COLLAGEN/($ZnTiO_3$/SiO_2) COMPOSITES OF AN WIDE SPECTRUM ANTIMICROBIAL ACTIVITY

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ABSTRACT

The aim of this study is to prepare collagen composites of an increased wide spectrum antimicrobial activity using zinc titanate embedded in a silane matrix ($ZnTiO_3$/SiO_2). Aiming to improve the predictability between the patient outcome and the in vitro antimicrobial testing, four Gram-negative, two Gram-positive bacterial strains, two fungi and seven mixed cultures are used in this study. A higher antimicrobial activity of Collagen/($ZnTiO_3$/SiO_2) composites as compared to that of Collagen/$ZnTiO_3$ ones is found against all test mono-microbial strains. Collagen/($ZnTiO_3$/SiO_2) composites demonstrate also an well expressed action against the mixed test bacterial cultures, in the most cases a better one compared to that against the bacteria participating in the corresponding mixed bacterial cultures. The increased antimicrobial effect is due to a more homogeneous distribution of the submicron $ZnTiO_3$ aggregates embedded in a silane matrix along the collagen fibrils and a formation of a specific, snail-like structure depicted by SEM. The specific activity against different microbial cells and mixed bacterial cultures is ascribed to features of the microbial cells: size, shape, cell membrane and walls as well as to different exopolymeric substances (EPSs) and their concurrent adsorption in cases of mixed bacterial cultures, as well as the EPSs concurrent adsorption being similar to the concurrent protein adsorption from blood plasma known as Vroman's effect. The wide spectrum high activity against fungi, mono- and multi-specie bacterial cultures makes the studied Collagen/($ZnTiO_3$/SiO_2) composites a promising antimicrobial biomaterial.

Keywords: embedded in a silane matrix zinc titanate ($ZnTiO_3$/SiO_2), collagen/($ZnTiO_3$/SiO_2) composites, antibacterial activity, mono-specie bacterial strains, multi-species bacterial cultures, antifungal activity.

INTRODUCTION

During the last decades, an extensive use of antibiotics worldwide led to a threatening situation referring to the resistance of a large number of bacteria. This requires the development of new effective antimicrobial compounds and treatments to solve the problem. The broad-spectrum antimicrobial agents are of special interest since the infections are usually caused by microbial mixes.

Collagen is a natural polymer frequently used in a variety of medical applications because of its excellent biocompatibility, bioresorbability and hemostatic activity. In different forms (powders, hydrogels, solutions, films, matrices, sponges), collagen is reported as an effective carrier of bioactive components [1 - 3]. The collagen-based biomaterials of an antimicrobial activity are attractive candidates for wound dressing and healing, tissue engineering, coatings, components of implantable devices, etc. One of the easiest and most effective ways to add an antimicrobial activity to the biomaterials,

among the large variety of known approaches, is the development of composites containing antimicrobial agent(s). Our idea is to exploit this easy approach for development of new antimicrobial collagen biomaterials using some newly synthesized chemical compounds of an expected antimicrobial activity and employing sol-gel cryogen drying technology to keep the natural biological activity of the collagen. Former publications describe the preparation, characterization and antimicrobial activity of collagen/$ZnTiO_3$ [4], collagen/RGO [5], collagen/(Ag/RGO) and collagen/(Ag/RGO/SiO_2) [6] porous nanocomposites. This investigation is focused on the preparation and evaluation the antimicrobial activity of collagen composites loaded with embedded in a silane matrix zinc titanate ($ZnTiO_3$/SiO_2) as an antimicrobial agent.

It is known that porous SiO_2 materials can easily adsorb various ions and organic molecules in their pores and on the surface [7]. Based on its chemical reactivity, tetraethoxysilane (TEOS) is used as a crosslinking agent of silicone polymers; a precursor to silicon dioxide core-shell nanoparticles production for drug delivery systems [8] and encapsulation of bioactive ingredients [9]. TEOS is often used to improve the dispersion of fillers, for example ultra-high dispersion of graphene in polymer composites [10] and of multi-walled carbon nanotubes [11]. SiO_2 particles are used as a component improving the functional performance of a number of other nano-composites, such as TiO_2/SiO_2 [12], SnO_2/SiO_2 [13] and others. Fibrous silica sponges are fabricated for tissue engineering [14]. SiO_2 is accepted as one of the most promising carriers for development of high performance antibacterial and bactericidal materials, such as Ag-loaded SiO_2 (Ag/SiO_2) [15]. The ability of the silica matrix to improve the dispersion and hence to reduce the agglomeration of the nanoparticles is also known [15-17]. There are reports on the good antimicrobial activity of nano-composites, consisting of silver nanoparticles (AgNPs) embedded in a matrix of amorphous silicon dioxide (SiO_2). Such nano-composites inhibit the microbial growth due to a surface contact with the silver/silica particles [18, 19]. Ag/SiO_2 nanoparticles and Ag/TiO_2 embedded in TEOS matrix demonstrate an improved antibacterial activity [20, 21]. An increased antimicrobial action of Ag/RGO nanoparticles embedded in TEOS is reported earlier for Collagen/(Ag/RGO/SiO_2) composites compared to that of Collagen/Ag/RGO ones [6].

The promising bread spectrum biological activity of Collagen/$ZnTiO_3$ composites is demonstrated in a previous investigation [4]. No literature reports are found on collagen composites containing zinc titanate ($ZnTiO_3$/SiO_2) embedded in a silane matrix although they hold promise for a higher antimicrobial activity of a wide spectrum, based on the known effect of the silane matrix. The *in vitro* antimicrobial testing is usually made against mono-specie microbial strains, most often against one Gram-negative and one Gram-positive bacterium but the clinical infections are most often caused by microbial mixtures. For increased predictability of the patient outcome and the *in vitro* antimicrobial testing, an employment of test mono- and multispecies bacterial cultures and fungi is required together with an evaluation of the cytotoxicity observed. Unfortunately, the scientific data on the preparation and use of multi-specie microbial cultures is scarce.

