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A comprehensive approach to *in vitro* functional evaluation of Ag/alginate nanocomposite hydrogels

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ABSTRACT

In this work, we present a comprehensive approach to evaluation of alginate microbeads with included silver nanoparticles (AgNPs) at the concentration range of 0.3–5 mM for potential biomedical use by combining cytotoxicity, antibacterial activity, and silver release studies. The microbeads were investigated regarding drying and rehydration showing retention of ~80–85% of the initial nanoparticles as determined by UV–vis and SEM analyses. Both wet and dry microbeads were shown to release AgNPs and/or ions inducing similar growth delays of *Staphylococcus aureus* and *Escherichia coli* at the total released silver concentrations of ~10 μg/ml. On the other hand, these concentrations were highly toxic for bovine chondrocytes in conventional monolayer cultures while nontoxic when cultured in alginate microbeads under biomimetic conditions in 3D perfusion bioreactors. The applied approach outlined directions for further optimization studies demonstrating Ag/alginate microbeads as potentially attractive components of soft tissue implants as well as antimicrobial wound dressings.

1. Introduction

In recent decades, a constant increase in the number of microorganisms resistant to existing antibiotics revived the clinical use of silver (Monteiro et al., 2009). Variety of silver-containing products have been developed and utilized especially for treatments of infections in burns, open wounds and chronic ulcers. However, there are still contradictory results in literature about the effectiveness of different silver-containing dressings or topical agents in treatments of different wound types (Atiyeh, Costagliola, Hayek, & Dibo, 2007; Lo, Chang, Hu, Hayter, & Chang, 2009; Storm-Versloot, Vos, Ubbink, &Vermeulen, 2010) and it was stressed that knowing the properties of these products is needed for appropriate selection of the most effective treatment procedure (Atiyeh et al., 2007). Thus, the ultimate goal in this area is development of products with superior antimicrobial activity while inducing no host cell cytotoxicity (Atiyeh et al., 2007). Silver nanoparticles (AgNPs) are known to exhibit stronger antimicrobial activity as compared to silver ions and induce broad inhibitory biocide spectra for variety of viruses (Xiang, Chen, Pang, & Zhenga, 2011), bacteria (Guzman, Dille, & Godet, 2012; Radzig et al., 2013), and fungi (Panáček et al., 2009). Panáček et al. (2006) have shown that chemically synthesized AgNPs in solutions of different saccharides exhibited antibacterial activity against ten different bacterial strains with the smallest nanoparticles of about 25 nm in diameter being the most effective. However, one of the key problems for application of AgNPs is strong tendency of these particles to agglomerate due to high specific surface area and surface energy. To overcome this problem, polymer solutions or hydrogels are used to stabilize AgNPs such as poly(*N*-vinyl-2-pyrrolidone), polyvinyl alcohol, polyacrylamide, etc. (Hong, Park, Sul, Youk, & Kang, 2006; Jovanović et al., 2011; Sharma, Yngard, & Lin, 2009).

Among these polymers, alginates are already in medical use, especially in variety of wound dressings, due to effective regulation of moisture levels that leads to rapid granulation and reepithelization of the damaged tissue (Paul & Sharma, 2004; Queen, Orsted, Sanada, & Sussman, 2004). These dressings are easily removed and replaced without causing much trauma due to the highly hydrophilic alginate gel nature. In addition, alginate hydrogels are widely investigated for regeneration and engineering of a number of tissues and organs, including skeletal muscle (Kamelger et al., 2004), blood vessels (Lee, Peters, & Mooney, 2003), nerve (Hashimoto et al., 2005), pancreas (Korbutt, Mallett, Flashner, & Rajotte, 2004), liver (Glicklis, Shapiro, Agbaria, Merchuk, & Cohen, 2000), and cartilage (Fragonas et al., 2000). High water content in

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these gels supports efficient transport of nutrients and gases and provides aqueous environment comparable to that in soft tissues (Lum & Elisseeff, 2003). Also, alginate gels could be introduced into the body to fill irregularly shaped defects by a minimally invasive procedure (Lee & Mooney, 2012). All of these properties make alginate hydrogels attractive and potentially applicable as soft tissue implants (Hashimoto et al., 2005; Suzuki et al., 2000).

Recently, AgNPs were synthesized in alginate solutions by chemical reduction (Seo et al., 2012), irradiation (Liu, Chen, Zhong, & Wu, 2009), photochemical reduction (Saha et al., 2009) and simply by heating where alginate served both as a reducing agent and a nanoparticle stabilizer (Sharma, Sanpui, Chattopadhyay, & Ghosh, 2012). In addition, we have previously shown that AgNPs can be successfully produced in alginate solutions by electrochemical synthesis (Jovanović, Stojkovska, Obradovic, & Mišković-Stanković, 2012). Ag/alginate colloid solutions could be further either crosslinked to form nanocomposite hydrogels (Jovanović et al., 2012; Obradovic, Mišković-Stanković, Jovanović, & Stojkovska, 2010; Seo et al., 2012) or mixed with solutions of other polymers followed by subsequent processing to obtain nanocomposite dry films (Marie Arockianathan, Sekar, Sankar, Kumaran, & Sastry, 2012; Sharma et al., 2012) or hydrogels (Obradovic, Stojkovska, Jovanovic, & Mišković-Stanković, 2012). The obtained films and hydrogels were investigated mostly regarding antimicrobial activity and cytotoxicity, while films containing alginate and sago starch impregnated with AgNPs were studied *in vivo* in a rat wound healing model showing significantly shorter healing periods as compared to non-treated controls (Marie Arockianathan et al., 2012).

Yet, successful development of nanocomposite biomaterials such as those containing AgNPs, for potential biomedical applications, requires comprehensive studies that will connect different aspects of biomaterial functionality, including physical and biomechanical properties, as well as antimicrobial activity, cytotoxicity and silver release kinetics. In this fashion, Travan et al. (2009) investigated synthesis and stabilization of AgNPs in a chitosan-derived polysaccharide solution and antibacterial properties of the obtained solution in direct contact in suspensions of four bacterial strains. In addition, production of nanocomposite hydrogels by gelation of mixtures of the obtained solution and sodium-alginate was described accompanied with investigations of antibacterial activity of the hydrogel surface and cytotoxicity studies on different eukaryotic cell lines in direct contact and using hydrogel extracts in monolayer cultures. The study has indicated firmly embedded AgNPs within the hydrogel exhibiting bactericidal surfaces and negligible silver release without cytotoxicity although possible formation of AgCl in the presence of chloride ions in the cell culture medium and saline solution was not considered.

In addition, investigations under conditions mimicking those found *in vivo* could potentially provide relevant source for prediction of biomaterial behavior upon application/implantation. Previous findings suggest that biomimetic bioreactor systems present valuable tools for physiologically-relevant biomaterial evaluation and studies of cell-biomaterial interactions that could potentially reduce the size of necessary tests on animals (*e.g.* Engelmayr, Hildebrand, Sutherland, Mayer, & Sacks, 2003; Mobasheri & Lewis, 2013; Stojkovska, Bugarski, & Obradovic, 2010; Wang et al., 2013).

