Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 2, April - June 2013, p. 869 - 876

ANTIMICROBIAL AND BIOCOMPATIBILITY ASSAY OF NEWLY FABRICATED MATERIALS BASED COPPER OR ZINC ALGINATE AND SiO_2 NETWORK

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Here we report the synthesis and bio-evaluation of a newly crosslinked Cooper and Zinc Alginate with/without SiO_2 network exhibiting significant antimicrobial activity against *Enterococcus faecalis*, in significantly lower concentrations than the plain Copper or Zinc solutions ($p<0.05$). Furthermore, the obtained hybrid materials did not exhibit any cytotoxic effect against eukaryotic cells, proving their good biocompatibility and enabling them to be used as drug delivery systems and scaffolds for tissue engineering, providing prolonged, effective antimicrobial activity.

(Received March 29, 2013; Accepted May 31, 2013)

Keywords: Crosslinked alginate, Hydrogels, Improved MIC, Silica network;

1. Introduction

Known as a commensal intestinal inhabitant, Gram-positive *Enterococcus faecalis* (*E.faecalis*) can also cause life-threatening infections in humans, especially in the nosocomial environment, where antibiotic resistance reaches highlevels.*E. faecalis* often cause endocarditis and bacteremia, urinary tract infections (UTI), meningitis, and other infections in humans [1]. Antibiotic-resistant enterococci are major causes of hospital-acquired infections [1] and therefore represent a serious public health problem. *E. faecalis* is resistant to many commonly used antimicrobial agents, as aminoglycosides, aztreonam, cephalosporins, clindamycin, the semisynthetic penicillins: nafcillin,oxacillin and trimethoprim-sulfamethoxazole. High-level gentamycin resistance correlated with other virulence factorslocated on the same plasmid is associated with a five-fold increase in risk of death in human bacteremia patients [2]. Resistance to vancomycin in *E. faecalis* is also becoming more common [3,4]. Treatment options for

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vancomycin-resistant *E. faecalis* include linezolid and daptomycin, although ampicillin is preferred if the bacteria are still susceptible [5]. This opportunistic pathogen is also known for its ability to form biofilms *in vivo* and *in vitro* [6,7]. Biofilm bacteria are differentiated from their free-swimming (planktonic) counterparts, exhibiting biofilm-specific phenotypes such as enhanced tolerance to antibiotic treatment and increased levels of genetic exchange [7]. *E. faecalis in vitro* biofilms have been observed to exhibit enhanced tolerance to vancomycin and teicoplanin [8], limiting the therapeutic possibilities, and requiring alternative methods for handling enterococcal infections.

Alginates are hydrophilic polysaccharides derived from brown algae known as *Phaeophyceae* [9-11]. They are sodium salts of alginic acid composed of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate(G) units. The most important property of alginate polymer is the ability to react with polyvalent metal cations to produce strong gels or low soluble polymer [12-14]. The crosslinking with polyvalent cations can improve characteristics likewater resistance, mechanical resistance, barrier properties, cohesiveness and rigidity [15, 16]. Alginate crosslinked with calcium has frequently been used to make scaffolds for cutaneous wound dressings [17, 18].

The SiO_2 materials have been widely investigated and used due to their porous structure and high surface area in various applications [19]. The SiO_2 as an inorganic carrier proves to be an ideal supporting material, not only because of its physico-chemical properties, but also because of its fast fabrication and high stability in most chemical and biological environments [20]. The main advantages of SiO_2 are its biocompatibility and hydrophilicity, which is due to the presence of Si-O−functional groups. Because of these characteristics, studies revealed that SiO_2 can be involved in low clearance of certain therapeutic compounds [21,22]. Our previous works revealed that the SiO_2 network has improved the kanamycin activity on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 strains, with a significant decrease of the minimum inhibitory concentration [23], as well asthe ability of the polymer-silica network to maintain or improve the efficacy of the following antibiotics: piperacillin-tazobactam, cefepime, piperacillin, imipenem, gentamicin, ceftazidime against *Pseudomonas aeruginosa* and of cefazolin, cefaclor, cefuroxime, ceftriaxone, cefoxitin,trimethoprim/sulfamethoxazole against *Escherichia coli* [24].

