PREPARATION AND ANTIMICROBIAL ACTIVITY OF FUCOIDAN CONTAINING COLLAGEN/($ZnTiO_3$/SiO_2) COMPOSITES

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ABSTRACT

The aim of this investigation was to develop collagen based composite biomaterial with improved antimicrobial activity using a combination of antimicrobial agents consisting of zinc titanate embedded in a silane matrix, ($ZnTiO_3$/SiO_2) and fucoidan at varied concentrations. The morphology of the investigated porous collagen/($ZnTiO_3$/SiO_2)/fucoidan composites was observed by SEM and their antimicrobial activity was evaluated against four Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Pseudomonas putida, Salmonela holeresius), two Gram-positive bacteria (Staphylococcus epidermidis, Bacillus cereus) and two fungi (Candida Lusitania, Saccharomyces cerevisiae) by disk diffusion test.

Broad-spectrum antimicrobial activity of the studied porous collagen/($ZnTiO_3$/SiO_2)/fucoidan composites was demonstrated, specific toward the different test microbial strains and dependent on the fucoidan concentration. The specific activity toward different microbial cultures was ascribed to the features of the microbial cells (size, shape, cell wall and membrane) and differences in the composition of the secreted exopolymeric substances. It was found that both, the formed interconnected open porous structure of the mixed collagen/fucoidan matrix with fine dispersed submicron $ZnTiO_3$/SiO_2 particles along the marix fibrils and the own antibacterial activity of the fucoidan, contribute to the increased wide spectrum antibacterial activity compared to that of similar collagen composites do not containing fucoidan.

Keywords: collagen/($ZnTiO_3$/SiO_2)/fucoidan composites, morphology, antimicrobial activity.

INTRODUCTION

As a natural polymer with excellent biocompatibility, bioresorbability and hemostatic activity, collagen is frequently used in a variety of medical applications. Collagen-based biomaterials with antimicrobial activity are attractive candidates for wound dressing and healing, tissue engineering, coatings, components of implantable devices, etc. One of the most easiest and effective ways to add antimicrobial activity of biomaterials, among the large variety of known approaches, is the development of composites with participation of antimicrobial agents.

Sharply increasing microbial resistance to currently used antibiotics and multidrug treatments rises a need to look for new antimicrobial agents. With the idea to exploit this easy approach for development of antimicrobial collagen biomaterials, we started a serial investigation, using some newly synthesized chemical compounds, natural substances and their combinations with expected biological activity as new antimicrobial agents.

Currently, many antimicrobial compounds and nanoparticles are known whose activity is often associated with the presence of some metal ions, such as silver-, zinc-, titanium- and others [1 - 9]. New in this respect, collagen/$ZnTiO_3$ porous nanocomposites were developed at the start of our serial investigation and for the first time was demonstrated their broad-spectrum antimicrobial activity, combined with a low cytotoxicity to eukaryotic cells at optimal loading level of the new antimicrobial agent, $ZnTiO_3$ [10].

SiO_2 is accepted as one of the most promising carriers for development of high performance antibacterial and bactericidal materials [11]. The ability of the silane matrix to improve the dispersion of embedded nanoparticles and hence to reduce their agglomeration is well known [11 - 21]. No literature reports were found about collagen composites containing embedded in a silane matrix zinc titanate ($ZnTiO_3/SiO_2$) as a new antimicrobial agent although they hold promise for improved antimicrobial activity. In a following investigation, it was demonstrated that embedding of $ZnTiO_3$ in a silane matrix guaranties a more homogeneous distribution of its submicron aggregates along the collagen fibrils, organized in spiral snail-like structures. It was supposed that both, the finer dispersion of the antimicrobial agent ($ZnTiO_3/SiO_2$) and the specific porous structure contribute to the increased antimicrobial activity of the collagen/($ZnTiO_3/SiO_2$) composites, compared to that of similar collagen composites, containing $ZnTiO_3$ without silane matrix [22].

