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Antibacterial properties of elastomers modified with chitosan

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Bacterial infections pose a serious threat to human health. For many years, there has been a search for materials that would inhibit their development. It was decided to take a closer look at various elastomeric materials with the addition of chitosan. Mixtures based on silicone, silicone with a platinum catalyst, acrylonitrile-butadiene rubber, natural rubber, and ethylene-propylene-diene rubber were developed and tested for antibacterial and physico-mechanical properties. The dispersion of chitosan in the elastomer was also investigated using a scanning electron microscope. Of the tested mixtures, three were selected, characterised by the best antibacterial and physico-mechanical properties and a very good dispersion of chitosan in the matrix. The mixtures were based on silicone, silicone with a platinum catalyst and natural rubber. Tests were performed to measure the release of compounds into water for these mixtures. Furthermore, cytotoxicity with L929 cells and cytocompatibility in direct contact with MG63 cells were investigated for silicone samples. The results showed that these materials were not toxic to mammalian cells and supported their growth. The best bactericidal properties against *E. coli* and *S. aureus* strains compared to the other tested materials (>99.0–99.9% of killed bacteria) were shown by samples made of silicone and silicone with a platinum catalyst and added chitosan. At the same time, the best physico-mechanical properties were found for the samples with chitosan based on silicone with added platinum and natural rubber. Developed materials appeared to be good candidates for manufacturing medical equipment on which the adhesion and growth of bacteria should be prevented.

Key words: *chitosan, elastomers, antibacterial properties, physico-mechanical properties, cytotoxicity*

1. Introduction

Bacterial infections and the constant increase in bacterial resistance pose a serious threat to human health. Therefore, more materials that can inhibit the growth of bacteria are highly sought after. It was decided, as part of the current work, to develop materials that have antibacterial properties.

Industries such as medical, construction, automotive and footwear widely use various types of elastomeric elements used in hospitals, offices, nurseries, schools and clinics for different equipment, e.g., carpets, wipers, air conditioning elements, medical devices (gloves, hearing aid elements, drains, catheters, intubation tubes, oxygen tubes, and stethoscope tips). These are places that are particularly susceptible to the development of pathogenic microorganisms.

Various biocidal substances are currently used in elastomeric mixtures: silver in ionic compounds or in the form of nanoparticles, copper, gold, zinc oxide, titanium dioxide, antibiotics embedded in carriers, quaternary ammonium salts, ionic liquids, carbon nanotubes, halloysite, bentonite, graphene oxide [7], [20], [34]. Of these, the most common additive is silver and its compounds. Elastomers with biocidal properties are primarily polymers that release biocides [28].

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Silver has very good antimicrobial properties, but also has a few disadvantages such as its high cost, bioaccumulation as well as the risk of inducing bacterial resistance. Some studies highlight the possible immunotoxic potential of silver nanoparticles. In addition, silver when embedded within elastomers loses its activity, so its antibacterial effect can be impeded. Taking the increased resistance of bacteria to silver into account, it is necessary to develop alternative forms of biocides for elastomeric materials [5], [19], [25], [26], [28], [32].

Chitosan is a well known natural polymer with valuable physicochemical and biological properties [2], [4], [17], [23], [29], [35]. It is a co-polymer of N-acetyl--D-glucosamine and D-glucosamine linked by β-1,4--glycosidic bonds. It is a derivative of chitin produced by its deacetylation and is soluble in diluted acid solutions. Chitosan is biodegradable, biocompatible and non-toxic. It has coating properties along with antioxidant and antimicrobial activity [16], [22]. Its strong positive charge disrupts the bacterial cell membrane, making chitosan by nature, an antibacterial polymer that inhibits growth and kills bacteria [10], [14]. According to some studies, the amino groups in high molecular weight chitosan interact with carboxyl groups in bacteria, preventing bacteria from accessing nutrients. An alternative hypothesis suggests that low molecular weight chitosan can infiltrate cells and bind to DNA, inhibiting RNA transcription and finally synthesis of proteins [15].

In the literature, there are many reports regarding the antibacterial properties of various elastomers modified with chitosan. Natural rubber with chitosan has been the subject of studies carried out in several articles [12], [13], [21], [33] to provide these materials with antibacterial activity.

There are not many articles related to ethylene-propylene-diene rubber (EPDM) in the literature. The authors of the study [1] analysed the effect of chitosan and silica on EPDM mechanical properties. The tensile strength of EPDM vulcanisate/chitosan/silica was found to increase when the concentration of chitosan was increased to 5 phr (parts per hundred grammes of rubber), but the elongation at break decreased. The antimicrobial activity of EPDM nanocomposites was measured against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) in terms of zone inhibition and yielded satisfactory results.

Infections resulting from the use of medical equipment are one of the most common health complications [6]. Commonly used biomaterials, such as polydimethylsiloxane (PDMS), used to manufacture medical devices, can be problematic. This is because of the fact that their abiotic surfaces are susceptible to microorganism colonisation and promote biofilm growth [24], [27]. The formed biofilm provides adhered bacteria with specific properties that bacteria in a planktonic form do not possess.