The purposeof this work was to comparatively evaluate the antimicrobial properties of Copper and Zinc-Alginate coated/uncoated with SiO_2 network against *Enterococcus faecalis.*

2. Materials and Methods

2.1. Materials

The chemicals for the fabrication of materials were purchased from Merck and Sigma-Aldrich companies.

2.2. Fabrication of the crosslinked Copper-Alginate (AlgCu) and Zinc-Alginate (AlgZn) hydrogels biomaterials

For the synthesis of the crosslinked Copper-Alginate (AlgCu) 25 mL sodium alginate solution (1%) was dropped with the help of a syringe and under constant stirring onto a 25 mL aqueous solution of $CuSO_4$ (10%). Same amounts were used to fabricate crosslinked Zinc-Alginate (AlgZn) hydrogel. It was prepared by dropping sodium alginate solution onto a 10% aqueous solution of $ZnCl_2$. The resulting beads were hardened overnight in the same solution at 4 ^oC. Then they were filtered and the excess $CuSO_4$ and $ZnCl_2$ was washed with deionized water. Finally, the beads were freeze dried.

2.3. Fabrication of crosslinked Copper-Alginate@SiO_2 (AlgCuSi) and Zinc-Alginate@SiO_2 (AlgZnSi) hydrogels biomaterials

One gram of sodium alginate and 10 mL sodium metasilicate was dissolved in 100 mL ultrapure water to prepare AlgNa-Na_2SiO_3 solution. Also, it was prepared a solution consist of 25 mL $CuSO_4$ or $ZnCl_2$ (10%) and 25 mL H_2SO_4 (5%) and 50 mL ultrapure water. The AlgNa-Na_2SiO_3 solution was dropped with the help of a syringe and under constant stirring onto $CuSO_4$or

$ZnCl_2/H_2SO_4$ solution. The resulting beads (AlgCuSiand AlgZnSi) were hardened overnight in the same solution at 4 ^oC. Then they were filtered and the excess $CuSO_4$, $ZnCl_2$, $Na_2SO_4$and H_2SO_4 was washed with deionized water. Finally, the AlgCuSi and AlgZnSi hydrogels were freeze dried.

2.4. Characterization

2.4.1. FT-IR Analysis

A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI), connected to the OMNIC operating system software (Version 7.0 Thermo Nicolet) was used to obtain FT-IR spectra of hybrid materials. The samples were placed in contact with attenuated total reflectance (ATR) on a multibounce plate of ZnSe crystal at controlled ambient temperature (25^oC). FT-IR spectra were collected in the frequency range of 4000–650 cm^{-1} by co-adding 32 scans and at a resolution of 6 cm^{-1} with strong apodization. All spectra were ratioed against a background of an air spectrum.

2.4.2. Scanning Electron Microscopy (SEM)

The SEM analysis was performed on a HITACHI S2600N electron microscope, at 15 and 25 keV, in primary electrons fascicle, on samples covered with a thin silver layer.

2.5. Strains and growth conditions

The *Enterococcus faecalis* ATCC 29212strainwas grown routinely on nutritive agar and for MIC assay fresh overnight cultures grown in nutritive broth were used. Overnight cultures were diluted to ~10^8 CFU/ml (0.5 McFarland) in Muller Hinton (MH) broth (for the MIC assay).

2.6. Minimal inhibitory concentration (MIC) assay

The MICs were established by using an adapted quantitative two-fold dilution assay [25, 26] in MH broth (Oxoid UK). Briefly, all tested compounds were diluted to 1mg/mL in sterile MH brothand 100µL of this solution was used to perform further two-fold dilutions in inoculated medium placed in 96 well plates (NUNC). Concentrations used ranged 500- 0.4882812 µg. Samples were incubated for 24hours at 37^oC in a static incubator. After incubation optical density (OD 600 nm) of each sample was read using a spectrophotometer (Jenway 6300). The MIC values were established considering the last lowest concentrationsignificantly inhibiting the growth of bacteria in the liquid medium which remain clear, similarly to the negative control represented by the sterile culture medium. Experiments were performed in triplicate and repeated on at least three separate occasions.