In particular, perfusion bioreactors were shown to support engineering of various tissues by efficient mass transport by convection and/or exposure of cells to controlled levels of hydrodynamic shear stresses. Thus, perfusion bioreactors were successfully used for cell seeding onto porous scaffolds (Radisic et al., 2003; Wendt, Marsano, Jakob, Heberer, & Martin, 2003) and tissue engineering of cardiac muscle (*e.g.* Carrier et al., 2002; Radisic et al., 2006), cartilage (*e.g.* Demarteau, Jakob, Schafer, Heberer, & Martin, 2003; Pazzano et al., 2000; Osmokrovic, Obradovic, Bugarski, Bugarski, & Vunjak-Novakovic, 2006), bone (*e.g.* Bancroft et al., 2002; Grayson et al., 2008; Sikavitsas et al., 2005; Zhao, Chella, & Ma, 2007) and liver (Catapano & Gerlach, 2007; Powers et al., 2002).

We have previously shown that nanocomposite Ag/alginate microbeads could be easily produced by electrostatic extrusion of alginate colloid solutions containing electrochemically synthesized AgNPs (Jovanović et al., 2012; Obradovic et al., 2012). The microbead form is advantageous for controlled release of immobilized active substances due to the large surface to volume ratio (De Vos, Faas, Strand, & Calafiore, 2006). In tissue engineering applications, particulate cell supports provide short diffusion distances enabling uniform cell distribution and tissue regeneration with structures allowing for development of vasculature between individual particles as well as possibilities for minimally invasive implantation by injection (Orr & Burg, 2008).

The aim of this work was to develop a comprehensive approach to investigate potentials for utilization of alginate microbeads with incorporated AgNPs in biomedical applications focusing on wound treatment and soft tissue implants. We have concentrated on Ag/alginate microbeads as a suitable form for efficient release of silver nanoparticles and/or ions as well as for drying possibilities so to potentially produce antimicrobial powders. Next, we have made an effort to couple studies of antibacterial activity and investigations of cytotoxicity of the obtained wet and dried Ag/alginate microbeads. In addition, cytotoxicity studies were performed in parallel in conventional monolayer cultures and in 3D bioreactor cultures of bovine calf chondrocytes in order to mimic the physiological environment upon biomaterial implantation.

2. Materials and methods

2.1. Materials

Low viscosity sodium alginate (A-2158), calcium chloride dehydrate and 3-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) were supplied from Sigma (St. Louis, MO). In addition, low viscosity alginate purchased from AppliChem (A3249, Darmstadt, Germany) was used in cytotoxicity studies in monolayer cell cultures and in studies of silver release. $AgNO_3$ was purchased from M. P. Hemija (Belgrade, Serbia), KNO_3 from Centrohem (Stara Pazova, Serbia), $Ca(NO_3)_2 \times 2H_2O$ from Alkaloid (Skopje, Macedonia), sodium citrate from Himedia (Mumbai, India), isopropanol and nitric acid (65%) from Zorka Pharma (Šabac, Serbia), ammonium hydroxide (25%) from NRK Inzenjering (Belgrade, Serbia) and collagenase type II from Worthington (Freehold, NJ). Dulbecco's Modified Eagle Medium (DMEM), HEPES, Fetal Bovine Serum (FBS), penicillin, streptomycin, proline, all used for cell cultures, were obtained from Sigma (St. Louis, MO), and ascorbic acid was bought from Galenika (Belgrade, Serbia). NaCl was purchased from Superlab (Belgrade, Serbia), tryptone from Torlak (Belgrade, Serbia), yeast extract from bioMerieus (Madrid, Spain) and *Staphylococcus aureus* TL was obtained from the Faculty of Technology, Leskovac, University of Niš (Leskovac, Serbia) while *Escherichia coli* (ATCC 25922) was obtained from American Type Culture Collection (Manassas, VA). Water from Milli-Q system (Millipore, Billerica, MA) was used in all experiments and N_2 gas was of high purity (99.5%).

2.2. Production of Ag/alginate microbeads

Sodium alginate solutions with AgNPs were produced by a novel electrochemical method as described previously (Obradovic et al., 2010; Jovanović et al., 2012). In brief, aqueous solution containing 0.1 M KNO_3, 2% w/v Na-alginate, and 3.9 mM $AgNO_3$ was used for electrochemical synthesis of AgNPs stabilized by

alginate. Electrochemical synthesis was performed galvanostatically, under continuous stirring at the current density of 50 mA/cm^2 and during the implementation time of 10 min in an electrochemical cell containing two Pt electrodes, and a reference, saturated calomel electrode (SCE) using Gamry Reference 600 Potentiostat/Galvanostat/ZRA (Gamry Instruments, Warminster, PA). The solution was exposed to N_2 for 20 min prior the synthesis, as well as during the synthesis.

Alginate colloid solutions with different concentrations of AgNPs (0.5 and 1 mM [Ag^0]) were obtained by dilution of the initially synthesized colloid solution with 1.9% w/v Na-alginate solution. In order to substantially reduce the bioburden, the obtained solutions were heat-treated by boiling for 30 min (Stojkovska, Zvicer, Jovanović, Mišković-Stanković, & Obradović, 2012). Furthermore, to assure the absence of bacterial and fungal spores, 1 ml samples were mixed each time with nutrition agar and malt extract agar, respectively, in Petri dishes and incubated for five days at 30 °C. Neither bacterial nor fungal colonies were observed in any of the experiments.

Electrostatic droplet generation was used to produce Ag/alginate microbeads as well as control alginate microbeads without AgNPs as described earlier (Jovanović et al., 2012; Obradovic et al., 2010; Stojkovska et al., 2012). Briefly, Ag/alginate colloid solutions or just Na-alginate solutions were extruded by a syringe pump through a positively charged blunt edge stainless steel needle (23 gauge, 6.6 kV applied electrostatic potential) positioned at a distance of 2.5 cm between the needle tip and the grounded gelling bath (1.5% w/v $Ca(NO_3)_2 \times 2H_2O$). Resulting droplets were collected in the gelling bath forming insoluble microbeads. The microbeads were left in the bath for additional 30 min in order to complete gelling, followed by washing in distilled water to remove any residual silver ions.

For each experimental series Ag/alginate colloid solutions and/or Ag/alginate microbeads were freshly prepared.

2.3. Production and rehydration of dry Ag/alginate microbeads

Drying of Ag/alginate microbeads with different AgNP concentrations until the constant weight was performed in air at room temperature. Possibilities for rehydration of the obtained dry Ag/alginate microbeads (4.87 ± 0.05 mM AgNP [Ag^0] concentration) were investigated in two experimental series using physiological saline solution (0.9% w/v NaCl) and distilled water. In the first experimental series, Ag/alginate microbeads (0.2 g) were dried at room temperature and then immersed in 5 ml saline solution at room temperature. Wet Ag/alginate microbeads (0.2 g) in the saline solution served as a control. In the second experimental series, Ag/alginate microbeads (0.1 g) were dried at room temperature and subsequently immersed in 3 ml of distilled water. Rehydration of microbeads was examined by measuring weights and microbead diameters.

