and the lower the TSI value, the more stable the system. The smallest TSI value is achieved for all analysed systems after 2 h. After 24 h, the TSI parameter increases in all systems. The TSI data after 2 and 24 h confirm the discussion based on the transmission/backscattering light changes.

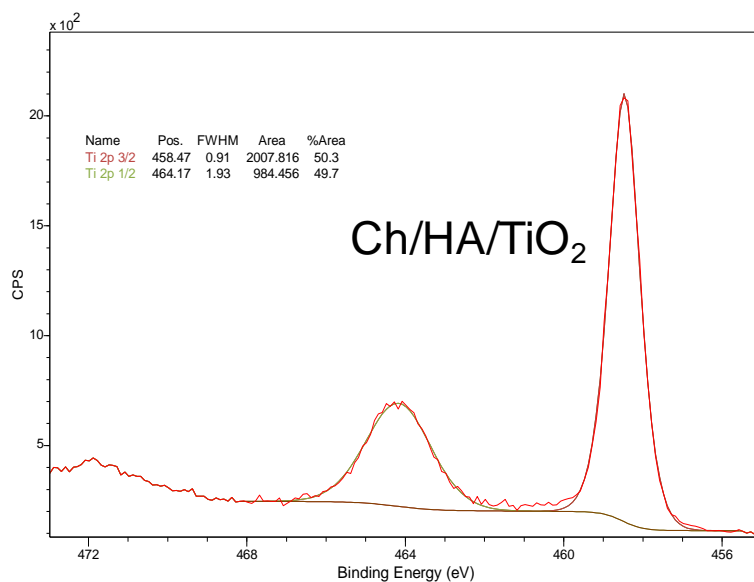
For the physicochemical characterisation in terms of the structure and mutual interactions between components, Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy (XPS) were used. Comparison of the data obtained for the mixture of chitosan and titanium(IV) oxide (Figure 5) in relation to the data for individual components suggests the occurrence of interactions between Ch and TiO<sub>2</sub>. Still, neither XPS nor FTIR analysis can confirm or exclude the type of interaction. Changes in the position and shape of signals characteristic of functional groups were interpreted as the effect of the formation of hydrogen bonds. The hydroxyl and amino groups from chitosan and the carboxyl and hydroxyl groups from hyaluronic acid may participate in this process, with the simultaneous presence of titanium(IV) oxide particles in the system (Figure 5).

Based on FTIR spectra analysis, the positions of stretching vibrations corresponding to C–O, amino, and hydroxyl groups indicated a strong association of polymers with TiO<sub>2</sub> nanoparticles. The new bands in the 385 - 900 cm<sup>-1</sup> region and at 3350 cm<sup>-1</sup> confirm the presence of O–Ti–O and N–H–O–Ti bonds, respectively. The last one confirms the formation of an electrostatic interaction between TiO<sub>2</sub> and Ch in the Ch–TiO<sub>2</sub> composite. Moreover, a new band appeared at 1000 cm<sup>-1</sup>, which corresponds to the Ti–O–C bond, demonstrating that TiO<sub>2</sub> can also be chemically bonded to Ch, not only physically adsorbed onto the Ch matrix.

The application of chitosan prevented TiO<sub>2</sub> from agglomerating during the CS-TiO<sub>2</sub> formation. The biopolymer reduced the effect of van der Waals interactions on TiO<sub>2</sub> nanoparticles due to their immobilisation in the biopolymer matrix.



**Figure 5.** XPS spectra and electrons' binding energies from various orbitals confirming the presence of Ti in the representative systems: TiO<sub>2</sub>, Ch/TiO<sub>2</sub>, HA/TiO<sub>2</sub> and Ch/HA/TiO<sub>2</sub>.



**Figure 5.** (continued) XPS spectra and electrons' binding energies from various orbitals confirming the presence of Ti in the representative systems: TiO<sub>2</sub>, Ch/TiO<sub>2</sub>, HA/TiO<sub>2</sub> and Ch/HA/TiO<sub>2</sub>.