Therefore this study is aimed at the preparation of new Collagen/($ZnTiO_3$/SiO_2) composites using $ZnTiO_3$ embedded in a silane matrix as an antimicrobial agent and the evaluation of their biological activity against mono- and multi-specie bacterial cultures (Gram-negative, Gram-positive bacteria and their mixed cultures) and fungi as well as the cytotoxicity to different types eukaryotic cells.

EXPERIMENTAL

Preparation of zinc titanate ($ZnTiO_3$/SiO_2) embedded in a silane matrix

$ZnTiO_3$ used in this investigation was in the form of a powder of self-synthetized aggregates whose characteristics referred to those described in refs. [22,4]. The sol-gel method was employed for its embedding in a silane matrix by mixing $ZnTiO_3$ dispersed in distilled water and ethanol (98 %, Aldrich) solution of tetraethyl orthosilicate (TEOS, 98 %, Aldrich) followed by gelation and drying to obtain $ZnTiO_3$/SiO_2. Briefly, 25 wt. % $ZnTiO_3$ dispersion in distilled water was prepared under ultra-sonication for 2 h. 1:1 (v:v) solution of tetraethyl orthosilicate (TEOS, 98 %, Aldrich) in ethanol (98 %, Aldrich) was prepared under magnetic stirring for 10 min. 75:25, wt. % mixture of water dispersion of $ZnTiO_3$ and ethanol solution of TEOS was obtained under magnet stirring for 10 min, and then a catalyst (di-butilthin di-laurat (Aldrich) was added (1:10 with TEOS) to cause

gelation within 1 h at a room temperature. The final product was obtained after vacuum drying.

Preparation of Collagen/($ZnTiO_3/SiO_2$) porous composites

Type I fibril collagen gel of a concentration of 2.64 wt. % was extracted from calf hide using a technology previously described [23]. The concentration of the collagen gel was adjusted at 1 % and pH of 7.3 (corresponding to the physiological medium) using 1 M sodium hydroxide. $ZnTiO_3/SiO_2$ powder was added at ratios Collagen: ZnTiO3/SiO2 = 2:1, 2:0.8, 2:0.6, 2:0.4, or 2:0.2 (wt.: wt.). Then the obtained Collagen/($ZnTiO_3/SiO_2$) composites were cross-linked with 0.5 % glutar aldehyde (to dry collagen) at 4 °C for 24 h and lyophilized at −40°C to obtain a sponge material within 48 h using a Martin Christ freeze-dryer following the procedure described in ref.[24].

SEM observations of the porous composites

JEOL SEM, model JSM-35 CF, Japan apparatus was used to observe the morphological features of the studied Collagen/($ZnTiO_3/SiO_2$) composites. The samples were gold-sputtering coated and viewed in the second electron mode with a field emission gun. This provided the observation of the composites in absence and presence of microbial cells. Those containing microbial cells were subjected to preliminary drying within a week at a room temperature

Antimicrobial activity testing

The test microbial strains: Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853; *Pseudomonas putida*, ATCC 10536; *Esherihia coli*, ATCC 10536, *Salmonela holeresius,* DSMZ 4224), Gram-positive bacteria (*Staphylococcus epidermidis*, ATCC 12228; *Bacilus cereus*, ATCC 11778 and fungi (*Candida lusitaniae* 74-4 – laboratory strain; *Saccharomyces cerevisiae* ATCC 9763) were provided by the National Bank of Microorganisms and Cell Cultures (NBIMCC), Bulgaria and cultured in the most suitable medium. *E. coli, S. epidermidis*, *S. enterica* were grown in a nutrient broth and a nutrient agar (NB, NA - Conda, Spain) respectively at 37°C and 180 rpm for 18 h. *B. cereus*, *C. lusitaniae* were propagated in a nutrient medium #14 NBIMCC (Meat extract 10.0; peptone 10.0; NaCl 5.0; Agar 20.0; Distilled water 1 L; pH = 7.2 - 7.4) and YGC (VWR Prolabo Chemicals) at 30°C and 120 rpm, respectively. *P. putida* was cultivated in a synthetic liquid medium (ISO10712) at 22°C - 23°C and 180 rpm for 12 h. Test cultures were prepared in an exponential phase after three consecutive cultivation in a liquid nutrient medium at180 rpm and 24°C for *P. putida*, 30°C for *B. cereus* and 37°C, respectively. The pure culture of every strain was prepared as a bacterial suspension in an exponential phase with OD 0.5 Mc Farland. 100 µL quantities of each were introduced drop wise and randomly distributed in a solid agar nutrient medium. Then discs of the investigated material were put on them. The plates were left for 20 h at 4°C - 6°C to afford the nanoparticles diffusion and after that cultivated for 24 h at 37°C, 30°C and 24°C, respectively. The formed sterile zones around the disks samples (diameter of 12.0 mm; thickness of 3 mm) were measured in mm (±0.5). All results are average of 6 measurements.

Preparation of mixed bacterial test cultures

The following mixed bacterial cultures were selected for this investigation: *E. coli* + *P. aeruginosa* (representatives of *Gracilicutes* bacteria); *B. cereus* + *S. epidermidis* (belonging to *Firmicutes* bacteria); *E. coli* + B. *cereus* and *E. coli* + *S. epidermidis* (combinations of bacteria with two types cell wall structure); and combination of *P. aeruginosa* with Gram-positive bacteria, both *B. cereus* and *S. epidermidis*). Unfortunately, no standard exists for the preparation of a mixed bacterial test culture. Therefore the mixed bacterial cultures used in this investigation were prepared following the protocol described above referring to mono-species bacterial cultures. The mix culture was prepared using 100 µl of every pure bacterial culture in an exponential phase and randomly spread on Nutrient agar.