2.7. Mesenchymal stem cells culture and cytotoxicity assay

$2x10^5$ mesenchymal cells were seeded in each well of a6 well plate (Nunc), containing a 20 mm / 20 mm sterile glass coverslip, in triplicate, using α-minimal essential medium (GIBCO, USA) supplemented with 10 % foetal bovine serum (GIBCO, USA) and 10 ng/mL basic fibroblast growth factor (bFGF) (Sigma, USA). Plateswere cultured at 37^oC in a humidified atmosphere containing 5 % CO_2 until 70%–80% confluence was reached. Confluent cells were treated with 1mg/mL solutions of tested compounds for 24hours, at 37^oC in a humidified atmosphere containing 5 % CO_2. After incubation coverslips containing adherent cells were removed, fixed in 70 % ethanol and stained with 50 µg/mL propidium iodide. The morphology of live or dead stained cells was observed using an Observer.D1 Carl Zeiss microscope in order to evaluate cytotoxic potential of tested compounds. Cytotoxicity was also evaluated using Trypan blue staining. Briefly,treated cells were disrupted from plate's wells using 0.25% trypsin – EDTA. 50 µL cell suspensions were mixed with an equal volume of 0.4% Trypan Blue staining solution. Stained cellular suspensions were spread onto a microscope slide and covered with a coverslip. Nonviable cells appear blue-stained. At least 200 cells were counted per each treatment.

2.8. Statistics

Data were analyzed using GraphPadInStat and Prism softwares, by applying One-way Analysis of Variance (ANOVA) test. P values lower than 0.05 were considered significant.

3. Results and discussion

A major topic in the synthesis of inorganic-organic compounds is the combination of active components. A hybrid material is a material that includes two moieties blended on the molecular scale [27]. Polymer composites obtained on the basis of polymers and metals are of practical interest, providing the opportunity for the development of new materials with potential applications for the biomedical field. The complexation of alginate with bivalent cations, *i.e.* Mg(II), Ca(II), Sr(II), Mn(II), Co(II), Cu(II), and Zn(II) has been previously studied both by computer based and experimental approaches [28, 29]. Alginate could facilitate the complexation of bivalent ions to their matrix (by decreasing the mobility of the molecular chains), because of the extensive hydroxyl groups on their structure, which provide more inter- and intra-molecular interstices that can selectively trap metals. It was demonstrated that the Na alginate could act as a size controller and stabilizing agent of Cu nanoparticles, due to its ability to bind strongly to the metal surface, demonstrating its wide application as an important biopolymer [30].