Research on PDMS modified with chitosan has been ongoing for years. There are studies on grafting chitosan with silicones [11]. Article [8] discusses the preparation and properties of chitosan-montmorillonite nanocomposites grafted with PDMS with different proportions of montmorillonite. Chitosan was intercalated into sodium montmorillonite and PDMS was grafted with that chitosan under UV radiation. The obtained material was characterised using conventional techniques, such as X-ray diffractometry, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy (^{13}C NMR), thermogravimetric analysis and differential thermal analysis. The sorption behaviour of the materials was measured by the degree of swelling, and the interactions of biopolymers and montmorillonite were also discussed. The results showed a high water retention by chitosan. In paper [30], the preparation and properties of PDMS modified with chitosan was detailed. A series of PDMS samples were made, first by acid-catalysed ring-opening octamethylcyclotetrasiloxane (D4) polymerisation to obtain PDMS prepolymers. The addition of PDMS to chitosan was found to increase the hydrophobicity of resulting material, as indicated by the decrease in the water vapour permeability of chitosan containing PDMS compared to that of unmodified chitosan. In another study [18], the chitosan-g-polydimethylsiloxane copolymer was prepared by grafting PDMS prepolymers with chitosan. PDMS prepolymers were prepared by octamethylcyclotetrasiloxane (D4) ion ring-opening polymerisation using *n*-butyl-lithium (BuLi). Tensile strength and elongation of the obtained chitosan-g-PDMS copolymer were mostly constant, regardless of the grafting percentage of PDMS prepolymer.

Additive manufacturing can be also used to process chitosan. In study [24], antimicrobial hydrogel mesh made from chitosan and a biosurfactant was manufactured using 3D printing. The 3D structure is designed for coating polydimethylsiloxane-based medical devices, in order to prevent infection. Furthermore, the printing of coatings based on surfactants and chitosan was optimised and the obtained 3D structure was characterised (wettability, presence of functional groups using FTIR-ATR, antimicrobial activity and biocompatibility were all tested).

Works on materials with antibacterial properties are on-going. Recent trends are such that to enhance the antibacterial effect, several biocides are used simultane-

ously to modify the raw material. In articles [9] and [3] the researchers added copper, copper compounds together with chitosan to enhance the antibacterial effect of the resulting material. Authors of paper [2] used lysine as an antibacterial coating for silicone materials. The authors of paper [36] synthesised silver-chitosan, silver--chitosan/clay and polymer/silver-chitosan/clay nanocomposites to be applied as biomedical catheter materials, and investigated their antibacterial activity against different bacteria strains. The obtained PDMS/clay--chitosan-silver nanocomposites with great antimicrobial properties could be used as biomedical materials for controlled release of drugs in urological catheters.

There are no reports on acrylonitrile-butadiene rubber modified with chitosan – 1945GRN nor silicone materials with vinyl groups PDMS (HTV 4001/60) obtained with the use of a new catalyst.

The aim of the present study was to introduce chitosan into different elastomers (natural rubber, silicone, acrylonitrile-butadiene rubber, ethylene-propylene-diene rubber) and to investigate the antibacterial and physico-mechanical properties of the obtained composites. The new silicone material with PDMS vinyl groups (HTV 4001/60) obtained with a new catalyst was also tested. We wanted to check whether the addition of chitosan to selected elastomeric materials allows for the preparation of a composite material with antibacterial properties, beneficial physico-mechanical properties and not displaying cytotoxicity towards mammalian cells. In the future, the materials chosen for further research could be used in areas where the growth of pathogenic microorganisms should be prevented.

2. Materials and methods

2.1. Materials

Five types of elastomers that may have medical applications – natural rubber type light crepe (LC), acrylonitrile-butadiene rubber (NBR), ethylene-propylene-diene monomer rubber (EPDM), silicone (S) and silicone with a platinum catalyst (S-Pt) – were selected for the study. The filler, which acted as a composite additive, was chitosan, from Sigma-Aldrich (CAS 9012-76-4).

Two rubber blends were prepared from two types of silicones:

1) made of silicone polydimethylsiloxane with vinyl groups cross-linked with bis(2,4-dichlorobenzoyl) peroxide with the trade name HTV 401/60, Siliconnet (S),
2) made of silicone polydimethylsiloxane with vinyl groups with a platinum catalyst with the trade name HTV 4001/60, Siliconnet (S-Pt).

Moreover, 15 phr (phr – parts per hundred rubber) of chitosan was introduced into selected silicones (S-Ch, S-Pt-Ch). The composition of the other rubber blends is shown in Tables 1–3.

Table 1. Composition of a natural rubber compound with and without the addition of chitosan

Composition	LC/phr	LC-Ch/phr
Natural rubber type light crepe	100	100
Stearin	1.5	1.5
Zinc oxide	5	5
Paraffin wax (liquid)	15	15
Titanium oxide	20	20
Chalk	20	20
Anti-ageing agent (4010NA)	2	2
Sulphur	0.3	0.3
Disulphide tetramethylthiuram (TMTD)	2	2
Paraffin wax	2	2
Chitosan	–	15

Table 2. Composition of an acrylonitrile-butadiene rubber compound with and without the addition of chitosan

Composition	NBR/phr	NBR-Ch/phr
Acrylonitrile-butadiene rubber (1945GRN; Arlanxeo)	100	100
Arsil	15	15
Kaolinite	15	15
Stearin	1	1
Zinc oxide	5	5
N-cyclohexylbenzothiazol-2--sulphenamide (CBS)	1.2	1.2
Sulphur	1.5	1.5
Chitosan	–	15

Table 3. Composition of an ethylene-propylene-diene monomer rubber compound with and without the addition of chitosan

Composition	EPDM/phr	EPDM-Ch/phr
Ethylene-propylene-diene monomer rubber (Keltan 4450S)	100	100
Perkadox 14	6	6
Silicon dioxide	15	15
Paraffin wax (liquid)	20	20
Chitosan	–	15

2.2. Methods

2.2.1. Sample preparation

The rubber blends were prepared using a Blere two-roller machine with a roller diameter of 110 mm and length of 200 mm. Cross-linking was carried out on a press made by ZUP Nysa, type NS12-20/320-40/1.8. Chitosan was added in a measured amount along with the other ingredients using a two-roll Blere machine. The characteristics of the vulcanisation process was determined on a non-rotating rheometer (MDR 2000 Monsanto) to determine the optimal vulcanisation time. Appropriate parameters were achieved for each elastomer. The rubber blends were then vulcanised and annealed in a dryer.