Marine organisms deliver bioactive substances with a high potential to act as anti-microbial agents against microbial pathogens causing infections in human body. This potential is not enough utilized so far. The antimicrobial activity of fucoidans is well known [23 - 28], but not fully explored. Being natural, practically non-toxic and with wide range biological activities, all depending on the source, chemistry and derivation technology, the fucoidans are considered now as promising antimicrobial substances that could replace some of the currently used antibiotics. Crude fucoidan extracts, derived from brown seaweeds, are commercially available as nutritional supplements [23]. The fucoidan antimicrobial effect in collagen biomaterials was not studied and practically not explored so far. This motivated us to try to additionally improve the antimicrobial activity of earlier developed by us, promising as antimicrobial biomaterial, collagen/($ZnTiO_3/SiO_2$) composites by use a fucoidan as a boosting antimicrobial agent. Thus, the development of collagen based composite biomaterials with improved antimicrobial activity by exploring a combination of antimicrobial agents consisting of zinc titanate embedded in a silane matrix, ($ZnTiO_3/SiO_2$) and fucoidan, got the aim of this study.

EXPERIMENTAL

Antimicrobial agent

The used in this investigation antimicrobial agent was a combination of embedded in a silane matrix zinc titanate, $ZnTiO_3/SiO_2$ and fucoidan. The embedded in a silane matrix zink titanate ($ZnTiO_3/SiO_2$) was the same as the used in a former investigation and prepared, as it was described earlier [22]. Brifly, sol-gel method was employed for embedding, that includes mixing a dispersed in distilled water $ZnTiO_3$ and ethanol (98 %, Aldrich) solution of tetraethyl orthosilicate (TEOS, 98 %, Aldrich), followed by gelation under room temperature (using di-butilthin di-laurat (Aldrich) as a catalyst) and finally vacuum drying. The fucoidan was extract from *Undaria pinnatifida,* 95 % (Sigma Aldrich).

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

Two collagen composites with low cytotoxicity to eukaryotic cells (osteoblast, MG 63; fibroblast, 3T3 and kidney epithelial, MDCK II) and moderate wide spectrum antimicrobial activity [22], were selected as basic matrixes for this investigation, namely collagen/($ZnTiO_3/SiO_2$) = 1.0 : 0.2 wt./wt. and collagen/($ZnTiO_3/SiO_2$) = 1.0 : 0.3 wt./wt. The loading level of fucoidan was varied as it is described below.

Type I fibrilar collagen gel with concentration of 2.64 wt. % was extracted from calf hide using previously described technology [29]. The concentration of the

collagen gel was adjusted at 1 % and then the pH was adjusted at 7.3 (that is of the physiological medium) by 1 M sodium hydroxide; $ZnTiO_3/SiO_2$ powder was added at constant ratio Collagen:($ZnTiO_3/SiO_2$) = 1 : 0.2 wt/wt; or Collagen:($ZnTiO_3/SiO_2$) = 1 : 0.3 wt/wt and fucoidan was added at varied ratios to them : 0.1; 0.2; 0.3; 0.4; 0.5 or 0.6. Then the obtained Collagen/($ZnTiO_3/SiO_2$)/fucoidan composites were cross-linked with 0.5 % glutar aldehyde (to dry collagen) at 4°C for 24 h and lyophilized at −40°C for 48 h to obtain a sponge material, using a Martin Christ freeze-dryer, as it was previously described [30].

The numbering of the in this way prepared samples is as follows:

Sample 1 - collagen : ($ZnTiO_3/SiO_2$) = 1 : 0.2, wt./wt.
Sample 2 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2 : 0.1, wt./wt./wt.
Sample 3 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2 : 0.2, wt./wt./wt.
Sample 4 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2: 0.3, wt./wt./wt.
Sample 5 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2 : 0.4, wt./wt./wt.
Sample 6 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2 : 0.5, wt./wt./wt.
Sample 7 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.2 : 0.6, wt./wt./wt.
Sample 8 - collagen : ($ZnTiO_3/SiO_2$) = 1: 0.3, wt./wt.
Sample 9 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.1, wt./wt./wt.
Sample 10 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.2, wt./wt./wt.
Sample 11 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.3, wt./wt./wt.
Sample 12 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.4, wt./wt./wt.
Sample 13 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.5, wt./wt./wt.
Sample 14 - collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.6, wt./wt./wt.

Increasing the cytotoxicity of the above low toxic collagen/($ZnTiO_3/SiO_2$) composites was not expected due to the including of non-toxic marine algae derived fucoidan. Therefore cytotoxicity testing was not performed.

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/($ZnTiO_3/SiO_2$)/fucoidan antimicrobial composites. The samples were gold-sputtering coated and viewed in the second electron mode with field emission gun. Both, composites without microbial cells and with microbial cells, were observed in this way, the second ones after drying at room temperature for a week.