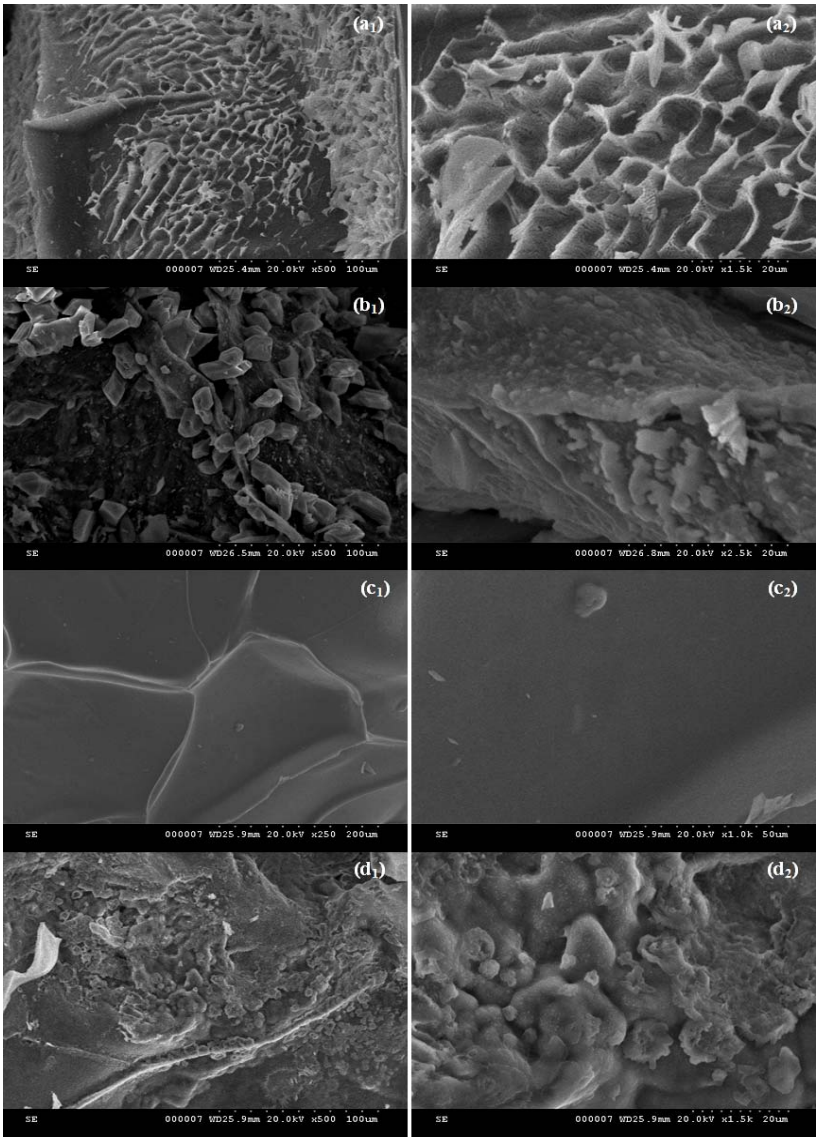

Fig. 1: SEM micrographs of AlgCu (a), AlgCuSi (b) AlgZn (c), AlgZnSi (d)

The mechanically stable silica network with controllable size pores has been used for many applications, including biomolecules separations, adsorption and controlled release of different active compounds [31-34]. Bioactive glass materials, or composites of bioactive glass and polymers, have been tested as delivery systems for antibiotics and other antibacterial agents, anti-inflammatory drugs, fluoride ions, vascular endothelial growth factor, bone morphogenetic proteins, and nitric oxide [35].

We report here the fabrication of alginate complexes with copper and zinc, with and without SiO_2 network. The morphology of the obtained hybrid materials is shown in Fig 1. The SiO_2 can be clearly distinguished on the surface of alginate matrix (Figs. 1b,d). As it can be seen from figures 1b and 1d, a highly porous network with interconnected porosity was produced due to presence of SiO_2. Figure 1b revealed that the fabricated AlgCu exhibit a rough surface with elongated micropores and a wrinkling structure. Instead, figure 1d reveals that the surface of the AlgZn is pellicular.

The IR analysis identified the SiO_2 organic shell on the surface of fabricated materials. Two sharp bands at 2915 and 2848 cm^{-1} were attributed to the asymmetric CH_2 stretching and the symmetric CH_2 stretching, respectively. In the IR spectraof AlgCu and AlgZn there are two specific strong absorption bands at 1601 cm^{-1} and 1410 cm^{-1} attributed to asymmetric and symmetric stretching vibrations of COO− groups on the polymeric backbone [36].Both of the above mentioned absorption bands were observed in the AlgCuSi and AlgZnSi. The silica network of AlgCuSi and AlgZnSi showed IR bands in the region of 1250-1000 cm^{-1}, which was assigned to Si–O–Si (stretch mode). The presence of residual silanol (Si–OH) groups is frequently observed in many sol-gel derived materials, reflecting the incomplete polycondensation [37].

Microbial adhesion to biomaterials is a major problem often resulting in infection and medical device failure. Several strategies have been employed to modulate eukaryotic cell adhesion and to hamper bacterial adherence to polymeric biomaterials, one of them being the incorporation of non-antibiotic antimicrobial agents into the biomaterial matrix that may reduce bacterial adhesion [38].The antifungal properties and cytotoxicity of alginate fibers prepared by replacing Na(I) with Ca(II), Zn(II), or Cu(II) were investigated to widen their application in tissue engineering. The results from inhibition-zone test and shake-flask test as well as the scanning electron micrographs showed that zinc alginate fibers have the most significant antifungal action, followed by that of copper alginate fibers, determining the occurrence of scraggly biofilms after the interaction between *C. albicans* and zinc alginate fibers [39]. In this study, we have investigated the influence of Zn and Cu alginate with or without silica networks on the growth of *Enterococcus faecalis* strains. To our knowledge, this is the first study reporting the antimicrobial activity of this kind of hybrid material against Gram-positive bacterial strains.