2.4. Cytotoxicity studies

2.4.1. Cell isolation

Full thickness articular cartilage was harvested aseptically from the femoropatellar grooves of either 6 or 12 month old bovine calves within 8 h of slaughter (Fig. 1a). The isolated cartilage was digested by collagenase type II as described previously (Freed et al., 1993) and the obtained chondrocytes were resuspended in the culture medium consisting of DMEM with 4.5 g/l glucose and 0.584 g/l glutamine, supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4 mM proline, and 50 μg/ml ascorbic acid.

Primary chondrocytes obtained from the cartilage of 6 month old calves were used for cytotoxicity studies in monolayer cultures. Primary chondrocytes isolated from the cartilage of 12 month old calves were directly immobilized in alginate microbeads and used for 3D cell cultures in perfusion bioreactors as it is described in next sections.

2.4.2. Monolayer cultures

Cytotoxicity of Ag/alginate microbeads with different concentrations of AgNPs was determined first in monolayer chondrocyte cultures (Fig. 1b) using the standard MTT test (Mosmann, 1983). Cultures with pure Ca-alginate microbeads without AgNPs were also established in order to verify alginate biocompatibility, while monolayer cultures alone served as a control. Two experimental series were performed using 0.3 g Ag/alginate microbeads per well (AgNP [Ag^0] concentrations of: 0.33 ± 0.02 mM, 0.99 ± 0.07 mM, and 3.75 ± 0.04 mM) and 0.6 g Ag/alginate microbeads per well (AgNP [Ag^0] concentrations of: 0.87 ± 0.05 mM, 1.82 ± 0.05 mM, and 3.76 ± 0.02 mM). Briefly, the cells were seeded in 12-well plates at the concentration of 1×10^6 cells in 3 ml culture medium in each well and incubated for 24 h at 37 °C and 5% CO_2 to allow cell attachment. Then, in each experimental well 0.3/0.6 g of either Ag/alginate or pure alginate microbeads was added and the cells were incubated for 48 h at 37 °C and 5% CO_2. MTT tests were performed by a standard procedure described in Supplementary information.

Cell survival is defined as the ratio of the number of cells grown in the presence of the investigated agent and the number of cells in the control. Since the number of live cells is directly proportional to the absorbance, the cell survival, S, can be calculated as

$$S \quad [\%] = \frac{A_u}{A_c} \times 100 \tag{1}$$

where A_u is the absorbance of cells grown in the presence of alginate or Ag/alginate microbeads and A_c is the absorbance of control cells. Cytotoxicity was rated as following: non-cytotoxic (>90% cell survival), slightly cytotoxic (60–90% cell survival), moderately cytotoxic (30–59% cell survival), and severely cytotoxic ($\leq$30% cell survival) (Meriç, Dahl, & Eystein Ruyter, 2008). Experiments were performed in triplicates.

In parallel studies, release of AgNPs/ions from the same Ag/alginate microbeads (0.3 and 0.6 g per well) was determined after 48 h under the same conditions while using physiological saline solution (0.9% w/v NaCl) instead of the culture medium.

2.4.3. 3D cell cultures in perfusion bioreactors

In order to examine cytotoxicity of Ag/alginate microbeads under conditions that imitate the physiological environment upon potential implantation *in vivo*, we have established 3D cultures of bovine calf chondrocytes immobilized in alginate microbeads in perfusion bioreactors (Fig. 1c). The isolated cells were mixed with 2.2% w/v sodium alginate solution in WFI water to obtain final concentrations of 1.5% w/v alginate and 21×10^6 cells/ml. Alginate microbeads with immobilized chondrocytes were produced by electrostatic droplet generation as described in Section 2.2 using 6.3 kV electrostatic potential, 3 cm electrode distance, and 1.5% w/v $CaCl_2$ gelling solution (Stojkovska et al., 2010). The obtained microbeads were left for additional 30 min in the gelling solution after which period they were transferred into the culture medium and subsequently in perfusion bioreactors.

Perfusion bioreactors, designed for cartilage tissue engineering, consisted of two glass parts, fitted into a piece of silicone tubing to form a 1 ml chamber, as described previously (Osmokrovic et al., 2006). Built-in sintered glass plates prevented outflow of microbeads or other cultivated material from the chamber. Each bioreactor was connected to a separate recirculation loop consisting of a medium reservoir, silicone tubing for gas exchange and two syringes for medium exchange.

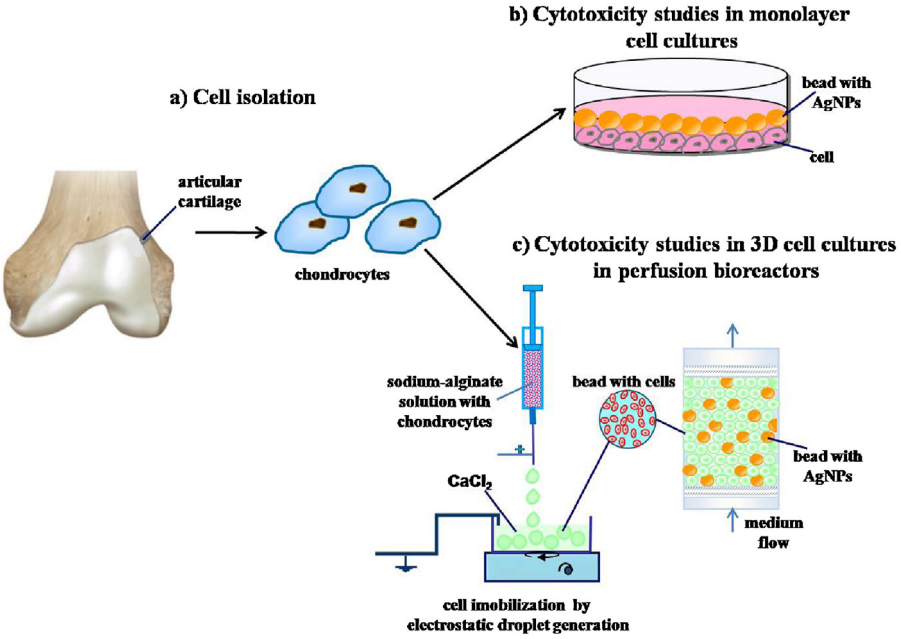

Fig. 1. Experimental set-up for chondrocyte isolation and cytotoxicity studies. (a) Chondrocytes were isolated from bovine calf articular cartilage. (b) Cytotoxicity studies in monolayer chondrocyte cultures with Ag/alginate microbeads. (c) Cytotoxicity studies in 3D bioreactor cultures: chondrocytes were immobilized in alginate microbeads by electrostatic extrusion and cultivated in perfusion bioreactors together with Ag/alginate microbeads.