2.2.2. Dispersion of chitosan

Dispersion studies of the filler (chitosan) in the elastomeric material were carried out using a Zeiss ULTRA Plus field emission scanning electron microscope with a GEMINI column, equipped with two secondary electron detectors: a standard one located in the SE2 chamber and an InLens in-column. Sections of 20 mm × 20 mm were cut from the samples and coated with a gold coating 1 nm thick gold coating. The images were taken at different magnifications and an electron accelerating voltage of 30 kV.

2.2.3. Physical and mechanical tests

Shore A hardness determination was made in accordance with the standard ISO 48-4:2018. Measurement was made with a hardness tester. 7206.100, based on the penetration of the tapered tip against the calibrated spring. Three strips of vulcanised sheets 2 mm thick were stacked on top of each other. The hardness was measured three times and the average value was calculated. The resulting Shore A value was read after 3 s.

Tensile strength and elongation at break, together with Young's modulus, were tested in accordance with the standard PN-ISO 37:2007. Tests were carried out for type 2 dumbbells. The dumbbell-shaped test pieces were cut from moulded sheets previously conditioned for 16 h at room temperature. Tensile tests were performed using Zwick 1445 machine at a crosshead speed of 500 mm/min at ambient temperature. Each measurement was taken after five tests for each sample. Tensile modulus (M100, M200), tensile strength, and elongation at break (Eb) were obtained from the tests.

2.2.4. Microbiological tests

2.2.4.1. Antimicrobial activity

Antimicrobial activity was tested using a quantitative method for the determination of germicidal properties of rubber according to the standard ISO 22196:2011: Measurement of antibacterial activity on plastic and other nonporous surfaces. Two strains of model bacteria were used for the study: *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538P). The test samples had dimensions of 50 mm × 50 mm. The covering layer was a polyethylene film (PE-LD, Malen E FGNX 2-D022, USA, measuring 40 mm × 40 mm and 0.06 mm thick). The volume of inoculum was 0.4 ml. Based on the results, the antimicrobial activity "R" was determined using Eq. (1):

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t, \qquad (1)$$

where:

U_0 – average of the logarithm of a number of CFU/cm^2, which was recovered from control samples (materials without chitosan – LC, S, S-Pt, NBR, EPDM) immediately after inoculation;

U_t – average of the logarithm of a number of CFU/cm^2, which was recovered from control samples (materials without chitosan – LC, S, S-Pt, NBR, EPDM) tested after 24 h;

A_t – average of the logarithm of a number of CFU/cm^2, which cell was recovered from samples tested after 24 h.

2.2.5. Global migration tests

Global migration tests from rubber samples to distilled water at 40 °C after 24 h and after 10 days were carried out according to the standard EN 1186 [31] and test procedure QPB.35/PLC developed by the laboratory "LABGUM" of The Łukasiewicz Research Network – Institute for Engineering of Polymer Materials and Dyes. The test consisted of extracting the rubber sample in distilled water at a specified temperature and time, and then drying the resulting extract to a constant weight. The amounts of the migrating substance were determined using Eq. (2):

$$X = 10(m - m_0)/P, \qquad (2)$$

where:

m – mass of the dry residue of the model solution [g],

m_0 – mass of the dry residue after evaporation of the pure model solution [g],

P – total area of the sample [cm^2].

According to the standard ISO 1186 and test procedure QPB 35/PLC, the acceptability criterion is ≤20 mg/dm^2 for 24 h and ≤50 mg/dm^2 for 10 days.

2.2.6. *In vitro* tests with mammalian cells

Cytotoxicity studies of the materials were performed in accordance with ISO 10993-5 (2009) using extracts and in direct contact. To prepare the extracts the weighed samples were incubated with an appropriate amount of cell culture medium (DMEM, PAN BIOTECH, Germany) with the addition of 10% foetal bovine serum (FBS, Biowest, France) and 1% antibiotics (penicillin/streptomycin, PAA, Austria) with a proportion of 10% wt./vol. for 72 h at 37 °C. Then the extracts were sterilised by filtration using syringe filters (0.22 μm). Undiluted extracts, i.e., 10%, and diluted extracts, i.e., 5, 2.5, 1.25 and 0.625%, were used in the study, and culture medium was used to dilute the extract. As a reference DMEM cell culture medium was used.