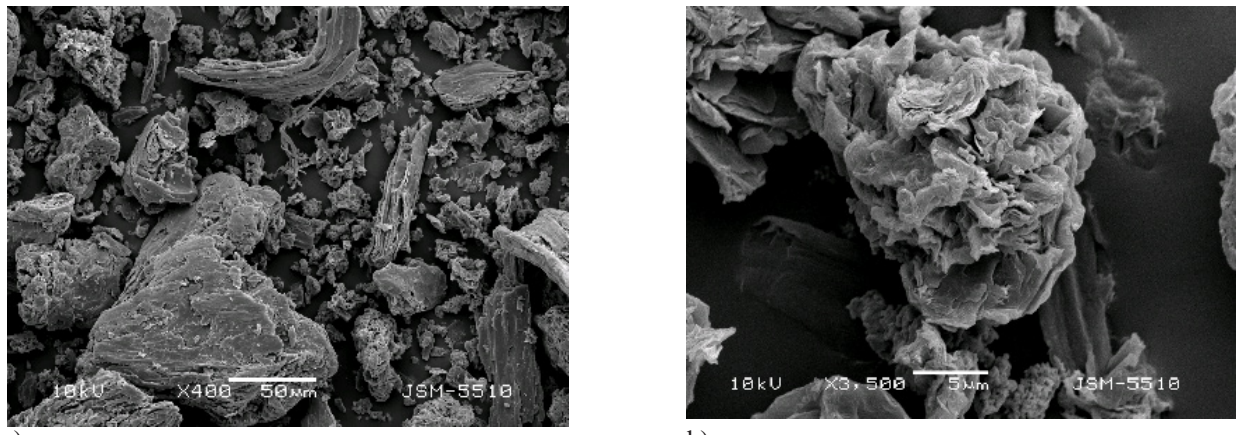
Antimicrobial activity testing

The test microbial strains: Gram-negative bacteria (*Esherihia coli*, ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853; *Pseudomonas putida*, ATCC 10536; *Salmonela holeresius DSMZ 4224*), Gram-positive bacteria (*Staphylococcus epidermidis*, 3486; *Bacillus cereus*, 1095) and fungi (*Candida lusitaniae* 74-4; *Saccharomyces cerevisiae* ATCC 9763) were provided by the National Bank of Microorganisms and Cell Cultures (NBIMCC), Bulgaria and cultured in the most suitable for each one media as it was described earlier [22]. The pure culture of every strain was prepared as a bacterial suspension in exponential phase with OD 0.5 Mc Farland. 100 μL of each were dropped and randomly distributed on solid agar nutrient medium, than discs of investigated material were put on them. Plates were left for 20 h at 4-6^0 C to afford diffusion of ~~the~~ some nanoparticles from the test sample and after that cultivated for 24 h at 37^0, 30^0 and 24^0 C respectively. The formed sterile zones around the disks samples (diameter of 10.0 mm; thickness of 2 mm) were measured in mm (± 0.5). All results are average of 5 measurements.

EXPERIMENTAL

SEM images

Since the antimicrobial activity determines to some extent, by the morphology of the porous collagen composites, SEM observation was performed of the studied materials. Fig. 1 presents the morphology of the used in this investigation $ZnTiO_3$ embedded in a silane matrix ($ZnTiO_3/SiO_2$), as a component of the studied two component antimicrobial agent: ($ZnTiO_3/SiO_2$)/fucoidan. Fig. 1 depicts the submicron $ZnTiO_3/SiO_2$ particles that form relatively large aggregates, more clearly seen at higher magnification, Fig. 1(b). Practically observed easy destruction of the aggregates



a) b)

Fig. 1. SEM images of $ZnTiO_3/SiO_2$ aggregates at different magnifications: (a) - x400; (b) - x3500.

under weak pressure, provides a fine dispersion of the $ZnTiO_3/SiO_2$ particles during the preparation of the collagen/($ZnTiO_3/SiO_2$) composites.