### 3.2. Biological Characteristic of TiO<sub>2</sub>/Polysaccharide System

Another objective of this research was to evaluate the effect of selected compounds on their dispersions in terms of biocompatibility with the model components of biological membranes. It is supposed that a highly reactive ammonium moiety of chitosan can be partially balanced in the presence of TiO<sub>2</sub> and hyaluronic acid. As a result, the systems containing Ch shouldn't drastically affect or alter the behaviour and structure of model biological membranes of eukaryotic organisms, which may indirectly suggest sufficient biocompatibility [11]. As mentioned earlier, the biocompatibility in the concept of biomaterials refers to their several features, mainly surface topography, wettability, chemical composition, and surface free energy. Defining the surface characteristics of a biomaterial enables the elicitation of a desired biological response. Understanding these parameters forms the basis for the rational and controlled design of surface properties of expected biocompatibility [11].

Due to the difficulties in studying most biological processes resulting from the high complexity of biological membranes, basic research is most often performed using model membranes imitating real biological systems. In terms of biocompatibility of the Ch/HA/TiO<sub>2</sub> mixture, its effect on model membranes was analysed based on Langmuir monolayers (L) at the liquid-air interface obtained by the Langmuir technique [7–10].

Based on the compression isotherms of typical DPPC or DOPC phospholipid films and their changes in the presence of dispersion with a given composition, it was possible to characterise the nature of polysaccharide/TiO<sub>2</sub> interactions with biological membranes in terms of biocompatibility and the kind of phospholipid used [7–10, 14, 16, 17].

Registered compression isotherms are characterised by sections that illustrate the state in which the monomolecular phospholipid film occurs: gaseous (G), expanded liquid (LE), condensed liquid (LC) and solid state (S). The physical state (phase) of the monolayer, which changes during compression, is determined by the interactions between molecules

in the surface layer, the strength and range of which change as the molecules approach each other. Table 3 provides the lift-off area ( $A_0$ ) and limit area ( $A_{lim}$ ) values obtained for DPPC and DOPC monolayers as a function of subphase composition.

**Table 3.** Comparison of lift-off area ( $A_0$ ) and limit area ( $A_{lim}$ ) values obtained for DPPC and DOPC monolayers as a function of subphase composition (Ch - chitosan, HA - hyaluronic acid, AA - acetic acid).

Subphase	$A_0$ [ $\text{\AA}^2/\text{molecule}$ ]		$A_{lim}$ [ $\text{\AA}^2/\text{molecule}$ ]	
	DPPC	DOPC	DPPC	DOPC
AA	96.7	119.8	53.9	90.6
AA/Ch	99.1	123.4	55.7	95.1
AA/HA	93.5	118.5	51.0	89.6
AA/TiO <sub>2</sub>	101.3	122.4	55.8	91.6
AA/Ch/HA	99.4	129.5	53.6	97.9
AA/Ch/TiO <sub>2</sub>	100.4	131.1	55.0	98.0
AA/HA/TiO <sub>2</sub>	97.9	151.3	53.7	99.2
AA/Ch/HA/TiO <sub>2</sub>	102.8	134.7	55.6	99.8

The highest changes in the structure and packing of the DPPC monolayer occurred during its contact with TiO<sub>2</sub>, which suggests a very strong influence of this component. The addition of biopolymers (Ch or HA) to inorganic TiO<sub>2</sub> alleviates the changes caused by its presence, which is reflected in the reduction of differences in the parameters characterising the phospholipid films in relation to the standard (Table 3). The toning effect of Ch was more noticeable than that of HA. In the case of DOPC, there is no penetration of the components into the monolayer, but only their close proximity in the interphase area. Components do not disturb the structure of the monolayer, but increase the distance between the phospholipid heads due to competitive interactions with them, thus indirectly indicating their biocompatibility with the model membrane [9, 14].

The analysis of antibacterial activity using microbiological techniques confirmed the high bactericidal effectiveness of the dispersions used. However, significant differences in sensitivity to the compounds and methods used have emerged among bacterial species. The most active against *E. coli* were the dispersions containing chitosan. A cell mortality value of ca.  $85.0 \pm 4.0\%$  was achieved. A slightly lower level of bactericidal capacity against *E. coli* was noted for HA ( $61.0 \pm 4.5\%$ ) and TiO<sub>2</sub> ( $68.0 \pm 5.5\%$ ). However, synergy of antibacterial capacity up to 84.0% was observed due to the simultaneous use of these compounds in dispersions. Similarly, for Ch and TiO<sub>2</sub> at level  $82.5 \pm 4.5\%$ . The effect of Ch, HA and/or TiO<sub>2</sub> was also visible in the Langmuir monolayer tests, resulting in changes in the *E. coli* film structures.