Two bioreactor chambers were filled each with 0.37 g alginate microbeads with immobilized cells (21×10^6 cells/ml, 1.5% w/v alginate, 770 ± 30 μm in diameter) and 0.13 g Ag/alginate microbeads (1.9% w/v alginate, 790 ± 20 μm in diameter, 4.7 ± 0.6 mM AgNP concentration), connected to separate recirculation loops, each filled with 10 ml of the culture medium and placed in a humidified 5% CO_2 incubator at 37 °C. Perfusion rate in the system was accomplished by a multichannel peristaltic pump at 0.38 ml/min. During 2 weeks of cultivation, 40% of medium was exchanged twice a week.

In order to examine exposure of cultivated cells to released AgNPs/ions from Ag/alginate microbeads under experimental conditions applied in perfusion bioreactors, a separate study was performed in which alginate microbeads with and without AgNPs were used in the same ratio. Control alginate microbeads without AgNPs were produced by the same procedure described in Section 2.2 using 1.5% w/v sodium alginate, 6.3 kV electrostatic potential, 3 cm electrode distance, and 1.5% w/v $CaCl_2$ gelling solution (Stojkovska et al., 2010). Two bioreactor chambers were filled each with control alginate microbeads (0.37 g, 650 ± 40 μm in diameter) and Ag/alginate microbeads (0.13 g, 500 μm in diameter, 5.5 ± 0.4 mM AgNP [Ag^0] concentration) and connected to a separate recirculation loop as described above. The bioreactor systems were each filled with 10 ml of physiological saline solution, placed in a humidified 5% CO_2 incubator at 37 °C and continuously recirculated by a multichannel peristaltic pump at the flowrate of 0.3 ml/min. The experiment lasted for 3 days corresponding to the first medium exchange in the cell cultivation study and the microbeads and saline solution were analyzed for the silver content. The amount of released AgNPs and/or ions from Ag/alginate microbeads was determined based on measured silver concentrations in the initial microbeads and after the experiment. The experiment was repeated two times.

2.5. Antibacterial activity studies

Antibacterial activity of wet and dried Ag/alginate microbeads was estimated against one Gram-positive strain *S. aureus* TL and one Gram-negative strain *E. coli* ATCC 25922. Ag/alginate microbeads were produced sterile and a portion of the beads was dried at room temperature until the constant weight. The microbeads used in *S. aureus* suspensions were 390 ± 20 μm in diameter with AgNP [Ag^0] concentration of 1.2 ± 0.05 mM while microbeads used in *E coli* suspensions were 480 ± 20 μm in diameter and had AgNP [Ag^0] concentration of 1.7 ± 0.2 mM. In each flask, 4 g (with the accuracy of 1 mg) of wet microbeads or 180 ± 8 mg of corresponding dried microbeads (~200 μm in diameter) equivalent to the same wet weight (*i.e.* 4 g) were added followed by addition of 10 ml of sterile LB broth and aliquots of 0.1 ml precultured bacterial suspensions (as described in Supplementary information) so that the initial number of bacterial cells in the broth was approximately in the range 10^5–10^6 CFU/ml. The flasks were incubated in a shaking water bath at 37 °C for 24 h and bacterial cultures in LB broth without Ag/alginate microbeads were used as the control. After 1 h and 24 h of incubation, 0.1 ml of liquid sample was aseptically withdrawn from each flask and transferred to prepared Petri dishes containing agar medium and nutrients and formed colonies were counted after 24 h as described in Supplementary information. The experiments were performed in duplicates and results are expressed as CFU/ml.

In parallel studies, concentrations of released AgNPs/ions from wet and dried Ag/alginate microbeads (1.9 ± 0.05 mM AgNPs [Ag^0]) were measured after 1 and 24 h of incubation under the same conditions while using 10 ml of physiological saline solution (0.9% w/v NaCl) instead of LB medium. The experiment was performed in triplicates and repeated two times.

2.6. Analytical methods

2.6.1. UV–vis spectroscopy

Presence of AgNPs in nanocomposite microbeads was examined by UV-3100 spectrophotometer (Mapada, Japan) after dissolution of either wet or dried microbeads in 2% w/v sodium citrate solution using the ratio based on the wet hydrogel weight of 0.1 g in 1 or 2.9 ml of the citrate solution depending on the AgNP concentration. AgNP concentration was determined from the linear dependence

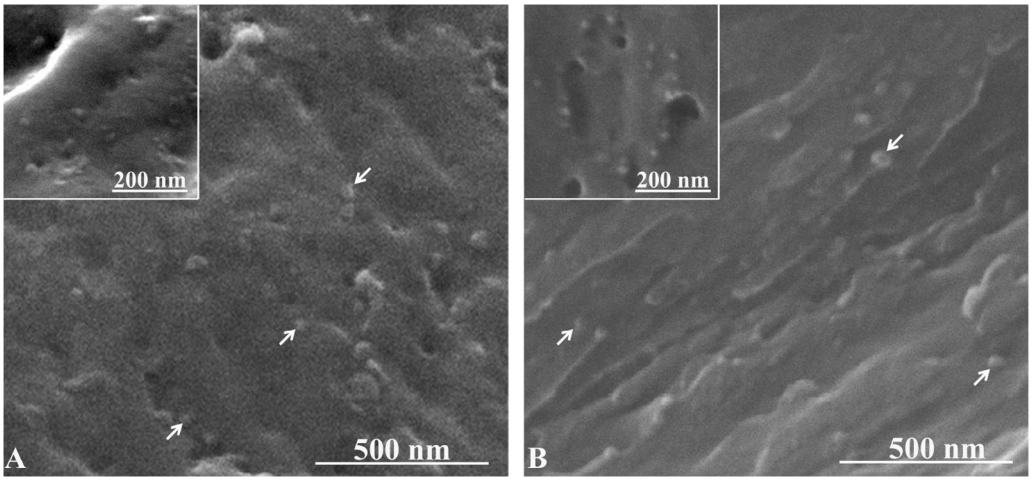

Fig. 2. FE-SEM micrographs of cross-sections of a wet (a) and dried (b) Ag/alginate microbead; white arrows designate individual silver nanoparticles.

of the maximum absorbance on AgNP concentration as described previously (Stojkovska et al., 2012).