Fig. 2 illustrates the porous structure of: (a) collagen sponge without antimicrobial agents; (b) collagen composite containing $ZnTiO_3$ (weight ratio collagen : $ZnTiO_3$ = 1 : 0.3); (c) collagen composite containing $ZnTiO_3/SiO_2$ (weight ratio collagen : $ZnTiO_3/SiO_2$ = 1:0.3) and (d) collagen composite containing combined ($ZnTiO_2/SiO_2$)/fucoidan antimicrobial agent (weight ratio collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.3). All composites were prepared under one the same conditions as it was described in the experimental section. The pictures of the other collagen/($ZnTiO_3/SiO_2$)/fucoidan composites: collagen : ($ZnTiO_3/SiO_2$) : fucoidan = 1 : 0.3 : 0.1; 1 : 0.3 : 0.2; 1 : 0.3 : 0.4; 1 : 0.3 : 0.5 and 1 : 0.3 : 0.6, wt.:wt.:wt as well as collagen : ($ZnTiO_3/SiO_2$): fucoidan = 1 : 0.2 : 0.1; 1 : 0.2 : 0.2; 1 : 0.2 : 0.4; 1 : 0.2 : 0.5 and 1 : 0.2 : 0.6, wt.:wt.:wt) were similar. Therefore they were not presented here.

The open and interconnected relatively homogeneous, porous structure of the collagen is seen Fig. 2(a). The distribution of $ZnTiO_3$ particles along the fibrils of the collagen matrix is presented in Fig. 2(b), whereas Fig. 2(c) depicts the specific spiral organization of the collagen fibrils in snail-like structures, with incrusted fine submicron ($ZnTiO_3/SiO_2$) particles at the collagen/($ZnTiO_3/SiO_2$) composite. This morphology was ascribed to the effect of incrustation of embedded in silicon matrix submicron zink titanate ($ZnTiO_3/SiO_2$) particles. Both, the specific morphology and the more fine $ZnTiO_3/SiO_2$ particles distribution as compared to the $ZnTiO_3$ particles were supposed to be a reason for the increased antimicrobial activity of the collagen/($ZnTiO_3/SiO_2$) composites compared to collagen/$ZnTiO_3$ ones [22].

The morphology of collagen/($ZnTiO_3/SiO_2$)/fucoidan composite, Fig. 2(c), is quite different compared to that of the same composite in absence of fucoidan, i.e. porous collagen/($ZnTiO_3/SiO_2$) composite, Fig. 2(b), although that both were prepared following one the same way and under one the same conditions. The specific spiral organization of the collagen fibrils in snail-like structures (due to incrustation of $ZnTiO_3/SiO_2$ submicron particles on their surface), characteristics for the collagen/($ZnTiO_3/SiO_2$) composite, lack at the similar composite, containing fucoidan, Fig. 2(c). It indicates a significant fucoidan influence on the structuring of the collagen composite. As it was expected, the water-soluble fucoidan dissolves during the fabrication of the composite and most probably thus alters the structuring conditions. The dissolved fucoidan engulfs by the collagen gel and against an open and interconnected, relatively homogeneous, porous structure forms, similar to that of collagen/$ZnTiO_3$ composites but with relatively homogeneous encrusted, fine, submicron $ZnTiO_3/SiO_2$ particles along the fibrils, maybe made by collagen/fucoidan mixture, Fig. 2(e).

SEM observation of test bacterial cells on the studied collagen/($ZnTiO_3/SiO_2$)/fucoidan composites demonstrated no presence of microbial cells, that could be due to their total destruction and therefore pictures are not presented here.