Cytotoxicity testing

In vitro cytotoxicity was tested following the requirements of ISO 10993 – 5 standards. A crystal violet assay was employed to quantify the viability of three types of eukaryotic cells on the collagen/($ZnTiO_3/SiO_2$) composites studied. The eukaryotic cells: osteoblast, MG-63; fibroblast, 3T3 and kidney epithelial, MDCK II, were provided from the NBIMCC, Bulgaria. The eukaryotic cells were maintained at standard conditions in humidified atmosphere with 5 % CO_2 at 37°C in F12 or DMEM (SIGMA) medium. For assessment of cytotoxicity, the samples were embedded in 96 wells-

plates and hydrated with 200 µl culture medium for 12 h - 24 h. Then the cells were seeded at a concentration of $1x10^5$cells/ml. After 24 h, the cytotoxicity was evaluated on the ground of the crystal violet test [6]. The residual cell monolayer was washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde in PBS for 15 min. After that the plates were washed with water and 200 µl 0.1 % crystal-violet solutions were added to every well. After 20 min incubation at a room temperature, the plates were washed with water and the protein-bound dye (which is corresponding to the cell number) was solubilized with 200 µl 10 % acetic acid. The values of the optical density were read on a micro plate reader (EPOCH UV/VIS Spectrometer) at 570 nm wavelength. The number of the vital cells was calculated as a percentage on the ground of their total amount.

RESULTS AND DISCUSSION

SEM images

Fig. 1 depicts the morphology of $ZnTiO_3$ embedded in a silane matrix used as an antimicrobial agent in this investigation. The aggregation of $ZnTiO_3/SiO_2$ particles is clearly seen in Figs. 1(a), 1(b) and 1(c). The submicron particles building up the relatively large aggregates can be observed at a magnification high enough, x10000 (Fig. 1(c)). These aggregates are weak and easily destructing under some pressure. This provides their fine dispersion during the preparation of Collagen/($ZnTiO_3/SiO_2$) composites.

Fig. 2 illustrates the porous structure of a collagen composite at a weight ratio Collagen:$ZnTiO_3/SiO_2$ = 2:1. The pictures of the other Collagen/($ZnTiO_3/SiO_2$) composites of lower concentrations of the antimicrobial agent (Collagen: $ZnTiO_3/SiO_2$ = 2:0.8; 2:0.6; 2:0.4 and 2:0.2, wt.:wt.) are similar. Therefore they are not presented here. Fig. 2(a) depicts the open and interconnected, relatively homogeneous, porous structure of Collagen/($ZnTiO_3/SiO_2$) composite. Fig. 2(b) and Fig. 2(c) show a very interesting, specific spiral organization of the collagen fibrils in snail-like structures due to the submicron particles of zinc titanate (embedded in a silane matrix) incrusted on their surface. These submicron particles are very well seen at a higher magnification, x10000 (Fig. 1(d)). The morphology of Collagen/($ZnTiO_3/SiO_2$) composites is quite different as compared to that of porous collagen composites prepared following the same route but containing $ZnTiO_3$ with no silane matrix (4, Fig. 3). This indicates the significant effect of the silane matrix on the structuring of the collagen composite. This SEM observation demonstrates the lack of large aggregates of an antimicrobial agent observed earlier in case of Collagen/$ZnTiO_3$ composites. The zinc titanate, $ZnTiO_3/SiO_2$, embedded in a silane matrix is more homogeneously distributed in the collagen composites in the form of submicron particles on the collagen fibrils surface.

SEM observation is carried out of the surface of samples with seeded microbial cells after drying at a room

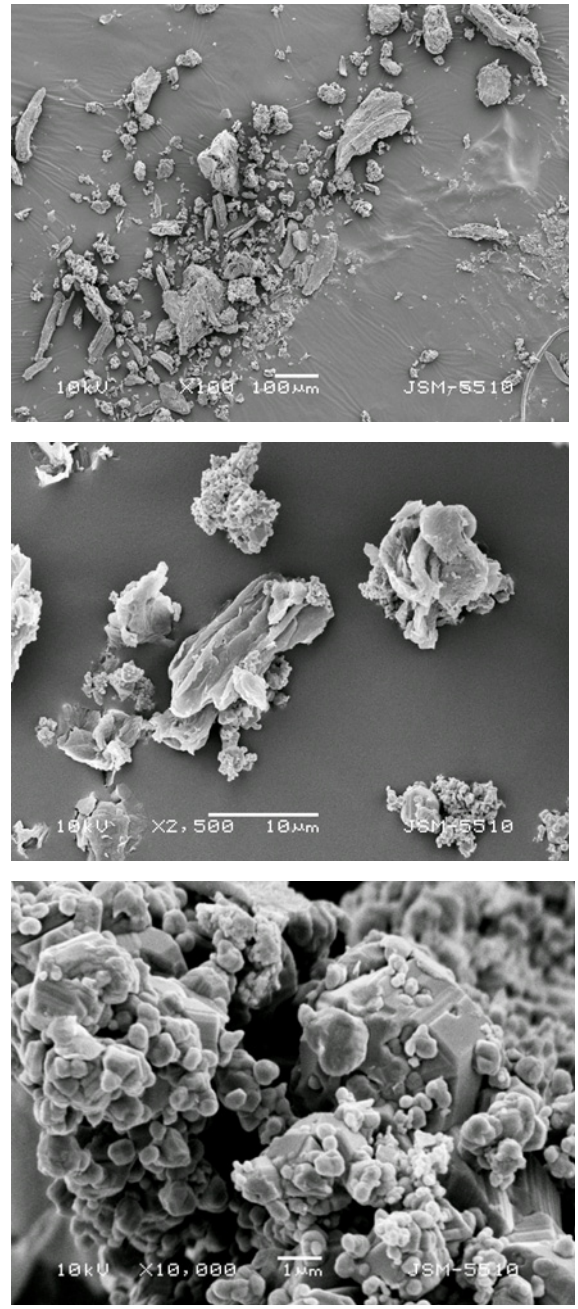


Fig. 1. SEM images of $ZnTiO_3/SiO_2$ aggregates at different magnifications: (a) - x100; (b) - x2500 and (c) - x10000.