2.6.2. Silver concentration

Silver content in Ag/alginate microbeads was determined upon oxidation of all AgNPs either by addition of the concentrated nitric acid (65 wt%) in excess (10–15 cm^3 of the acid per 1 g of microbeads) or after dissolution of microbeads in 2% w/v sodium citrate solution (using the ratio based on the wet hydrogel weight of 0.1 g in 3 ml of the citrate solution) by addition of ammonium hydroxide (5–10 cm^3 of the 25 wt% ammonium hydroxide per 0.1 g of microbeads). Free Ag^+ concentrations in physiological saline solutions were determined by direct measurements while the total silver content released in physiological saline solutions present as Ag^+ as well as AgCl was determined after addition of ammonium hydroxide (25 wt%) in excess (0.5 cm^3 of the ammonium hydroxide per 1 ml of medium) directly into the wells or flasks in which the experiments were performed in order to dissolve precipitated AgCl. Concentration of Ag^+ in all resulting solutions was then determined at four-digit accuracy by atomic absorption spectrometry (AAS) using Perkin Elmer 3100 spectrometer (Perkin Elmer, MA, USA).

2.6.3. Optical microscopy

Microbead diameters were determined by using an optical microscope (Olympus CX41RF, Tokyo, Japan). Wet microbeads were observed on slides in water. The average diameter was calculated from measurements of at least 20 microbeads using the image analysis program "Cell^A" (Olympus, Tokyo, Japan).

2.6.4. Field-emission scanning electron microscopy (FE-SEM)

A simple method for obtaining cross-sections of Ag/alginate microbeads for SEM analysis was developed, utilizing alginate solution/hydrogel as a microbead carrier. In specific, wet microbeads were mixed with 1.73% w/v alginate solution and the mixture was poured in a Petri dish covered with a filter paper saturated with the gelling solution (3% w/v $Ca(NO_3)_2 \times 2H_2O$). Another saturated filter paper was placed on the upper surface of the mixture and the gelling solution was then slowly added to fill the dish. After 24 h, an alginate gel sheet formed, which was then placed into a fresh gelling solution for the next 24 h. Final alginate gel sheet was used to punch out alginate discs (13 mm in diameter, 3 mm thick), which were allowed to complete gelation in fresh gelling solutions for the next 24 h. The obtained discs, which contained dispersed Ag/alginate microbeads, were fixed for 48 h in 2.5% glutaraldehyde and then washed sequentially in 3% acetic acid, 3% acetic acid and 25% ethanol, 3% acetic acid and 50% ethanol, and finally in 70% ethanol according to the modified fixation and staining protocol for histological analyses (Stevens, Qanadilo, Langer, & Shastri, 2004). Discs were then sliced into thin slices, mounted onto microscope glass slides cut in pieces, dried and used for SEM analysis.

In parallel, dried Ag/alginate microbeads were fixed in glutaraldehyde as described above and mixed with 1.73% w/v alginate solution. Then the mixture was poured into Petri dishes and dried at room temperature for 48 h to obtain dry films with incorporated dry Ag/alginate microbeads. Films were cut across the incorporated microbeads and used for SEM analysis.

SEM analyses were performed using MIRA 3 XMU Field Emission Scanning Electron Microscope (Tescan USA Inc., Cranberry Twp, PA).

3. Results and discussion

3.1. Production and rehydration of dry Ag/alginate microbeads

We have previously shown that Ag/alginate microbeads could be produced in a controlled manner by electrostatic extrusion of alginate colloid solutions containing electrochemically synthesized AgNPs (Jovanović et al., 2012; Obradovic et al., 2012; Stojkovska et al., 2012). By varying the synthesis and extrusion parameters, it is possible to produce spherical nanocomposite microbeads with diameters set in the range from 400 to 800 μm and AgNP concentrations in the range 0.2–4.5 mM (Jovanović et al., 2012; Stojkovska et al., 2012).

In the present work, we have investigated possibilities for drying and reswelling of the Ag/alginate microbeads, attractive for potential biomedical applications as antimicrobial powders for wound treatments. In the first experimental series, Ag/alginate microbeads (760 ± 30 μm in diameter) with the AgNP [Ag^0] concentration of 4.87 ± 0.05 mM were dried at room temperature until the constant weight yielding approximately 5% of the initial wet weight. Dried microbeads retained a relatively spherical shape (240 ± 30 μm in diameter) and preserved a greater part of AgNPs as verified by FE-SEM analysis (Fig. 2). It should be noted that we have developed a simple procedure for obtaining microbead cross-sections for FE-SEM analysis utilizing alginate hydrogel/solution as the microbead carriers. Thus, FE-SEM micrographs have shown microbead interiors where individual spherical AgNPs could be distinguished approximately 10–30 nm in diameter as previously determined by transmission electron microscopy of Ag/alginate colloid solutions

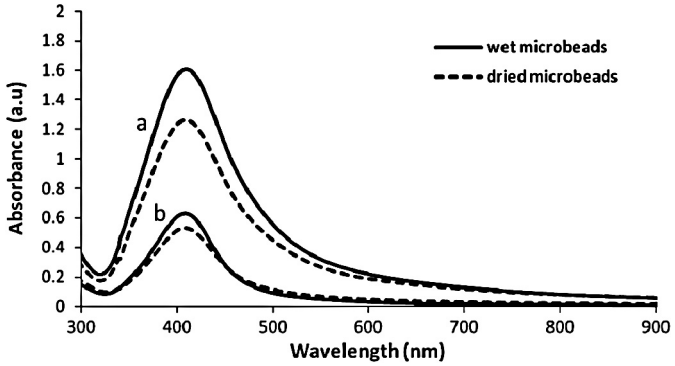

Fig. 3. Typical UV–vis absorption spectra of wet and dried Ag/alginate microbeads based on the same sample wet weight with different silver concentrations in the initial wet microbeads: (a) 4.2 ± 0.4 mM, (b) 1.2 ± 0.05 mM (data represent average of $n \geq 2$; standard deviations (≤10%) are omitted from the graph in order for the spectra to be distinguishable).

(Jovanović et al., 2012). In addition, cross-sections of wet and dried Ag/alginate microbeads appeared similar showing only occasional aggregation of AgNPs in the latter case. Comparison of UV–vis spectra of the initial wet and then dried microbeads with different AgNP concentrations, upon dissolution in Na-citrate, has shown an unchanged absorbance peak position at approximately 405 nm yielding a difference in the absorbance value of approximately 15–20% with respect to the initial microbeads (Fig. 3). These losses could be attributed to nanoparticle aggregation during hydrogel network contraction over drying being slightly more pronounced at higher AgNP concentrations (Fig. 3). Thus, it could be assumed that dried microbeads retained ~80–85% of AgNPs initially present before drying.

Possibilities for alginate gel rehydration and release of AgNPs from dried Ag/alginate microbeads were investigated in the physiological saline solution at room temperature in order to mimic contact with wound secretions. The experiments were performed in parallel with wet and dried microbeads (initial AgNP [Ag^0] concentration in wet microbeads of 4.87 ± 0.05 mM). Rehydration of dried microbeads over 5 days is presented in Fig. 4. After 24 h in the saline solution, both initially wet as well as dried microbeads increased in size reaching similar average diameters (1000 ± 40 and 1020 ± 40 μm, respectively) while in both cases approximately 95% of the initially present AgNPs were lost as determined by the absorbance intensity at 405 nm (data not shown). Furthermore, after 5 days in the saline solution all microbeads appeared fractured and devoid of AgNPs, as expected, due to diffusion of AgNPs/ions and Cl^- ions and formation of AgCl.