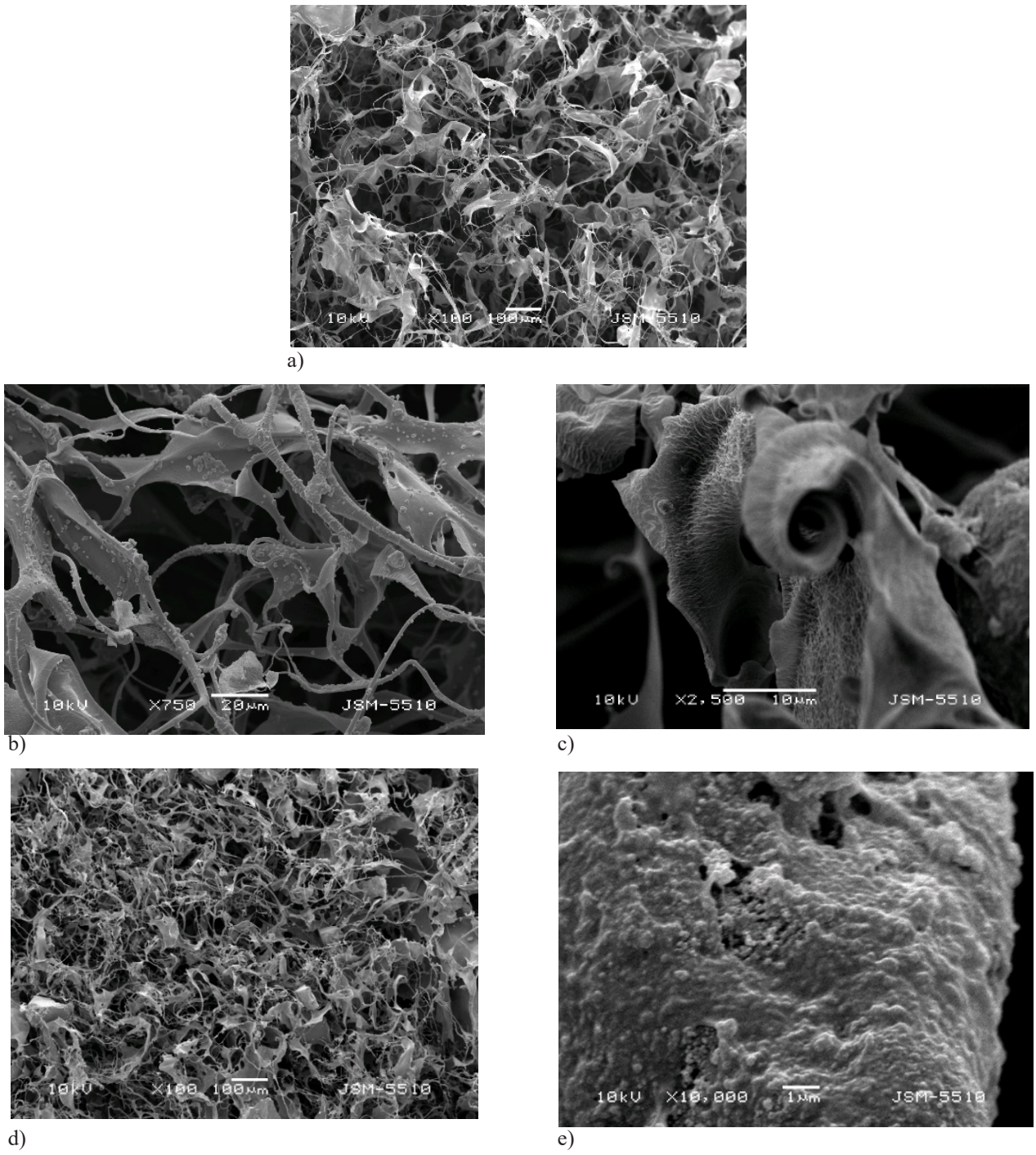


Fig. 2. SEM images of: (a) - porous collagen without antimicrobial agent (x100) and antimicrobial composites: (b) - collagen : $ZnTiO_3$ = 1 : 0.3, wt.:wt. (x750); (c) collagen : ($ZnTiO_3$/SiO_2) = 1:0.3, wt. : wt. (x2 500) ; (d) - collagen : ($ZnTiO_3$/SiO_2) : fucoidan = 1 : 0.3 : 0.3, wt. : wt. : wt. (x100); (e) - collagen : ($ZnTiO_3$/SiO_2) : fucoidan = 1 : 0.3 : 0.3, wt. : wt. : wt. (x10 000).

Antimicrobial activity

The antimicrobial activity (sterile zone, mm) of the studied collagen/($ZnTiO_3$/SiO_2)/fucoidan and collagen/($ZnTiO_3$/SiO_2) composites, for comparison, toward Gram-negative microbial strains is presented in Table 1. Table 2 presents the activity of the same composites toward Gram-positive bacteria and fungi.

The data in Table 1, Samples 1 and Sample 7

Table 1. Antimicrobial activity (sterile zone, mm) toward Gram-negative microbial strains of collagen/($ZnTiO_3$/SiO_2) = 1 : 0.2, wt./wt. (Sample 1) and similar composites containing different amounts fucoidan (Samples 2 - 7) as well as of collagen/($ZnTiO_3$/SiO_2) = 1 : 0.3 wt./wt. (Sample 8) and corresponding ones, containing different amounts fucoidan (Samples 9 - 14).