Regarding tests on *S. aureus*, for dispersions with Ch and TiO<sub>2</sub>, a significant reduction in the cell viability, equaling  $84.0 \pm 4.0\%$  and  $67.5 \pm 4.0\%$ , was observed, respectively. However, their multi-component dispersions showed even lower cell mortality, ca.  $42.0 \pm 1.5\%$ . On the other hand, for Ch/HA and Ch/HA/TiO<sub>2</sub> dispersions,  $61.0 \pm 2.5\%$  and  $41.5 \pm 2.5\%$  respectively, bactericidal activity against *S. aureus* was demonstrated. Thus, in the case of this bacterium, it wasn't easy to find synergy in antibacterial properties in multicomponent mixtures.

It was found that among the two methods applied in antimicrobial evaluation, the counting method was more sensitive than the fluorescent method. It can be assumed that with this combination of components in the dispersion, bacterial cells may enter a dormant state defined as viable but unsuitable for culturing. Thus, using several complementary techniques for assessing antibacterial activity and biocompatibility seems reasonable. Therefore, using the Langmuir technique, the significant effect of Ch (additionally enhanced by TiO<sub>2</sub> and/or HA) was also observed against *E. coli*/*S. aureus* monolayers tested. The limited miscibility and/or displacement of components from the monolayer were observed under the Brewster angle microscope as coexisting areas with packed (small bright domains) and expanded domains. This phenomenon was found for all monolayers of *E. coli* and *S. aureus*, both the standard one and those formed on the AA, AA/Ch, AA/HA and AA/Ch/HA subphases. Ch and HA drastically affected the behaviour of bacterial membranes made of lipids secreted from bacterial cells, indirectly indicating their antibacterial activity. This was manifested in changes in the course of the compression isotherms, causing an increase in the A<sub>0</sub> and A<sub>lim</sub> values, which consequently led to a decrease in the packing degree of films at a surface pressure of 30 - 35mN/m. These natural membrane domains have biological importance, so substantial modification of their properties may lead to cell death [13, 14].

#### **4. Conclusions**

The biomaterial composed of chitosan, hyaluronic acid, and titanium(IV) oxide does not disrupt the structure of biological membranes formed from phospholipids DPPC and DOPC, which indirectly confirms its biocompatibility. Using Ch/HA/TiO<sub>2</sub> dispersions reduces the elasticity of DPPC membranes and increases that of DOPC, which may ultimately enable the penetration and/or transport of active substances. The effects of Ch and TiO<sub>2</sub> are strongly dependent on the physical state of the monolayer. Both compounds cause an increase in the elasticity of monolayers in the LE state and a decrease in the elasticity of monolayers in the LC state. Dispersions containing Ch or TiO<sub>2</sub> influence the packing of DOPC molecules to a lesser extent than DPPC. The addition of hyaluronic acid to the TiO<sub>2</sub> and Ch/TiO<sub>2</sub> dispersions also improves their biocompatibility.

The developed biomaterial has been proven to possess antibacterial properties. Dispersions containing chitosan turned out to be the most active against *E. coli*. A slightly lower level of bactericidal ability against *E. coli* was recorded for HA and TiO<sub>2</sub>; however, as a result of the simultaneous use of these compounds in dispersions, synergy of antibacterial abilities was observed. The effect of HA and/or TiO<sub>2</sub> was also visible in Langmuir monolayer tests; their presence resulted in the higher elasticity of *E. coli* lipid films at the surface pressure corresponding to the lateral pressure of the biological membrane. A significant reduction in *S. aureus* viability occurred in the presence of Ch and TiO<sub>2</sub>. The effective action of Ch (additionally enhanced by the presence of TiO<sub>2</sub>) was also observed against *S. aureus* lipid monolayers. The obtained results confirmed the biocompatibility and antibacterial nature of the biomaterial and, with the appropriate composition, its relative mechanical stability. Additionally, the test carried out significantly contributes to a broader understanding of the interactions of components of different polarity with biological membranes (vs. the type of phospholipid) and thus expands the spectrum of potential applications of this type of system.

## 5. References

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