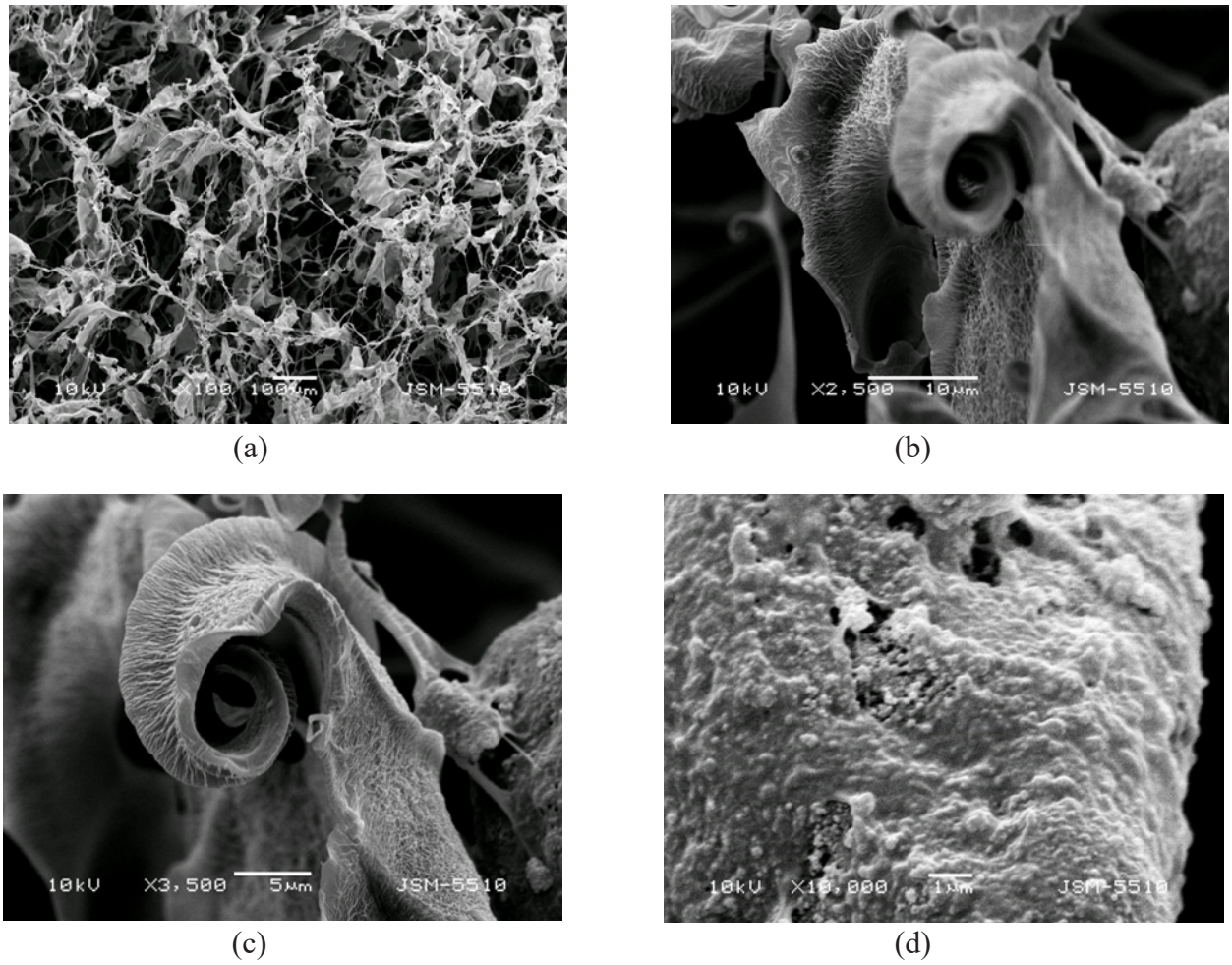


Fig. 2. SEM images of a porous collagen composite (Collagen : $ZnTiO_3/SiO_2$ = 2 : 1, wt. : wt.) at different magnifications: a) - x100; b) - x2500, c) - x3500; d) - x10000.