No	collagen : antimicrobial agent (wt./wt./wt.)	Sterile zone, mm			
		E. coli	*P. aeruginiza*	*P. putida*	*S. holeresius*
1.	coll./($ZnTiO_3$/SiO_2) 1 : 0.2	6.3 ± 0.2	5.6 ± 0.4	3.4 ± 1.2	5.9 ± 1.8
	coll./($ZnTiO_3$/SiO_2)/fucoidan				
2.	1 : 0.2 : 0.1	6.9 ± 1.0	7.3 ± 1.1	5.0 ± 0.7	8.3 ± 1.2
3.	1 : 0.2 : 0.2	7.0 ± 0.9	7.9 ± 1.4	5.4 ± 0.3	8.1 ± 0.2
4.	1 : 0.2 : 0.3	9.3 ± 2.0	9.9 ± 2.1	7.2 ± 1.0	10.6 ± 0.7
5.	1 : 0.2 : 0.4	9.2 ± 0.6	12.1 ± 0.6	9.1 ± 1.2	10.0 ± 1.3
6.	1 : 0.2 : 0.5	11.6 ± 0.3	13.4 ± 1.0	9.0 ± 2.1	9.2 ± 1.5
7.	1 : 0.2 : 0.6	10.9 ± 1.5	11.8 ± 0.3	12.3 ± 0.2	9.3 ± 1.1
8.	coll./($ZnTiO_3$/SiO_2) 1 : 0.3	10.8 ± 1.6	13.1 ± 1.0	10.2 ± 0.6	12.1 ± 1.1
	coll./($ZnTiO_3$/SiO_2)/fucoidan				
9.	1 : 0.3 : 0.1	10.9 ± 0.1	14.7 ± 1.0	12.8 ± 1.9	11.3 ± 0.3
10.	1 : 0.3 : 0.2	11.6 ± 0.9	13.9 ± 0.8	12.0 ± 0.3	13.6 ± 0.6
11.	1 : 0.3 : 0.3	12.9 ± 1.8	15.2 ± 0.7	13.9 ± 0.5	15.0 ± 1.0
12.	1 : 0.3 : 0.4	12.0 ± 0.3	16.8 ± 0.9	15.9 ± 1.6	17.1 ± 1.9
13.	1 : 0.3 : 0.5	13.9 ± 0.8	18.0 ± 1.6	15.9 ± 2.0	16.4 ± 0.7
14.	1 : 0.3 : 0.6	13.1 ± 1.0	16.6 ± 1.4	15.1 ± 0.6	16.0 ± 0.2

demonstrate the moderate antimicrobial activity toward the test bacterial strains of the control collagen : ($ZnTiO_3$/SiO_2) = 1: 0.2 wt./wt. and collagen : ($ZnTiO_3$/SiO_2) = 1: 0.3 wt. /wt. composites respectively, without fucoidan, better expressed at the second one. A comparison of the data for samples 2 - 7 to those for sample 1 and for samples 8 - 14 and sample 7, demonstrates that the including of fucoidan as a second bosting antimicrobial agent enlarge the sterile zone, i.e. increases the antimicrobial activity toward the four Gram-negative test bacteria (*E. coli, P. aeroginosa, P. putida, S. holereius*). A comparison of the data in the corresponding columns of Table 1 demonstrates the dependence of the antibacterial activity on the amount of fucoidan, presenting in the corresponding collagen/$ZnTiO_3$/SiO_2/ fucoidan composite. However, the loading of one the same fucoidan amount causes different enlargement of the sterile zone for the different Gram-negative bacterial strains (Table 1- compare the data in the corresponding rows). This is most probably connected to the specific characteristics of the microbial species like shape, size, cell membrane and others.

The data in Table 2 demonstrate that the presence of fucoidan in the collagen/($ZnTO_3$/SiO_2)/fucoidan composites enlarges more or less (depending on the fucoidan concentration) the sterile zone for the two Gram-positive test bacteria (*S. epidermidis* and *B. cereus*) (Table 2 - compare data in the corresponding columns). The data in the last two columns of Table 2 demonstrate insignificant influence of fucoidan on the biological activity of the studied composites toward the test fungal strains (*C. lucitania* and *S. cerevisiae)*.