As an analogous study, rehydration of dried microbeads was performed in distilled water showing only moderate rehydration after 48 h reaching approximately 20% of the initial wet weight and half of the initial wet microbead size (250 ± 20 μm as compared to the initial diameter of 550 ± 30 μm). Yet, these microbeads retained ~30% of AgNPs initially present in the wet microbeads as determined from the absorbance peak intensity at ~415 nm (data not shown).

The obtained results indicate potentials for use of dry Ag/alginate microbeads, which successfully retained AgNPs while demonstrating similar swelling behavior in the physiological saline solution as wet microbeads. On the other hand, low rehydration of dried Ag/alginate microbeads in distilled water is expected and explained by the formation of egg-box multimers during drying, which are stable in pure water (Vreeker, Li, Fang, Appelqvist, & Mendes, 2008). Nevertheless, in the presence of Na^+ ions in the saline solution, Ca^{2+} ions within the gel are exchanged with Na^+, which ultimately causes chain relaxation and enhances the gel swelling, as well as the release of substances entrapped within the alginate matrix (Bajpai, Shukla, Bhanu, & Kankane, 2008; Vreeker et al., 2008).

3.2. Cytotoxicity studies

In order to evaluate *in vitro* cytotoxicity of Ag/alginate microbeads, two types of experimental studies were carried out: monolayer cultures of bovine calf chondrocytes and 3D cultures of the same cell type immobilized in alginate microbeads in perfusion bioreactors.

3.2.1. Cytotoxicity studies in monolayer cell cultures

The first experimental study of Ag/alginate microbead cytotoxicity was performed according to the standard protocol of monolayer cultures exposed to direct contact with the investigated agent followed by determination of cell viability after 48 h by the MTT test. A separate study was performed under the same conditions using physiological saline solution instead of the cell culture in order to determine concentrations of the released silver nanoparticles and/or ions into the medium to which the cell monolayers were exposed. In specific, we have used Ag/alginate microbeads with different AgNP concentrations added to monolayers in two weight ratios, while the saline solution was analyzed for free Ag^+ ions as well as for the total content of the released silver as Ag^+ and AgCl. Due to very low solubility of AgCl, concentrations of free Ag^+ ions were not significantly different in all solution samples amounting to 0.5 ± 0.1 μg/ml (data not shown). However, when the total silver content was measured after dissolution of the precipitated AgCl significant differences among the experimental groups were detected, as expected. In Table 1 summary of experimental conditions for cytotoxicity determination and measured concentrations of total silver content released in saline solutions is presented.It could be seen that over 48 h Ag/alginate microbeads release 40–50% of the initial silver content yielding total silver concentrations in the solution in the range 2–40 μg/ml in the present study. Effects of these concentrations on the cell survival, *S*, in the presence of alginate and Ag/alginate microbeads is presented in Fig. 5. It could be seen that presence of alginate and Ag/alginate microbeads with released silver concentrations of up to 5 μg/ml induced negligible effects on bovine calf chondrocyte survival ($S > 90\%$). However,

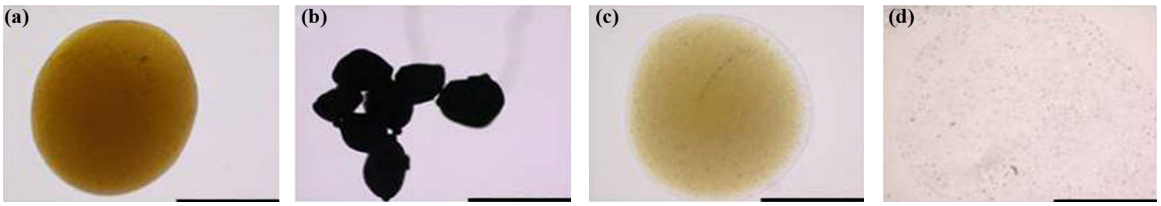

Fig. 4. Rehydration of dried Ag/alginate microbeads: (a) a representative wet microbead; (b) dried microbeads; (c) a representative dried microbead rehydrated for 24 h in the saline solution; (d) a representative dried microbead rehydrated for 5 days in the saline solution (scale bar: 500 μm).

Table 1
Silver release from Ag/alginate microbeads into the saline solution after 48 h.

AgNP [Ag^0] concentration in the initial microbeads (mM)	Microbead mass per well (g)	Released silver concentration in the saline solution (as Ag^+ and AgCl) (μg/ml)	Fraction of silver released into the saline solution* (%)
0.33 ± 0.02	0.3	2.0 ± 0.2	52
0.99 ± 0.07	0.3	5.0 ± 0.7	47
3.75 ± 0.04	0.3	22.9 ± 0.5	53
0.87 ± 0.05	0.6	6.8 ± 0.5	35
1.82 ± 0.05	0.6	17.6 ± 4.7	43
3.76 ± 0.02	0.6	40.0 ± 2.2	48

* Calculated based on the measured average silver concentrations in saline solutions, measured solution volumes, and measured average initial silver concentrations and weights of Ag/alginate microbeads.

when released silver concentration was increased to about 7 μg/ml it induced severe cytotoxicity ($S = 13.9 \pm 0.8\%$).

The obtained results are in agreement with other studies of the effects of different systems containing AgNPs on various mammalian cell types, where cytotoxic concentrations of AgNPs were reported to range from 1.6 to 50 μg/ml (Hussain, Hess, Gearhart, Geiss, & Schlager, 2005; Park, Yi, Kim, Choi, & Park, 2010). It should also be noted that it is well known that cytotoxicity of AgNPs depends on the concentration and size of nanoparticles, as well as on the cell type (Kim et al., 2012).

Overall, results of Ag/alginate microbead cytotoxicity studies in monolayer cell cultures have shown that Ag/alginate microbeads released approximately 40–50% of the initial AgNP amount and that the released silver concentrations in the culture medium up to 5 μg/ml were not cytotoxic for chondrocytes.