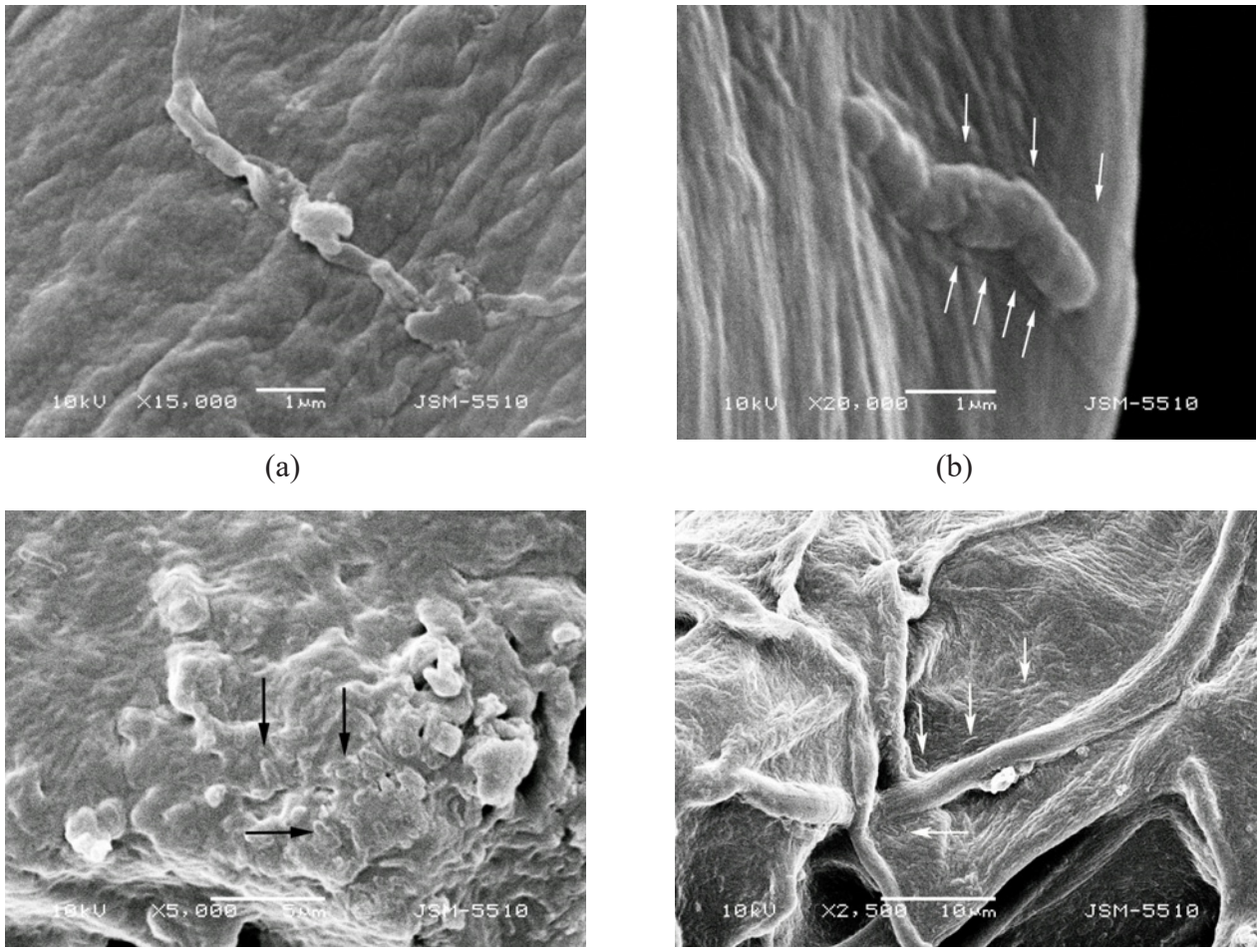
temperature expecting that it would provide understanding in respect to the interaction of the test microbial cultures with the Collagen/($ZnTiO_3/SiO_2$) composites studied. Fig. 3 presents some of the most characteristic appearances of the test bacterial cells: Fig. 3(a) and Fig. 3(b) depict 2 Gram-negative bacteria, *P. aeruginosa* I and *E. coli*, respectively. Fig. 3(c) depicts the Gram-positive bacterium, *B. cereus*, while Fig. 3(d) presents a mixed culture of Gram-positive, *B. cereus* and Gram-negative, *P. aeruginosa*.

The deformed chain of *P. aeruginosa* cells is seen in Fig. 3(a), while the disrupted cell surface of *E. coli* and the leaked material of the cells content are observed in Fig. 3(b). The arrow's heads points at the surrounding material as a halo and a tail around and after the bacterial chain. A lot of deformed cells of *B. cereus* are seen in Fig. 3(c). Fig. 3(d) shows cells near the collagen fold at the first plane. They are without volume, with a big concavity in the middle and aperture at some of them. The arrows point at the perforated cells in the fold at the left and the right side of the picture. Although not all pictures are presented here, the SEM observation demonstrates deformed bacterial cells, disrupted bacterial cell membranes and cell content leaked on the collagen surface. These effects can result from some helation of the metal ions and/or an interaction of the bacterial cells with reactive oxygen species formed with participation of the antimicrobial agent, $ZnTiO_3/SiO_2$.

Antimicrobial activity

Activity against monocultures of bacteria and fungi

The antimicrobial activity of the studied Collagen/($ZnTiO_3/SiO_2$) composites (as a sterile zone in mm) against mono species microbial strains, Gram-negative and Gram-positive as well as against some mixed



(a) (b)

(c) (d)

Fig. 3. SEM images of microbial cells on Collagen: $ZnTiO_3/SiO_2$ = 2 : 1, wt. : wt. composite: (a) and (b) - Gram-negative bacteria, *E. coli* and *P. aeruginosa*, respectively; (c) - Gram-positive bacterium, *B. cer*eus; (d) - mixed cell culture consisting of Gram positive (*B. cereus*) and Gram-negative (*P. aeroginosa*) bacterium.

bacterial cultures are presented in Tables 1, 2 and 3, respectively. The data referring to samples 1-5 in Table 1 show, as expected, a concentration dependent activity against 4 test Gram-negative microbial strains (*E. coli, P. aeroginosa, P. putida, S. holereius*) that is well expressed at Collagen:$ZnTiO_3/SiO_2$ ratios of 2:0.6 and above (wt./wt.). A comparison to the corresponding samples containing the same amounts of $ZnTiO_3$ (Table 1, samples 6-10) indicates the higher activity of those containing $ZnTiO_3$ embedded in a silane matrix ($ZnTiO_3/SiO_2$): the sterile zones for *E. coli* and *S. Enterica* are higher and an activity appears against *P. putida* in case of samples 1-5 (Table 1).