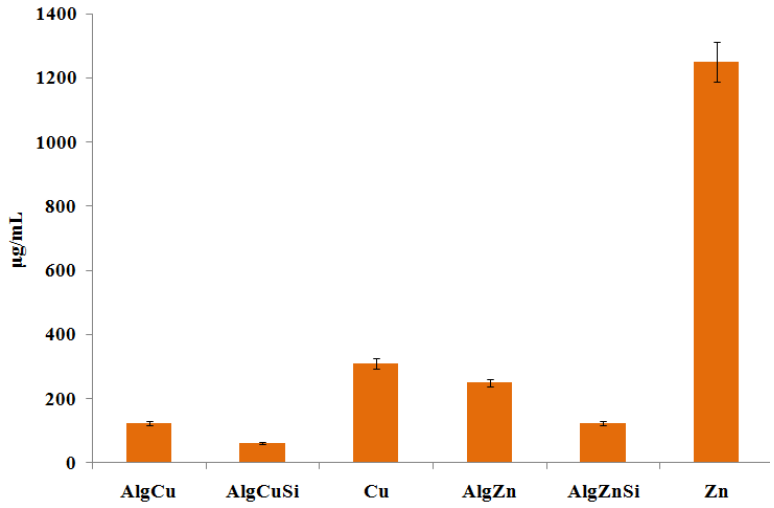

*Fig. 2. MIC values for $ZnCl_2$ (Zn), $CuSO_4$ (Cu), AlgZn, AlgCu, AlgZnSi and AlgCuSi on E. faecalis ATCC 29212 strain. *P<0.05 Alg samples vs. Zn, Cu control $CuSO_4$ (Cu), AlgCu and AlgCuSi*

The MIC assay demonstrated that all tested compounds become inhibitory for bacterial growth at different concentrations. Both AlgCuSi and AlgZnSi are more efficient inhibitors ofMIC values twice lower than those of AlgCu and AlgZn respectively. Zn based materials proved to be more efficient antibacterial agents than the copper ones (Fig. 2). Our results come into agreement with other previous studies, demonstrating the excellent antifungal property of zinc alginate fibers and their potential application in skin tissue engineering comparing with the copper alginate fiber [20].