Table 2. Antimicrobial activity (sterile zone, mm) toward Gram-positive bacteria and fungi of collagen/($ZnTiO_3/SiO_2$) = 1 : 0.2, wt./wt. (Sample 1) and similar composites containing different amounts fucoidan (Samples 2 - 7) as well as of collagen/($ZnTiO_3/SiO_2$) = 1 : 0.3 wt./wt. (Sample 8) and corresponding ones, containing different amounts fucoidan (Samples 9 - 14).

No	collagen : antimicrobial agent (wt./wt./wt.)	Sterile zone, mm			
		S. epidermidis	*B. cereus*	*C. lucitania*	*S. cerevisiae*
1.	coll./($ZnTiO_3/SiO_2$) 1 : 0.2	10.8 ± 0.3	9.1 ± 1.0	10.3 ± 1.1	9.7 ± 0.6
	coll./($ZnTiO_3/SiO_2$)/fucoidan				
2.	1 : 0.2 : 0.1	10.0 ± 1.0	9.0 ± 0.4	10.2 ± 1.3	9.0 ± 0.8
3.	1 : 0.2 : 0.2	12.4 ± 0.9	10.7 ± 0,6	9.9 ± 1.1	9.9 ± 0.4
4.	1 : 0.2 : 0.3	16.0 ± 0.1	17.3 ± 0.9	10.6 ± 1.6	9.3 ± 1.4
5.	1 : 0.2 : 0.4	17.9 ± 1.2	18.1 ± 1.6	10.0 ± 1.2	10.0 ± 1.1
6.	1 : 0.2 : 0.5	18.0 ± 0.6	16.5 ± 1,5	9.7 ± 2.1	10.1 ± 1.3
7.	1 : 0.2 : 0.6	17.2 ± 0.3	14.9 ± 1.0	9.9 ± 0.9	9.8 ± 1.9
8.	coll./($ZnTiO_3/SiO_2$) 1 : 0.3	11.0 ± 2.0	12.6 ± 0.7	15.3 ± 1.1	12.9 ± 0.9
	coll./($ZnTiO_3/SiO_2$)/fucoidan				
9.	1 : 0.3 : 0.1	14.6 ± 1.6	12.0 ± 1.0	15.3 ± 0.9	13.0 ± 1.1
10.	1 : 0.3 : 0.2	14.3 ± 2.0	13.9 ± 0.3	14.9 ± 0.9	13.6 ± 0.6
11.	1 : 0.3 : 0.3	16.9 ± 0.7	16.0 ± 1.4	16.6 ± 1.3	12.1 ± 0.9
12.	1 : 0.3 : 0.4	18.6 ± 0.2	18.0 ± 0.9	15.2 ± 0.2	12.9 ± 0.6
13.	1 : 0.3 : 0.5	18.0 ± 0.9	17.5 ± 0.7	15.0 ± 0.6	12.0 ± 0.8
14.	1 : 0.3 : 0.6	18.0 ± 1.3	17.5 ± 1.0	16.0 ± 1.1	11.3 ± 1.2

Fortunately, the presence of fucoidan does not decrease the high antifungal activity of the collagen/($ZnTiO_3/SiO_2$) composites.

All experimental data show, that the antibacterial activity of the collagen/($ZnTiO_3/SiO_2$)/fucoidan composites, toward the used six test bacterial strains is increased, specific and dependent on the concentration of the fucoidan, whereas their antifungal activity toward the two test fungal strains remains almost the same as that of the corresponding composites do not containing fucoidan.

RESULTS AND DISCUSSION

Combined antimicrobial agent was employed for this study consisting of embedded in a silane matrix zinc titanate ($ZnTiO_3/SiO_2$) and derived from marine algae fucoidan. The combined antimicrobial agent was included in a porous collagen-based matrix by sol-gel cryogen drying to be obtained Collagen/($ZnTiO_3/SiO_2$)/fucoidan composites. No chemical interactions were expected under these conditions.

The antimicrobial activity of the studied collagen/($ZnTiO_3/SiO_2$)/fucoidan composites could be due to the presence of $ZnTiO_3/SiO_2$ nanoparticles and dissolved in the collagen gel water-soluble fucoidan. Unfortunately, the biological activity mechanisms of the $ZnTiO_3/SiO_2$ and fucoidan (as well as of natural polysaccharides generally) are not fully understood.