The data in Table 2 demonstrates the specific and concentration dependent activity of the Collagen/($ZnTiO_3/SiO_2$) composites studied (Table 2, samples 1 - 5) against 2 Gram-positive bacteria (*S. epidermidis, B. cereus*) and 2 fungi (*C. lucitania, S. cerevisiae*). It is very well expressed at samples 1-3 (Collagen:$ZnTiO_3/SiO_2$ = 2:1, 2:0.8 and 2:0.6, wt.:wt.). Very interesting is the activity against the test fungus cultures: *C. Lucitania* and *S. cerevisiae*. The activity against the same microbial species (bacteria and fungi) of the corresponding Collagen/$ZnTiO_3$ composites is also presented in this table (Table 2, samples 6-10) aiming a comparison. The activity of Collagen/($ZnTiO_3/SiO_2$) composites (samples 1 - 5) against *S. epidermidis, B. cereus* and *C. lucitania* is significantly higher than that of Collagen/$ZnTiO_3$ one (samples 6-10). The sterile zones are larger for the three Gram-positive test microbial species at all studied concentrations of the antimicrobial agent when it is embedded in a silane matrix, as is the case for the Gram-negative bacteria (Table 1). The increased activ-

Table 1. Antimicrobial activity (presented as a sterile zone, mm) of collagen composites, loaded with different amounts antimicrobial agent (AMA): $ZnTiO_3/SiO_2$ (Samples 1 - 5) or $ZnTiO_3$ (Samples 6 - 10), against Gram-negative microbial strains.

Sample No	Collagen : AMA (wt./wt.)	Sterile zone, mm			
		E. coli	*P. aerugin.*	*P. putida*	*S. holeresius*
	Coll./($ZnTiO_3/SiO_2$)				
1	2 : 1.0	14.9±1.5	14.0±1.4	16.1±1.3	17.9±1.9
2	2 : 0.8	16.9±1.1	12.2±0.8	-	16.1±0.6
3	2 : 0.6	11.8±2.6	13.0±1.0	12.2±0.6	14.0±1.1
4	2 : 0.4	8.0±0.3	-	0	7.2±2.1
5	2 : 0.2	2.1±1.2	-	0	1.3±0.2
	Coll./$ZnTiO_3$				
6	2 : 1.0	10.4±0.6	-	0	7.9±1.3
7	2 : 0.8	8.5±1.2	-	0	4.5±0,4
8	2 : 0.6	4.5±0.9	-	0	3.5±1.0
9	2 : 0.4	3.5±0.3	-	0	1.0±0.3
10	2 : 0.2	0	-	0	0

ity of the new collagen composites containing $ZnTiO_3$ embedded in a silane matrix against Gram-negative and Gram-positive microbial species demonstrates the significance of the silane matrix. Its positive effect could result from a relatively homogeneous distribution of the antimicrobial agent as finer submicron particles along the collagen fibrils leading to their spiral organization in specific snail-like structures observed by SEM (Fig. 2).

Activity against mixed bacterial cultures

As it is evident from the data in Table 3, the new developed Collagen/($ZnTiO_3/SiO_2$) composites demonstrate an antimicrobial activity which is concentration dependent and specific for the mix cultures. It is identical with that against the test monocultures (Table 1 and Table 2). The activity against the mix culture of two Gram-negative bacteria, *E. Coli+P. aeruginosa* (Table 3, the first column) is slightly lower compared to that against the mono cultures of *E. coli* and *P. aeruginosa* (Table 1), whereas for the mix culture of two Gram-positive bacteria, *S. epidermidis+B. cereus* (Table 3, the second column) the activity is significantly increased as compared to that against the monocultures of *S. epidermidis* and *B. cereus*. The activity against the double mixed cultures consisting of Gram-negative and Gram-positive bacterium (Table 3, columns 3-6) is dependent on the bacteria participating in the corresponding mixed culture: it is the lowest for the couple *E. coli+S. epidermidis* (the third column) followed by the couple *P. aeroginosa+S.epidermidis* (the fits column); it is higher for the couple *E. coli+B. cereus* (column fourth) followed by the couple *P. aeroginosa+B. cereus* (column sixth). It is interesting that the activity against the couples containing the Gram-positive bacterium *S. epidermidis* (Table 3, columns 3 and 5) is lower compared to the activity against the couples containing Gram-positive bacterium *B. cereus* (Table 3, columns 4 and 6). Most interesting is the highest and very well expressed activity against a mixed culture consisting of four bacteria (Table 3, the last column), the same Gram-negative and Gram-positive bacteria participating in the double mixed test cultures. The results on the antimicrobial activity against mixed test bacterial cultures indicate complicated interactions between the bacteria in the mixed cultures depending on the characteristics of the corresponding bacterium. Such interactions most probably define the different resistance to an identical antimicrobial composite.

Table 2. Antimicrobial activity (presented as a sterile zone, mm) of collagen composites, loaded with different amounts antimicrobial agents (AMA): $ZnTiO_3$/ SiO_2 (Samples 1-5) or $ZnTiO_3$ (Samples 6 - 10) against Gram-positive bacteria and fungi.