3.2.2. *Cytotoxicity studies in 3D cell cultures in perfusion bioreactors*

In order to investigate possible cytotoxic effects of the Ag/alginate microbeads under conditions imitating physiological environment in vascularized tissues *in vivo*, a 3D cell culture was established in perfusion bioreactors. In specific, bovine chondrocytes were immobilized at the concentration of $21.0 \pm 1.7 \times 10^6$ cells/ml in alginate microbeads with practically all cells being viable. Cell loaded microbeads were mixed with Ag/alginate microbeads (4.7 ± 0.6 mM AgNPs [Ag^0]) in the approximate ratio 3:1 and cultivated in perfusion bioreactors under continuous medium flow of 0.38 ml/min. The applied flowrate corresponded to the superficial medium velocity of ~100 μm/s, which is in the range of blood velocities in capillaries. After 2 weeks of bioreactor cultivation, microbeads slightly deformed, while the concentration of immobilized cells slightly but not significantly decreased ($16.4 \pm 3.5 \times 10^6$ cells/ml) with the viability preserved at 78% implying slight cytotoxic effects. In order to determine the maximal concentration of the released AgNPs and/or ions in the medium to which the cells were exposed, corresponding to the first medium exchange (*i.e.* 72 h), a parallel experiment was performed using Ag/alginate microbeads (5.5 ± 0.4 mM AgNP [Ag^0] concentration), pure alginate microbeads instead of cell loaded microbeads and saline solution instead of the cultivation medium. The concentration of free Ag^+ ions in the saline solution was 0.9 ± 0.3 μg/ml not significantly different from measured Ag^+ concentrations in the monolayer studies due to low solubility of AgCl. Thus, the total amount of released AgNPs and/or ions from Ag/alginate microbeads was determined based on measured silver concentrations in initial microbeads and after the experiment (5.5 ± 0.4 mM and 102 ± 16 μM, respectively). Based on the mass of Ag/alginate microbeads (0.13 g) and measured saline solution volumes the total silver concentration in the saline solution can be calculated as 9.3 ± 0.9 μg/ml. This concentration corresponds to strong cytotoxicity as determined in monolayer cultures (Fig. 5). However, under *in vivo* like settings in perfusion bioreactors and 3D environment, this concentration had only a negligible effect on cell viability. Consequently, cells in monolayers could be regarded as more sensitive to AgNPs and Ag^+ than cells surrounded by the alginate matrix in a 3D environment. Thus, these results stress the importance of the comprehensive biomaterial assessment including different aspects and settings, and demonstrate the utility of biomimetic bioreactors for functional biomaterial evaluation under *in vivo*-like conditions.

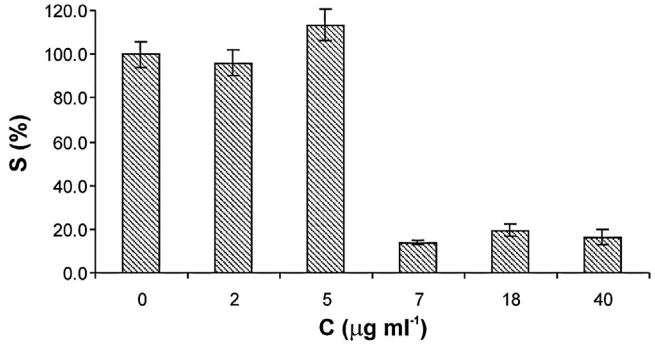

Fig. 5. Survival, *S*, of bovine calf chondrocytes cultured in the presence of alginate or Ag/alginate microbeads with different AgNP concentrations normalized to the control sample as a function of total released silver concentrations in the medium, *C* (Table 1; data represent average of $n = 3$).

3.3. *Antibacterial activity*

In order to demonstrate the potential utility of Ag/alginate microbeads, antibacterial activity of both wet and dried microbeads was investigated in suspensions of *S. aureus* and *E. coli* as frequently used model bacterial strains for Gram-positive and Gram-negative species, respectively. Ag/alginate microbeads used in *S. aureus* suspensions had AgNP [Ag^0] concentration of 1.2 ± 0.05 mM while microbeads used in *E coli* suspensions had AgNP [Ag^0] concentration of 1.7 ± 0.2 mM. Part of the microbeads was dried to the constant weight yielding particles of ~200 μm in diameter. Antibacterial activity was investigated in bacterial suspensions based on the same microbead wet weights. Both microbead types demonstrated the release of AgNPs and/or ions inducing growth delay of both *S. aureus* and *E. coli* (Tables 2 and 3, respectively). After 1 h of incubation, bacterial concentrations in both microbead groups were lower than the initial concentrations (reduction of about 1 $\log_{10}$-unit) and slowly increased over the next 23 h reaching the colonies count of the order of 10^6 CFU/ml. Still these values were significantly lower than those measured in the control groups (reduction of almost 3 $\log_{10}$-units). It is interesting to note, that both wet and dried microbeads exhibited similar antibacterial activity against both investigated bacterial strains with the exception of the slightly stronger effect found in the *E. coli* culture after 24 h incubation with dried Ag/alginate microbeads (Table 3).

In order to investigate the release of silver nanoparticles and/or ions, which has caused the observed bacterial growth delays, corresponding experiments were performed in physiological saline

Table 2
Colonies count of *Staphylococcus aureus* TL in the control suspension and suspensions with wet and dried Ag/alginate microbeads at the concentration of 0.4 g/ml based on the microbead wet weight.

	Initial	1 h	24 h
Control (CFU/ml)	3.8×10^5	6.6×10^5	2.7×10^9
Wet microbeads (CFU/ml)	3.8×10^5	$(9.5 \pm 2.1) \times 10^4$	$(6.9 \pm 1.3) \times 10^6$
Dried microbeads (CFU/ml)	$(3.7 \pm 0.3) \times 10^5$	$(9.5 \pm 4.9) \times 10^4$	$(5.3 \pm 2.8) \times 10^6$

solutions using Ag/alginate microbeads with AgNP concentration of 1.9 ± 0.05 mM AgNPs [Ag^0] under the same conditions. Concentrations of released free Ag^+ ions in saline solutions were again not significantly different in all samples yielding the value of 0.53 ± 0.06 μg/ml, corresponding to the results determined in cytotoxicity studies and low solubility of AgCl. However, when the total contents of released silver as Ag^+ and AgCl in the saline solution were measured, values of 0.58 ± 0.09 μg/ml and 10.3 ± 3.0 μg/ml were obtained for wet microbeads after 1 and 24 h, respectively. Corresponding measured total released silver concentrations for dried microbeads were 1.67 ± 0.62 μg/ml and 2.44 ± 0.05 μg/ml, respectively, although the exhibited antibacterial activity was similar as compared to that of wet microbeads. These differences could be attributed to slight differences in alginates used in antibacterial and silver release studies, the latter of which was observed to swell at slower rates.

The obtained results are in agreement with other studies of different systems containing AgNPs against *S. aureus* and *E. coli*, where the minimum inhibitory concentrations of AgNPs were reported to range from 0.34 to 120 μg/ml for *S. aureus* and 0.26 to 180 μg/ml for *E. coli* (Panáček et al., 2006; Ruparelia, Chatterjee, Duttagupta, & Mukherji, 2008; Valodkar, Modi, Pal, & Thakore, 2011), depending on the size, shape and surface modifications of nanoparticles (Marius et al., 2011; Morones et al., 2005; Pal, Tak, & Song, 2007; Sondi & Salopek-Sondi, 2004). In comparison to our study, the surface of a Chitlac-alginate hydrogel with incorporated AgNPs was shown to be bactericidal against *E. coli*, *S. epidermidis*, *S. aureus*, and *P. aeruginosa* (Travan et al., 2009). Furthermore, Ag/alginate nanocomposite sponges (5.65 mM, 4.8 ± 0.1 cm in diameter) obtained by freeze–drying of Ag/alginate colloid solution followed by cross-linking in 0.2 M $CaCl_2$ solution, completely reduced the number of *S. aureus* colonies after 24 h of incubation (Seo et al., 2012). However, in this study bactericidal effects were related to the synergistic effect of bacteria entrapment within the alginate matrix and direct contact with AgNPs, while in the present investigations, antimicrobial activity could be attributed mainly to the release of AgNPs and/or ions.