2.2.6.1. Indirect testing

L929 cells (murine fibroblasts, NCTC clone 929, CCL-1, American Type Culture Collection) were cultured in DMEM medium (PAN BIOTECH, Germany) with the addition of 10% FBS (Biowest, France) and 1% antibiotics (penicillin/streptomycin, PAA, Austria). The culture was set at a temperature of 37 °C with 5% CO_2 and increased humidity. Cells were seeded in 48-well plates at a density of 10000 cells per well in 500 μl of medium. After 24 h of incubation, the medium above the cells was replaced with undiluted and appropriately diluted extracts (500 μl). After another 24 h, the metabolic activity of the cells and their viability were examined using the AlamarBlue test and live/dead staining.

For Alamar Blue, 150 μl of a 10% resazurin solution in medium was added to each well and incubated for 3 h. Then 100 μl of the solution was transferred from each well to a black 96-well plate and the fluorescence was measured (λ_{ex} = 544 and λ_{em} = 590 nm). To calculate the level of resazurin reduction, we used the following Eq. (3):

$$\%\ \text{resazurin reduction} = \frac{S_x - S_{\text{blank}}}{S_{\text{reduced}} - S_{\text{blank}}} \cdot 100\%, \tag{3}$$

where:

S_x – fluorescence of the samples,

S_{blank} – 0% reduction of resazurin,

S_{reduced} – 100% reduction of resazurin.

To perform live/dead staining, calcein AM (0.1%) and propidium iodide (0.1 were dissolved in phosphate buffered saline solution (PBS) %); all chemicals from Sigma-Aldrich. After that, 100 μl of the solution was added to each well and incubated for 20 min in darkness. Subsequently, with the use of a fluorescent microscope (ZEISS Axiovert 40 CFL with metal halide illuminator, Oberkochen, Germany), pictures of live and dead cells were taken.

2.2.6.2. Direct testing

In the study human osteoblast-like cells MG-63 (from the European Collection of Cell Cultures, Salisbury, UK) were used. Cells were cultured in MEM Eagle medium (PAN BIOTECH, Germany) with the addition of 10% fetal bovine serum (FBS, Biowest, France), 1% antibiotics (penicillin/streptomycin) and 0.1% amino acids and pyruvate (PAA, Austria). The culture was set at 37 °C with 5% CO_2 and increased humidity. Cells were placed directly on the materials at a density of 15000 per sample. As control cells were also cultured on tissue culture polystyrene (TCPS, i.e., the bottom of the wells, Nunclon). The metabolic activity of the cells and their viability were examined at after 1, 3 and 7 days, using the AlamarBlue assay and live/dead staining as described above.

2.2.6.3. Statistics

All data are expressed as the average ± standard deviation (SD). Normal distribution was verified using the Shapiro–Wilk test followed by a one-way analysis of variance (ANOVA) test with the LSD Fisher post-hoc test to determine statistical significance. Origin software (version 2022 SR1, OriginLab Corporation, Northampton, MA, USA) was used and we considered a probability value of less than 0.05 as statistically significant: * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

3. Results

3.1. Characteristics of the tested materials

Scanning electron microscopy analyses of selected samples with the best antibacterial properties were carried out to determine whether the materials were homogeneous on a microscale and whether chitosan was present throughout the whole sample. Examples of images are shown in Fig. 1. SEM images of the surface of the tested materials do not show any significant differences. An even distribution of the filler is observed.

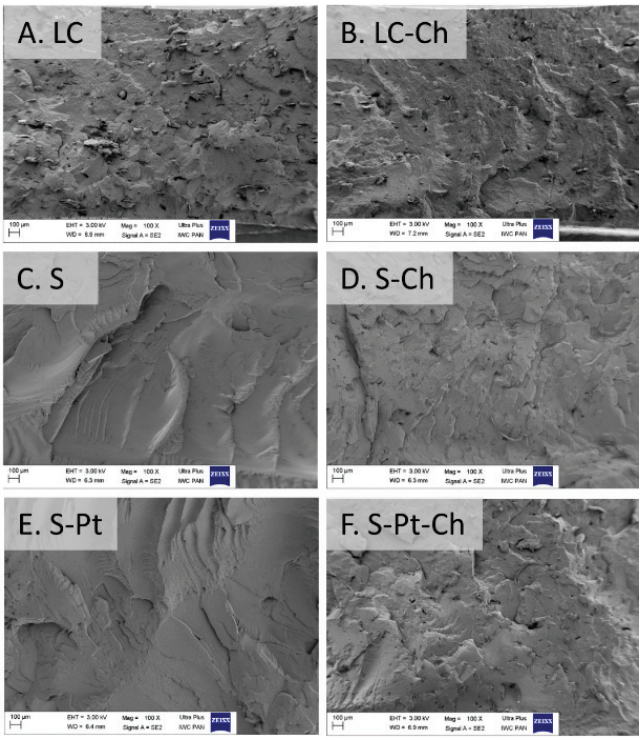

Fig. 1. SEM images for LC (A), LC-Ch (B), S (C), S-Ch (D), S-Pt (E), S-Pt-Ch (F)