Based on the knowledge up to date, it could be supposed, a chelation of metal ions from collagen/$ZnTiO_3SiO_2$/fucoidan composites and reactive oxygen

species formation due to interactions between the microbial environment and the antimicrobial agent. The sharp ages of the $ZnTiO_3/SiO_2$ particles were covered by a silane matrix, as it was observed earlier [22]. Therefore, mechanical demolition of the cell envelope and membrane [31] probably does not happens. Chelation of some metal ions from the $ZnTiO_3/SiO_2$ particles whit free oxygen and nitrogen electron couples in peptide bonds of collagen/fucoidan mixture maybe contribute to their biological activity. A negative charge of the microbial cell wall under the test conditions could be a reason for intake of released metal ions chelated with collagen/fucoidan mixture. Engulfed in sufficiently high concentrations, the metal ions cause toxicity to any cells [32, 33].

Formation of reactive oxygen species (ROSs) due to some interactions of the antimicrobial agent with the microbial envelop could contribute to the antimicrobial activity of the studied collagen/($ZnTiO_3/SiO_2$)/fucoidan composites [34 - 38]. According to some authors [39], exopolymeric substances (EPSs) could not protect cells from the ROSs and they penetrate in the slime around the cell to ruin 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 [39]. ROSs formation due to interactions between water molecules around and in the microbial cells from the one side and the containing fucoidan antimicrobial agent, from the other side can stipulate damages of any macromolecules of the cell [4]. In debt understanding of how the microbial cells interact with the studied collagen/($ZnTiO_3/SiO_2$)/fucoidan composites is difficult on a number of reasons. The interaction of cells and biomaterials is mediated by secreted EPSs that are a complex mixture of polysaccharides, proteins, nucleic acids, lipids and humic substances [40]. The EPSs are different not only for the different cells, but also for one the same cells on different surfaces [41].

The investigated collagen/($ZnTiO_3/SiO_2$)/fucoidan composites demonstrate specific activity toward Gram-negative, Gram-positive bacteria and fungi, Table 1 and Table 2. The specific antimicrobial activity could be connected, in addition to differences in the secreted EPSs, to the differences in the cell size, shape and wall structures: thin peptidoglycan layer and external membrane at Gram-negative bacteria: *E. coli*, *S. enterica* and *Pseudomonas spp.* and thick peptidoglycan layer at Gram-positive bacteria: *B. cereus, S. epidermidis*. The used in this study test fungi (*C. lusitanie and S. cerevisiae*) have very different cell wall structure, more rigid and robust, than bacteria [42]. The found higher antimicrobial activity of the Collagen/($ZnTiO_3/SiO_2$)/fucoidan composites as compared to that of Collagen/($ZnTiO_3/SiO_2$) ones is due to the presence of the natural bioactive component (dissolved in the collagen matrix). This alters the structuring conditions and formation of interconnected pore structure of the composite with relatively homogenies distribution of the fine submicron $ZnTiO_3/SiO_2$ particles along the collagen/fucoidan fibrils. The toxicity of the fucoidan for the bacteria contribute also to the increased antibacterial activity of the new collagen/($ZnTiO_3/SiO_2$)/fucoidan composites.

CONCLUSIONS

New collagen/($ZnTiO_3/SiO_2$)/fucoidan composites with relatively high, wide spectrum antimicrobial activity against bacteria and fungi were successfully prepared by sol-gel cryogen drying using a combined antimicrobial agent consisting of $ZnTiO_3/SiO_2$ and marine algae derived fucoidan.

The presence of fucoidan in the collagen/($ZnTiO_3/SiO_2$)/fucoidan composites increases significantly their activity toward bacteria and insignificantly toward fungi. The specific activity toward different microbial cultures was ascribed to the specific features of the microbial cells (size, shape, cell wall structure) and different composition of the secreted EPSs.

Both, the interconnected open pore structure of the mixed collagen/fucoidan matrix and the fine dispersion of the submicron $ZnTiO_3/SiO_2$ particles along the matrix fibrils, contribute to the increased wide spectrum antibacterial activity than that of similar collagen composites do not containing fucoidan.

The experimentally found high activity against Gram-negative and Gram-positive bacteria combined with a good antifungal one makes the new developed collagen/($ZnTiO_3/SiO_2$)/fucoidan composites promising wide spectrum antimicrobial biomaterial.

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