It should be also added that antibacterial effects depend on the amount of antibacterial agent applied as well as on the initial bacterial concentration. It was reported that growth of *E. coli* was inhibited at a larger extent as the initial bacterial concentration was decreased so that at the initial number of colony-forming units (CFU) of 10^4 after 24 hours of incubation the growth was completely suppressed (Sondi & Salopek-Sondi, 2004). Thus, it could be concluded that antibacterial activity of Ag/alginate microbeads should be further investigated in different experimental settings and against broader range of microbial strains while this study has shown underlying principle of antibacterial activity of both wet and dried Ag/alginate microbeads by the release of AgNPs and/or ions. Possible strategies to enhance antibacterial activity of Ag/alginate hydrogels at the same levels of silver release could be production of spongeous or fibrous wound dressings composed of Ag/alginate microfibers (Vidovic, Zvicer, Stojkovska, Miškovic-Stankovic, & Obradovic, 2012) with larger specific surface area as compared to that of microbeads, which could additionally provide bacterial entrapment as reported in literature (Seo et al., 2012).

In the present work we have also attempted to evaluate potential functionality of Ag/alginate microbeads by a comprehensive approach to jointly analyze aspects of different studies. If the results of antibacterial activity of Ag/alginate microbeads are compared to the results of cytotoxicity studies, it can be assumed that the release of silver nanoparticles and/or ions can be tuned so to induce antibacterial activity without causing cytotoxic effects. This assumption is supported by the finding that the total concentration of released silver present as AgNPs, Ag^+ and/or AgCl of about 9–10 μg/ml could induce bacteriostatic effects (*i.e.* growth delay) without affecting viability of cells embedded in a tissue-like matrix in perfusion bioreactors. On the other hand, if only results of cytotoxicity studies in monolayer cultures are considered it would be presumed that these silver concentrations would be highly cytotoxic for chondrocytes. It is now well known that 2D cell culture systems have severe limitations when representing *in vivo* environments since tissue-specific architecture, mechanical and biochemical signals, and cell–cell communication are lost in these systems (Mazzoleni, Di Lorenzo, & Steimberg, 2009). Furthermore, cell function and behavior were reported to significantly differ under 2D and 3D culture conditions (Mazzoleni et al., 2009) so that cytotoxicity studies in 2D cultures may not accurately reflect the actual toxicity of nanoparticles in the body (Lee, Lilly, Doty, Podsiadlo, & Kotov, 2009). Accordingly, cytotoxic effects of different agents were found to be more pronounced in 2D as compared to 3D cultures (*e.g.* Hashimoto et al., 2014; Lee et al., 2009; Movia, Prina-Mello, Bazou, Volkov, & Giordani, 2011) as also found in the present study. An additional key parameter determined in the present study is the form of released silver that is measured in antimicrobial and cytotoxicity studies. Chloride ions, necessarily present in all body fluids as well as in commonly used cell culture media and nutrient broths, react with AgNPs and/or ions resulting in poorly soluble AgCl formation. Thus, it should be taken care to distinguish concentrations and the effects between those of free Ag^+ ions and AgCl. In the present study cytotoxicity and antibacterial effects could be clearly related to total released silver concentrations in contrast to concentrations of free Ag^+, which were approximately an order of magnitude lower and not significantly different in all culture systems. Further studies should be performed in order to determine mechanisms and kinetics of AgNP release, oxidation and AgCl formation in different systems based on wet and dried Ag/alginate nanocomposites.

Table 3
Colonies count of *Escherichia coli* ATCC 25922 in the control suspension and in suspensions with wet and dried Ag/alginate microbeads at the concentration of 0.4 g/ml based on the microbead wet weight.

	Initial	1 h	24 h
Control (CFU/ml)	2.8×10^6	7.6×10^6	2.3×10^9
Wet microbeads (CFU/ml)	$(1.7 \pm 0.2) \times 10^6$	$(8.3 \pm 0.3) \times 10^4$	$(3.1 \pm 1.5) \times 10^6$
Dried microbeads (CFU/ml)	$(2.2 \pm 0.4) \times 10^6$	$(5.0 \pm 3.7) \times 10^5$	$(4.3 \pm 5.9) \times 10^3$

Overall, it could be assumed that wet Ag/alginate microbeads could be attractive for tissue engineering applications in combinations with cell scaffolds, constructs or soft tissue implants providing a sterile environment, either *in vitro* or *in vivo*. On the other hand, dried Ag/alginate microbeads could be applied as antimicrobial powders in wound treatments providing moisture capture by rapid alginate matrix reswelling and antibacterial effects due to the release of AgNPs and/or ions.

4. Conclusion

Development of novel biomaterials for potential biomedical applications requires comprehensive evaluation studies regarding different aspects of biomaterial functionality under *in vitro* conditions, which could in concert decrease the extent of necessary animal studies. In this work, we have attempted to use an integrated approach to assess nanocomposite alginate microbeads with incorporated AgNPs in wet as well as in dry hydrogel form. In specific, drying of the microbeads has shown successful preservation of ~80–85% of the initial nanoparticles while the hydrogel retained the capacity for reswelling and release of AgNPs and/or ions. Next, several studies have been combined with the aim to set a platform for optimization of silver release and antibacterial activity while preventing cytotoxic effects. In this respect it has been demonstrated that the use of 3D cultures and biomimetic bioreactors is valuable when developing novel biomaterials and predicting the biomaterial behavior upon implantation. In specific, bovine calf chondrocytes in monolayer cultures were shown to be more sensitive to released AgNPs and/or ions as compared to chondrocytes immobilized in alginate microbeads and cultured in perfusion bioreactors. Concentrations of released silver as AgNPs, Ag^+ and/or AgCl of 9–10 μg/ml were shown to be severely cytotoxic to cells in monolayer cultures while indistinctly affected cells within the alginate matrix and, on the other hand induced growth delay in suspensions of *E. coli* and *S aureus*. It was shown that care should be taken in measurements of released silver nanoparticles and/or ions in the presence of chloride ions leading to formation of poorly soluble AgCl. Overall, this study has demonstrated utility of combining different investigations and aspects of novel biomaterials in order to reveal underlying functional principles, determine directions for biomaterial optimization and evaluate application capabilities. In specific, in the present work novel alginate microbeads with incorporated silver nanoparticles in wet and dried forms were shown to exhibit potentials for biomedical applications as components of tissue engineering scaffolds or soft tissue implants as well as in antimicrobial wound dressings so to decrease the need for antibiotics.

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