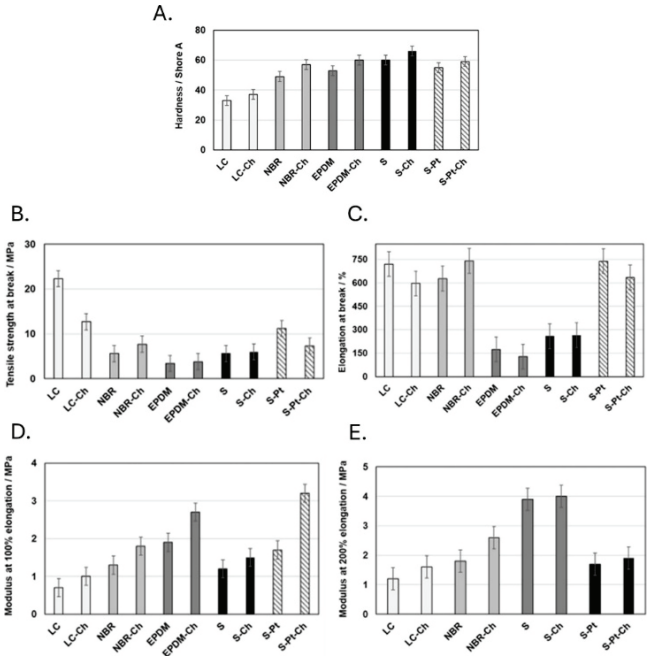

Fig. 2. Mechanical properties: Shore A hardness (A), tensile strength (B), elongation at break (C), Young's modulus at 100% elongation (D) and Young's modulus at 200% elongation (E) of LC, LC-Ch, NBR, NBR-Ch, EPOM, EPOM-Ch, S, S-Ch, S-Pt, and S-Pt-Ch rubber materials

In Figure 2, the results of the mechanical properties of the elastomeric materials investigated are presented. Hardness values and strength parameters for the tested materials such as tensile strength, elongation at break, and Young's modulus at elongation equal to 100% and 200% are presented. As observed, depending on the type of rubber used and the presence of chitosan, the parameters changed significantly.

The highest Shore A hardness values were found for the silicone-based material with chitosan (S-Ch) (Fig. 2A). For all samples, the hardness was higher when chitosan was added to the elastomer compared to those without chitosan. The greatest increase in hardness was observed for samples containing acrylonitrile-butadiene rubber (NBR) and ethylene-propylene-diene rubber (EPDM).

The tensile strength at break decreased for LC-Ch and S-Pt-Ch, compared to LC and S-Pt, respectively, while for the remaining samples, it increased by 10% to 28% (Fig. 2B). The elongation at break decreased for LC-Ch, S-Pt-Ch, and EPDM-Ch, and increased slightly for the other materials (Fig. 2C). Young's modulus measured at 100% and 200% elongation increased for all materials after the addition of chitosan (Fig. 2D, E). Interestingly, when the Young's modulus was measured for a higher elongation of 200%, its values were also higher than when measured for elongation of 100%.

3.2. Antimicrobial activity of rubber materials with chitosan

Antibacterial activity analyses were performed on two strains of bacteria: Gram-positive and Gram-negative. *E. coli* was selected as the Gram-negative strain. It is an anaerobic rod-shaped bacterium commonly found in the small intestines of warm-blooded organisms. Pathogenic strains of *E. coli* are responsible for urinary tract infections and stomach bugs. *S. aureus* was selected as the Gram-positive strain. This bacterium, among others, is usually found in the nasal-throat cavity or on the skin. Based on the analyses conducted, it was found that chitosan had a different effect on bacteria depending on the rubber used (Fig. 3).

3.3. Global migration for the selected rubber materials

For the most promising composite materials, that is, with light crepe, with silicone rubber and silicone rubber with a platinum catalyst, global migration of components from rubber to water was studied. The types of chemicals that can migrate from a material vary greatly and depend on the type of raw material from which it is made. The selected materials were charac-

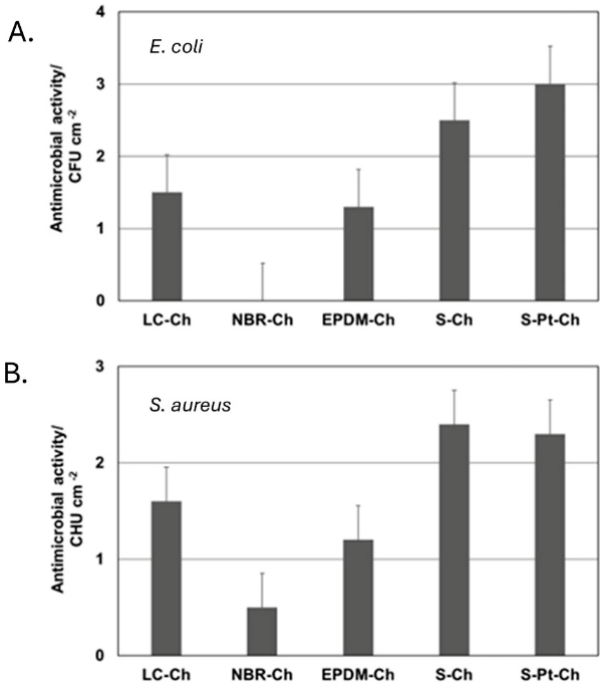

Fig. 3. Antimicrobial activity for tested rubber materials: LC-Ch, NBR-Ch, EPDM-Ch, S-Ch, S-Pt-Ch with *E. coli* (A), *S. aureus* (B)

terised by their ability to release substances into distilled water (Table 4). The results show that release of chitosan is the highest from LC-Ch, so it was excluded from the following studies.