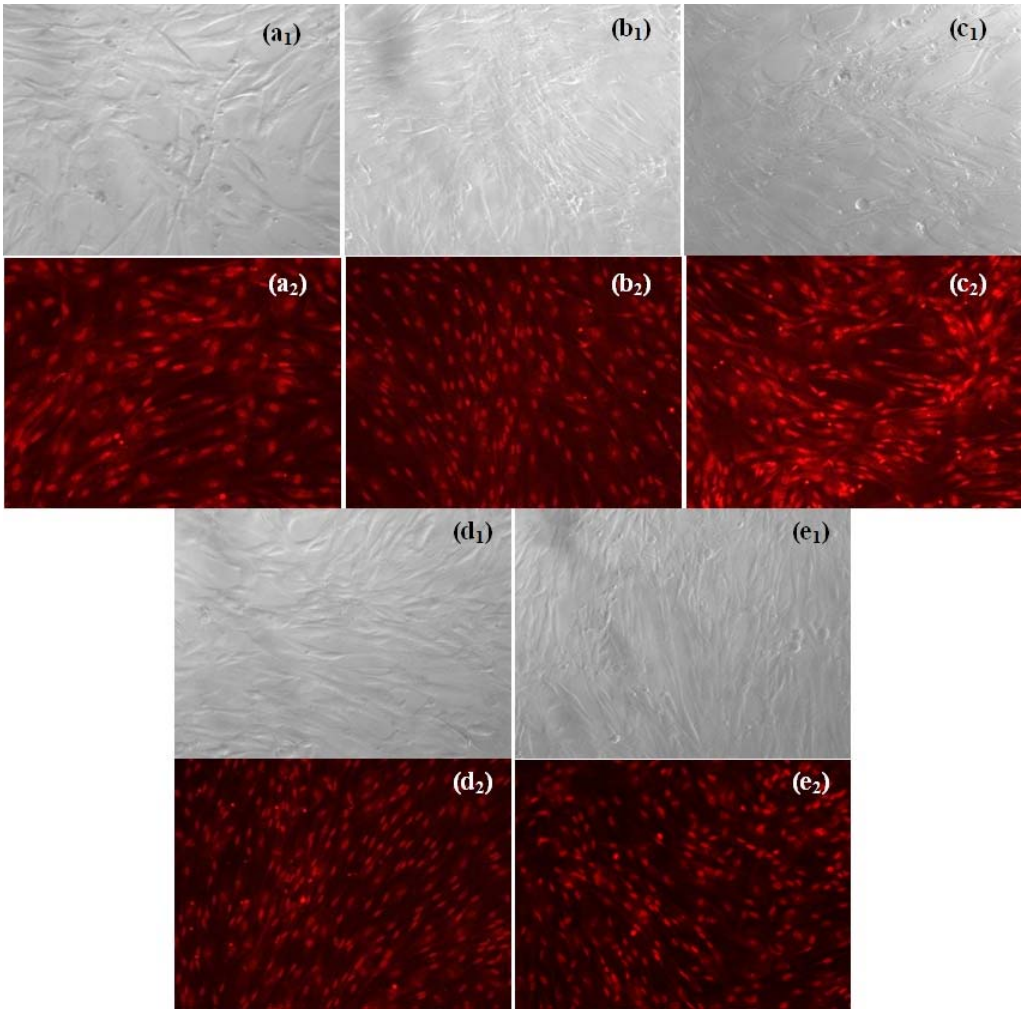

Fig. 3. Micrographs of AlgCu (a); AlgCuSi (b); AlgZn (c); AlgZnSi (d); MSC control (e). 1000x magnification, immersion oil.

Table 1. Percentage of viable mesenchymalcells after 24hours treatment with tested compounds

Compound (1mg/mL)	Viable cells (percentage)
AlgCu	**92%**
AlgCuSi	**94%**
AlgZn	**95%**
AlgZnSi	**89%**
untreated control	**97%**

The increased antimicrobial properties of the composites containing SiO_2 as compared to their cognate, non-reticulate counterparts could be explained by the fact that the silica matrix pore

architecture makes it suitable for hosting a broad variety of compounds and is flexible enough to allow the controlledrelease of the bivalent cations, which may help to avoid some negative effects that usually appear with the implantation of a material, *i.e.* infection, inflammation etc. [40,41].

Bearing in mind the potential applications of the obtained hybrid material for tissue engineering, the next goal of our study was to assess effects of the novel synthesized metallic alginate structures on eukaryotic cells. The obtained results revealed no cytotoxicity effect after treating eukaryotic cells with 1mg/mL solution of the tested compounds. Microscopy assay revealed that mesenchymal stem cells exhibit normal cellular morphology and adhered to the surface of fabricated materials (Fig. 3).

Microscopy results were confirmed by cells viability assay after Trypan blue staining, demonstrating that all treated cell cultures exhibit a high percentage of viable cells (at least 89% viability) (table 1).

4. Conclusions

This paper reports the fabrication and bioevaluation of a newly biocompatible crosslinked Alginate-SiO_2 network, in relation with the prokaryotic and eukaryotic cells. The SiO_2 containing alginate networks crosslinked with Zn and Cu exhibited a significantly higher antimicrobial activity against *E. faecalis* strain ($P<0.05$) than the metal salt solutions and also, proved to be highly biocompatible. The obtained hybrid systems, by the textural features of the silica matrix together with the incorporation of the antibacterial bivalent metals seem to perfectly modulate the eukaryotic cell adhesion and hamper bacterial adherence, properties enabling them to be used as drug delivery systems and as scaffolds for tissue engineering, providing prolonged, effective antimicrobial activity.

Acknowledgments

This paper is supported by the Sectoral Operational Programme Human Resources Development, financed from the European Social Fund, and by the Romanian Government under the contract number POSDRU/86/1.2/S/58146 (MASTERMAT).

This work was partially supported by the Romanian National Authority for Scientific Research, CNCS-UEFISCDI, project number PCCA 153/02.07.2012.

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