Sample No	Collagen : AMA (wt./wt.)	Sterile zone, mm			
		S. epidermidis	*B. cereus*	*C. lucitania*	*S. cerevisiae*
	Coll/($ZnTiO_3$/SiO_2)				
1	2 : 1.0	19.6 ±1.9	24.3 ±0.6	19.9±2.1	15.0± 0.4
2	2 : 0.8	21.0±5.0	12.0±1.2	14.1±1.0	26.8 ±1.2
3	2 : 0.6	12.0±2.0	12.6±0.7	15.3±0.7	12.9± 0.9
4	2 : 0.4	10.8±0.3	9.1±1.0	10.3±1.1	9.7±0.6
5	2 : 0.2	6.0±1.9	4.0±1.2	7.3±0.9	0
	Coll/$ZnTiO_3$				
6	2 : 1.0	12.5±1.0	9.5±0.5	13.3±0.2	-
7	2 : 0.8	9.9±0.5	9.0±0.9	11.0±1.6	-
8	2 : 0.6	10.0±1.2	8.0±1.0	12.3±1.3	-
9	2 : 0.4	6.2±1.8	5.0±1.3	2.2±0.6	-
10	2 : 0.2	5.1±1.5	3.5±0.3	5.5±0.3	-

Cytotoxicity

Crystal violet staining with colorimetric quantification is used for *in vitro* evaluation of the cytotoxicity of the studied Collagen/($ZnTiO_3$/SiO_2) nano-composites loaded with different amounts of the antimicrobial agent (Collagen : $ZnTiO_3$/SiO_2 = 2:1; 2:0.8; 2:0.6; 2:0.4, wt./wt.). The viability of three types of eukaryotic cells (osteoblasts, fibroblasts and epithelial) on the Collagen/($ZnTiO_3$/SiO_2) composites studied is presented in Table 4. It is evident that the viability of the different eukaryotic cells is different. It is the lowest and below 50 % in case of the osteoblast cells, MG63 at ratios Collagen: $ZnTiO_3$/SiO_2 = 2:1 and 2:0.8, wt./wt. (samples 1 and 2), whereas it is above 50 % at ratios Collagen:$ZnTiO_3$/SiO_2 = 2:0.6 and below, wt./wt (samples 3 and 4). The viability of fibroblasts, 3T3 and of epithelial cells, MDCK II is above 50 % on all composites studied (samples 1-4) excluding the viability of epithelial cells, MDCK II, on the composite of the highest concentration (sample 1). The data in Table 4 indicates that generally, the composites Collagen: $ZnTiO_3$/SiO_2 = 2:0.8 wt.:wt. and below should be preferable in view of a low cytotoxicity.

Crystalline $ZnTiO_3$ (with particles size of about 8 nm, containing around 13 % amorphous phase consisting of ZnO and TiO_2 in semi-equal amounts [4] embedded in a silane matrix is used as an antimicrobial agent in this study. It is entrapped in a porous collagen matrix by sol-gel cryogen drying to produce Collagen/($ZnTiO_3$/SiO_2) composites. No chemical interactions are expected under these conditions. The biological activity of such composites is due to the presence of $ZnTiO_3$ nanocrystal aggregates embedded in a silane matrix and small amounts of ZnO, TiO_2 and SiO_2 introduced through the antimicrobial agent. Unfortunately, the mechanism of the biological activity of $ZnTiO_3$, ZnO, TiO_2 and SiO_2 is not fully understood so far.

A mechanical demolition of the cell envelope and membrane, similar to that of titanium-doped ZnO at *E. coli* and *Staphylococcus sp*. [25], probably does not happen at Collagen/($ZnTiO_3$/SiO_2) composites because the sharp ages of the antimicrobial agent particles are hidden in a silane matrix as shown by SEM (Fig.1(c)).

Chelation of some Collagen/($ZnTiO_3$/SiO_2) composites metal ions with free oxygen and nitrogen electron

Table 3. Antimicrobial activity (presented as a sterile zone, mm) of collagen composites, loaded with different amounts antimicrobial agent $ZnTiO_3/SiO_2$, against mixed cultures: *E. Coli + Ps. aeruginosa* (both *Gram-negative*); *St. epidermidis + B. cereus* (both Gram-positive); *E. coli + St. epidermidis; E. Coli + B. cereus; Ps. aeruginosa +St. epidermidis; Ps. aeroginosa+B. cereus* (Gram-negative and Gram-positive bacteria); *E. coli+Ps. aeruginosa+S. epidermidis+B. cereus* (2 Gram-negative and 2 Gram-positive bacteria).

Sample No	Coll:$ZnTiO_3/SiO_2$ (wt.: wt.)	Sterile zone, mm / Mixed cultures						
		E.coli + Ps.aer. (- -)	*S. epid.+ B. cereus* (+ +)	*E. coli+ S. epid.* (- +)	*E. coli + B. cereus* (- +)	*Ps.aer. + S.epid.* (- +)	*Ps. aer.+ B. cereus (- +)*	*E. coli + Ps. aer. + S. epid. + B. cereus* (- - + +)
1	2 : 1	13.9±1.8	28.0±4.5	9±2.3	20.6±1.6	12,5±3.9	23.5±5.0	31,0±5.8
2	2 : 0.8	12.2±2.1	22.1.±5.0	9.5±3.1	21.9±2.2	12.0±4.3	21,5±6.2	36.1±3.6
3	2 : 0.6	12.8±1.3	27.3±4.8	9,5±3.9	11.1±2.6	17.0±2.9	9.0±3.9	12.9±5.0
4	2 : 0.4	7.6±1.5	13.1±3.6	6.1±4.1	18.7±1.2	-	12.3±4.1	16.9±3.5
5	2 : 0.2	2.6±1.6	3.9±2.8	2.1±1.1	-	-	3.9±1.8	2.0±1.42

couples in the peptide bonds may contribute to their biological activity. The negative charge of the microbial cell wall under the test conditions could be a reason for the intake of released metal ions chelated with collagen molecules. Engulfed in sufficiently high concentrations, the metal ions cause toxicity to any cells [26-28]. Some metal ions change the activity of succinate dehydrogenase or interact directly with MTT, leading to false results of an increased enzymatic activity [29]. This is found for Collagen/$ZnTiO_3$ composites [4].