Table 4. Amount of substance migrating from rubber to distilled water

Rubber materials	After 24 hours		After 10 days	
	[mg/dm^2]	[mg/kg]	[mg/dm^2]	[mg/kg]
S-Ch	2.6	223	1.9	161
S-Pt-Ch	1.3	116	1.6	143
LC-Ch	4.1	393	10.6	1031

3.4. *In vitro* cell culture tests of selected composite materials

The results of indirect tests show that all 10% extracts of materials are toxic to L929 cells, as shown by the AlamarBlue test (Fig. 4A). While 5% extracts of silicone samples (S) are still toxic, those of silicone with a platinum catalyst (S-Pt) and all samples modified with chitosan (S-Ch and S-Pt-Ch) are not toxic according to ISO 10993-5. According to the results shown in Fig. 4B the cell viability relative to the control conditions (cell culture in DMEM) for the 5% extracts of S-Pt, S-Ch and S-Pt-Ch are above the 70% threshold, which means that these extracts are not cytotoxic. The results of live/dead staining support the findings of AlamarBlue. The L929 cell cultures in 5, 2.5, 1.25 and 0.63% extracts are the same as when the cells were cultured in the control DMEM (Fig. 5). Only for 10% extracts are cell cultures were found less dense, which means that these extracts show significant cytotoxicity.

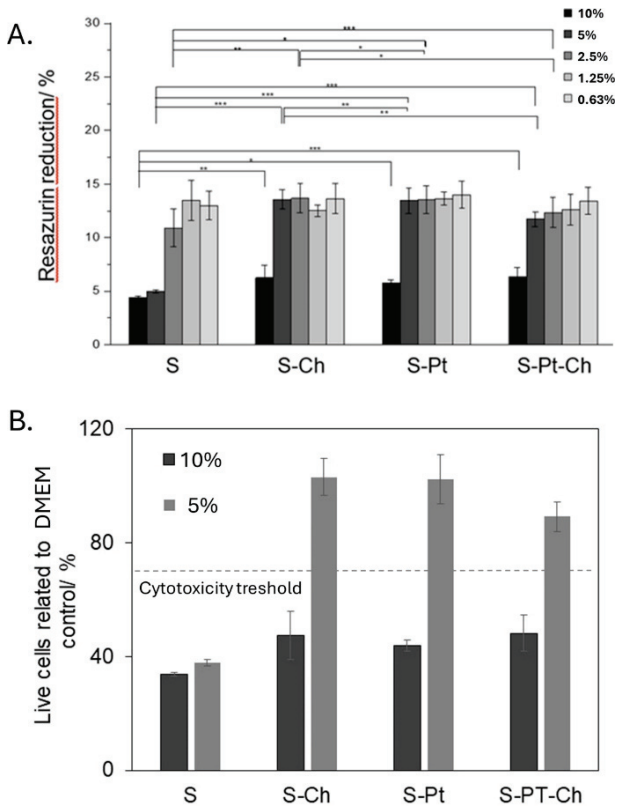

Fig. 4. Viability of L929 cells cultured in 10, 5, 2.5, 1.25 and 0.63% extracts for rubber materials tested: S, S-Ch, S, S-Pt-Ch determined by the Alamar Blue resazurin reduction metabolic activity assay; $n = 9 \pm$ SD, statistical significance was calculated by one-way ANOVA test with Fisher's post-hoc LSD test (A), viability of L929 cells presented as percentage of resasurin reduction of the cells cultured in control cell culture medium (DMEM), 70% threshold of cytotoxicity is shown as a dashed line (B)

The AlamarBlue results of MG-63 cells cultured directly on the materials for 1, 3 and 7 days are shown in Fig. 6. Although cell proliferation was significantly lower on all materials studied as compared to those on control TCPS, the cells exhibited a significant level of viability. Significantly higher cell proliferation on day 7 was found on non-modified samples than on modified with chitosan.

The results of live/dead staining show that on all materials cells were stained green, thus alive (Fig. 7).

On S, S-Ch and S-Pt samples, cell density and morphology were similar to those cells cultured on control TCPS. Interestingly, on S-Pt-Ch cells, for all time points, had the tendency to grow in separated spots.

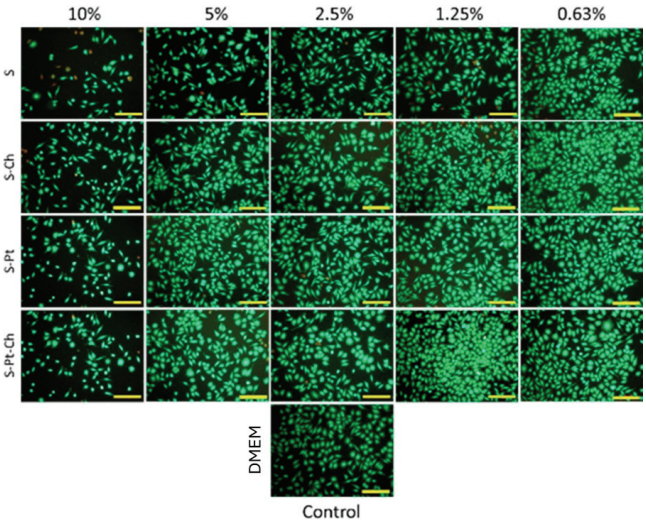

Fig. 5. Images of L929 cells cultured in extracts from the samples: S, S-Ch, S-Pt, S-Pt-Ch at concentrations of: 10, 5, 2.5, 1.25 and 0.63% and in control cell culture medium (DMEM); cells were stained by live/dead fluorescence staining: green – live, red – dead cells; scale bar = 200 µm

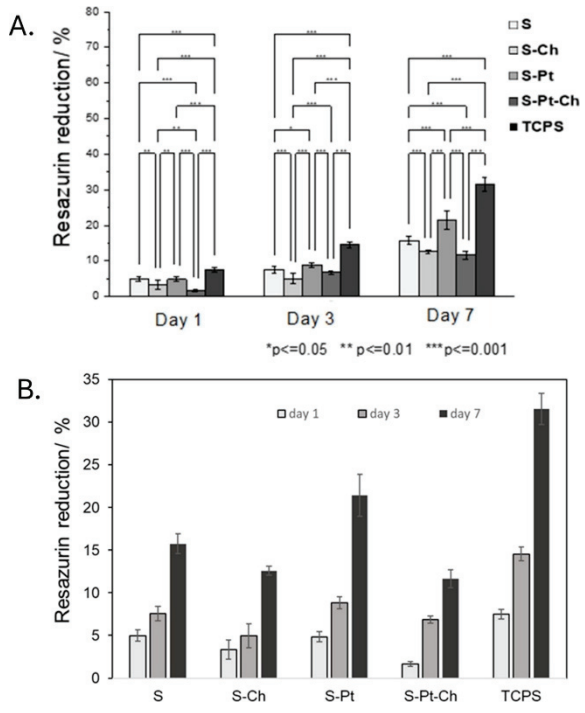

Fig. 6. Proliferation of MG63 cells after 1, 3, and 7 days of culture on the surface of the samples S, S-Ch, S-Pt, S-Pt-Ch determined by AlamarBlue resazurin reduction metabolic assay, $n = 9 \pm$ SD, statistical significance was calculated by one-way-ANOVA test with Fisher's post-hoc LSD test (A, B)

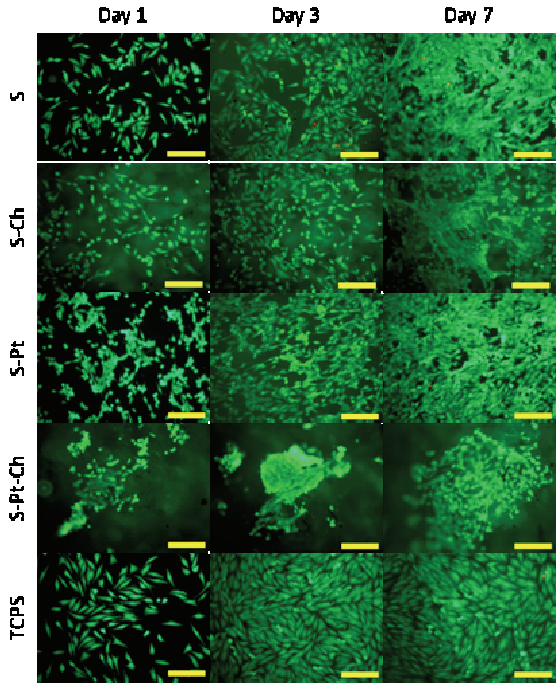

Fig. 7. Images of MG63 cells cultured on the samples: S, S-CH, S-PT, S-Pt-Ch and on control TCPS for 1, 3 and 7 days; cells were stained by live/dead fluorescence staining: green – live, red – dead cells; scale bar = 200 µm

4. Discussion

The results showed that the selected materials were characterised by a very good dispersion of chitosan on the whole surface. Good distribution of the components and no agglomerate formation was observed. The materials obtained were homogeneous (Fig. 1).

The hardness of the materials is an indicator of its stiffness against moderate stresses. The addition of chitosan to the tested rubber compounds resulted in an increase in their hardness (Fig. 2A). The greatest increase in hardness was observed for samples containing acrylonitrile-butadiene rubber (NBR) and ethylene-propylene-diene monomer rubber (EPDM). This was to be expected, since it is known that in rubber materials, an increase in hardness occurs with an increase in the amount of filler, and the added chitosan should be treated as a filler.

The effect of chitosan on the tensile strength and elongation at break of the rubber compounds was not clear (Fig. 2B, C). The different interaction of both rubber and additives present in the rubber material has an ambiguous effect on its strength properties. For samples containing either light crepe or silicone rubber with a platinum catalyst, the addition of chitosan

resulted in a reduction in both tensile strength and elongation at break. This is the result of a decrease in the molecular mobility of rubber chains, resulting in an observed increase in the hardness of these materials. However, for other blends with silicone and acrylonitrile-butadiene rubber, the best interaction of rubber with chitosan was observed, with a consequent increase in tensile strength and an increase in elongation at break. This may be caused by the different chemical structure of these materials and possible other bonds between them, and in the case of NBR, the presence of a nitrile group. In the case of ethylene-propylene-diene monomer rubber materials, there was no significant effect on their strength. For all materials tested, after the introduction of chitosan, an increase in Young's modulus at 100% and 200% elongation was generally observed compared to rubber materials without filler (Fig. 2D, E). For ethylene-propylene-diene monomer rubber materials with chitosan, it was not possible to measure the modulus at 200% elongation. Thus, the best strength properties were exhibited by materials with light crepe and silicone rubber with a platinum catalyst both with and without the presence of chitosan. These materials differed only significantly in hardness, which may affect another area of their application.