The formation of reactive oxygen species (ROSs) due to the interaction of the antimicrobial agent particles and the microbial envelop [30-33] could determine the antimicrobial activity of the Collagen/($ZnTiO_3/SiO_2$) composites studied. The formation of ROSs between zink oxide nanoparticles and bacterial cells is already reported [34]. According to some authors, EPSs could not protect cells from the ROSs. They could penetrate the slime around the cell, to ruin the cell wall, and to disintegrate not only the cell membrane but also large macromolecules such as nucleic acids inside the cell. Thus they stop the cells propagation [35]. ROSs formation due to the interactions between water molecules around and in the microbial cells on one hand and the antimicrobial agent on the other can stipulate damages of any macromolecules of the cell [36]. The SEM observation (Fig. 3) of test bacterial cells on the antimicrobial Collagen/($ZnTiO_3/SiO_2$) composites demonstrates deformed cells, holes in the cells and liquid around them that is an evidence for cells destruction due to a probable

Table 4. Crystal violet assay of eukaryotic cells: osteoblast, MG63; fibroblast, 3T3 and kidney epithelial, MDCK II on Collagen/($ZnTiO_3/SiO_2$) composites.

Sample No	Collagen : $ZnTiO_3/SiO_2$ (wt. : wt.)	Eukaryotic cells viability, %		
		MG 63	3T3	MDCK II
1	2 : 1.0	29±9	61±3	45±7
2	2 : 0.8	45±10	82±12	79±9
3	2 : 0.6	59±9	89±10	90±13
4	2 : 0.4	71±18	91±7	89±20

chelation of the metal ions and interaction with ROSs.

In debt understanding of the mechanism of the microbial cells interaction with the Collagen/($ZnTiO_3$/SiO_2) composites is difficult on a number of reasons: the interaction of the cells and the biomaterials is mediated by secreted EPSs that are a complex mixture of polysaccharides, proteins, nucleic acids, lipids and humic substances [37]; the EPSs differ not only for the different cells, but also on the different surfaces in case of identical cells [38]; different strains from identical bacterial or fungal species may exhibit different phenotypes significantly influencing their ability to form a biofilm on the surface [39 - 41]; the microorganisms can alter the surface roughness and topography through penetration of secreted EPSs [42]. The interactions of the microbial cells with the biomaterials are more complicated in case of multi-microbial infections because of interspecies interactions resulting in emergent properties of the system [43]. Therefore, an *in vitro* estimated antimicrobial activity against microbial mono-cultures is not relevant to the multi-microbial infections. Multispecies biofilms have usually higher tolerance towards antimicrobial agents, than that expected from assessment of mono-specie biofilms studied individually [44, 45], i.e. the activity against microbial mixtures is usually lower than that to mono specie bacterial strains. Concurrent absorption of EPSs at multi-microbial infections can contribute to such differences, similarly to the concurrent protein adsorption from blood plasma, known as Vroman effect [46].

The Collagen/($ZnTiO_3$/SiO_2) composites investigated demonstrate a specific antimicrobial activity to mono-specie microbial cultures both Gram-negative (Table 1) and Gram-positive (Table 2) and different toxicity to the different eukaryotic cells. The specific antimicrobial activity could be connected, in addition to the differences in the secreted EPSs, to the differences in the cell size, shape and wall structures: the thin peptidoglycan layer and the external membrane in case of Gracilicutes (Gram-negative bacteria: *E. coli, S. e*nterica and *Pseudomonas spp.*), and the thick peptidoglycan layer at Firmicutes (Gram-positive bacteria: *B. cereus*, Staphylococcus epidermidis). The yeasts as a section of fungi (*C. lusitanie* and *S. cerevisiae*) have a very different cell wall structure, more rigid and robust, than the bacterial one [47]. The differences in the composition of the secreted EPSs and their concurrent adsorption most probably contribute to the specific antimicrobial activity, observed also against mixed test microbial cultures.

The verified higher antimicrobial activity of the Collagen/($ZnTiO_3$/SiO_2) composites as compared to that of Collagen/$ZnTiO_3$ one is due to the embedding of the antimicrobial agent in a silane matrix. The antimicrobial agent particles embedded in a silane matrix are relatively homogeneously distributed along the collagen fibrils as fine submicron aggregates. This causes a specific spiral organization of the incrusted collagen fibrils in a specific, snail-like structure as demonstrated by SEM (Fig. 3). Both, the better dispersion of the antimicrobial agent in form of smaller aggregates and the special structuring of the fibrils contribute to the increased antimicrobial activity of the new Collagen/($ZnTiO_3$/SiO_2) composites.

CONCLUSIONS

New Collagen/($ZnTiO_3$/SiO_2) composites of a high wide spectrum antimicrobial activity against mono- and multi species bacterial cultures and fungi are successfully prepared by sol-gel cryogen drying using $ZnTiO_3$ embedded in a silane matrix as an antimicrobial agent.

Their activity against mixed bacterial cultures is even higher than that against the corresponding mono-specie bacterial strains.

The specific activity toward different mono- and mixed microbial cultures is ascribed to specific features of the microbial cells (size, cell wall and membrane), differences in the composition of the secreted EPSs and concurrent adsorption in case of mixed cultures.

The embedding of $ZnTiO_3$ in a silane matrix guaranties a more homogeneous distribution of its submicron aggregates along the collagen fibrils forming a specific snail-like structure of the porous Collagen/($ZnTiO_3$/SiO_2) composites. Both, the finer dispersion of the antimicrobial agent and the formed specific porous structure contribute to their increased wide spectrum antimicrobial activity when compared to that of similar collagen composites containing $ZnTiO_3$ but no silane matrix.

An optimal balance between the antimicrobial activity and cytotoxicity is found for the composites Collagen : $ZnTiO_3$/SiO_2 = 2 : 0.8 – 2 : 0.4, wt/wt.

The verified high antimicrobial activity against Gram-negative, Gram-positive bacteria, their mixed cultures and fungi makes the newly developed Collagen/($ZnTiO_3$/SiO_2) composites a promising antimicrobial biomaterial.

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