Different effects of antibacterial activity of chitosan were observed depending on the rubber used (Fig. 3). The most effective activity of chitosan against the bacterial strains *E. coli* and *S. aureus* was observed in samples containing silicone rubber and silicone rubber with a platinum catalyst. For these materials, 99.0–99.9% of both types of bacteria were killed compared to the control sample (materials without chitosan). The value of the $\log_{10}$ reduction of the tested samples was above 2, so such results can be considered satisfactory. This may have been influenced by the fact that there was no interaction of chitosan with other additives, as these silicones were commercially prepared with the crosslinking assembly and did not deactivate its antibacterial properties. The addition of platinum could also have been a factor in enhancing the antibacterial effect. A weaker effect of chitosan was observed in samples containing light crepe or ethylene-propylene rubber. These samples showed acceptable bactericidal properties ($R < 1.5$) against the strains of bacteria *E. coli* and *S. aureus* compared to the control samples. There was an observed reduction in viable *E. coli* and *S. aureus* cells of 95% and 96.2% for ethylene-propylene rubber and 96.3% and 97.2% for natural rubber, respectively. However, the introduction of chitosan into NBR-based rubber materials had no significant effect on its antibacterial properties. These samples did not show antibacterial activity against *E. coli* ($R = 0$) and very weak activity against *S. aureus* ($R = 0.5$). Most likely, as was observed in the strength tests, chitosan interacts strongly with NBR rubber, resulting in not only an increase in tensile strength and elongation at break a decrease in the antibacterial activity of chitosan in a given rubber material.

The tested materials, which have good antibacterial properties, were tested for release rates of substances into water. This variable is important for environmental and health aspects. The smallest amount of substance that migrates from the material into distilled water was determined for the silicone rubber material with a platinum catalyst (after 24 hours it was 1.31 mg/dm^2 and after 10 days it was 1.62 mg/dm^2). On the contrary, the highest amount of migrating substance was observed for light crepe material (after 24 hours it was 4.13 mg/dm^2 and after 10 days it was 1031.03 mg/dm^2). This may be due to insufficient binding of the components in the mixture, as well as dissolution of the components in water at 40 °C.

Indirect cell culture tests showed that L929 fibroblasts grew well in twice diluted extracts from modified silicone rubber-based materials, i.e., at a concentration of 5%, with the exception of the nonmodified silicone where low cell viability was observed (Fig. 4A). Cell viability in the 5% extract is above 70% as compared to the TCPS control material TCPS (Fig. 4B). According to ISO 10993-5, this result proves that the material is not cytotoxic. Live/dead staining is consistent with data obtained from resazurin reduction analyses (Fig. 5). In the case of 10% extract, a slightly lower number of live cells stained green, and a single dead cell stained red, can be seen. As the dilution of the extract increases, the number of live cells increases and the number of dead cells decreases. The most cytotoxic material was with silicone (S). The cell morphology for all materials was similar to that of the control sample – the cells were well flattened and polygonal in shape.

Studies of the materials in direct contact with MG63 osteoblast-like cells of MG63 also showed that the cells grew well on all materials and their number increased in the days they were cultured (Fig. 6). Modification of materials with chitosan was observed to deteriorate cell viability, these results were statistically significant. Live/dead staining confirmed the observations derived from metabolic activity tests (Fig. 7). In the following days, an increase in the number of cells was observed. Cells were flattened and spindle-shaped, resembling cells cultured on the TCPS control sample. In the samples with chitosan, where the polymerisation initiator was platinum, the cells showed a tendency for aggregation and growth in the spots. This

might be due to the synergistic effect of chitosan and platinum.

5. Conclusions

This publication described characteristics of the structure and properties of various elastomers modified with chitosan. We evaluated their mechanical properties, hardness and migration of substances from the samples into distilled water, along with their antibacterial properties, cytotoxicity, cytocompatibility and biological properties when in contact with bone cells. On the analyses conducted and the results obtained, it was found that:

1. The greatest increase in hardness was observed in samples containing acrylonitrile-butadiene rubber (NBR) and ethylene-propylene rubber (EPDM).
2. For mixtures with silicone and acrylonitrile-butadiene rubber, better interaction between the rubber and chitosan was observed. Consequently, this resulted in an increase in tensile strength and elongation at break.
3. The highest strength was obtained for materials with light crepe and silicone rubber with a platinum catalyst – both with and without the addition of chitosan. The samples varied significantly in terms of hardness, which could influence their areas of application.
4. Elastomeric materials with the best antibacterial properties were characterized by very good chitosan dispersion.
5. The materials obtained can come into contact with water, which would be of great importance, among others, in the case of medical applications.
6. The silicone rubbers modified with chitosan represent much better cytocompatibility with model cells in both indirect and direct tests.
7. The best bactericidal properties against *E. coli* and *S. aureus* bacterial strains compared to the other tested materials (>99.0–99.9% killed bacteria) were shown in samples with chitosan based on silicone (S-Ch) and silicone with a platinum catalyst (S-Pt-Ch).
8. The tested LC-Ch sample showed acceptable (R = 1.5–2.0) bactericidal properties against *E. coli* and *S. aureus* strains of bacteria compared to the control sample, reducing the number of viable bacteria. According to the calculations, the test sample reduced the number of viable *E. coli* and *S. aureus* cells by 96.3% and 97.2%, respectively, for each strain.
9. The other samples tested showed poor or no biocidal properties against the selected bacteria used in the analyses.

The results obtained indicate good antibacterial and physico-mechanical properties in silicone-based materials (S-Ch and S-Pt-Ch), along with acceptable biological properties in contact with mammalian cells. This indicates the possibility of applying these materials to produce different medical devices and use them in the medical environment, where the adhesion and growth of bacteria should be prevented.

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