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In vitro osteogenic potential of collagen/chitosan-based hydrogels-silica particles hybrids in human bone marrow-derived mesenchymal stromal cell cultures

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ABSTRACT

The aim of this study was to assess osteogenic potential of three groups of biopolymeric hydrogel-based surfaces made of plain collagen, chitosan or collagen/chitosan, crosslinked with genipin or all three biopolymers modified with silica particles of two sizes (S1 = 240 nm and S2 = 450 nm). Biocompatibility and osteoinductive properties of the resulting composites were analyzed in the human bone marrow-derived mesenchymal stromal cells (hBMSCs) *in vitro* cultures. It was revealed that all tested materials are biocompatible and significantly enhance ALP activity in hBMSCs which was particularly pronounced for collagen/chitosan based hybrids. Gene expression (*RUNX-2*, *COL-I*, *OC* and *VEGF* mRNA) analyses performed in hBMSCs cultured at collagen/chitosan materials showed that ColChS1 hybrid the most effectively promotes osteogenic differentiation of hBMSCs. SEM and EDS analyses of materials carried out after 20 days of hBMSCs culturing on ColCh-based hydrogels revealed that the hybrid materials enhanced hBMSCs-mediated mineralization of ECM. Our studies revealed that collagen/chitosan hydrogels modified with silica particles of smaller sizes (ColChS1) exhibit high pro-osteogenic properties without the need of applying any additional osteogenic inducers. That suggests that ColChS1 having the intrinsic osteoinductive activity holds great potential as material of choice for bone regeneration procedures, especially in regeneration of small bone losses.

1. Introduction

Regenerative medicine faces many challenges regarding selection of biomaterials which could be useful for bone tissue engineering which aims to meet the growing needs for therapies of trauma-, diseases- or aging-affected bones [1,2]. Three-dimensional scaffolds, structurally similar to naturally occurring extracellular matrix (ECM) have been particularly extensively studied for bone tissue engineering purposes [3]. However, preparation of a scaffold that would meet all the rigorous criteria for maintaining cell growth and enabling their differentiation is highly challenging. Scaffold should serve as a temporary ECM architecture-mimicking template which can guide the cells, facilitate their growth and provide a mechanical support for a developing tissue. Plethora of materials have been tested for scaffold designing application and it has been shown that their proper choice is essential for the success of a future bioimplant. Among them ceramics (hydroxyapatite, bioglasses), polymers (collagen, chitosan, hyaluronic acid and polyglycol) as well as varied composites have been most often used [4–6]. The review of the literature indicates that biodegradable, injectable hydrogels are getting considerable interest as versatile candidates for tissue engineering scaffolds [7]. It was also demonstrated that highly hydrated hydrogels obtained by *in situ* gelation of sols can better mimic both the chemical and physical environments of ECM and thus create an ideal cellular microenvironment for cell proliferation and differentiation. Most importantly, injectable hydrogels have a similar microstructure to the extracellular matrix hence may provide good physical integration with the bone defect [8]. Moreover, the supplementation of sols with additional components enables preparation of hydrogels with highly desirable properties such as bioactivity, osteoinductivity which altogether make a scaffold display demanded therapeutic characteristics [9,10].

Silica based materials with favorable biocompatibility are considered as an excellent candidates for applications in various biomedical fields [11,12]. Previous studies have shown that silicon plays an important role in bone formation and conditions and it has been suggested

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that it is involved in collagen synthesis as well as in matrix mineralization [13].

Yang et al. [14] investigated the dose- and size-effect of silica NPs on osteogenic differentiation of hMSCs and found that the NPs with three different sizes (50 nm, 200 nm and 400 nm) did not affect the cell viability (even up to a concentration of 500 μg ml^{-1}). They also examined the effect of silica NPs on both early and late marker of osteogenic differentiation and established that nanoparticles promote osteogenic differentiation of hMSCs however that was observed in osteogenic medium (OM) with the presence of dexamethasone, β glycerophosphate disodium and ascorbic acid. Beck et al. demonstrated that silica based NPs possess strong biological activities including stimulation of osteoblast differentiation and mineralization and suppression of osteoclast differentiation *in vitro* as well as enhance bone mineral density *in vivo* [15].

Moreover, the composite based on mesoporous silica nanoparticle/chitosan nanofibers have been also found to promote the osteoblast attachment, proliferation, osteogenic differentiation and bone formation [16]. Keller et al. [17] revealed that 3D chitosan-based scaffolds loaded with silica nanoparticles promoted *in vitro* cell proliferation and the formation of osteoblastic spheroids, which was not observed in case of 3D scaffolds based on PCL or collagen. The effect of an inorganic silica supplement added to an Na-alginate matrix have been also studied [18]. Bone- and osteoblast-like SaOS-2 cells were embedded into polymeric matrix which was supplemented with prehydrolyzed TEOS (a source of ortho-silicate). It was found that after cells exposure to silica their ability to synthesize the hydroxyapatite crystallites noticeably increase, but the experiment was performed in a mineralization activation cocktail (composed of β glycerophosphate, ascorbic acid and dexamethasone).

In our previous papers [19,20] we have described the preparation of the novel hybrid organic-inorganic materials based on collagen and collagen-chitosan hydrogels containing, *in situ* prepared, silica nanoparticles of two various sizes and presented the studies on their bioactivity as well as on physicochemical and biological properties. In *in vitro* experiments carried out under simulated body fluid conditions we have demonstrated that the hybrids developed by us efficiently induced mineralization process. Importantly, it was also shown that due to the addition of silica particles to the sols, the hydrogel-silica hybrid system enhanced mineralization not only at the surface but also in the bulk of the material.

The main goal of the studies presented in this paper was to evaluate the osteogenic potential of different organic-inorganic hybrids based on biopolymer hydrogels containing silica particles of two sizes. Our experiments were performed in cultures of human bone marrow-derived mesenchymal stromal cell (hBMSCs), the type of cells holding great potential in regenerative medicine and bone-targeted therapies [21]. We have investigated viability as well as ALP activity of hBMSCs cultured on collagen, chitosan and collagen/chitosan based hybrids. Gene expression (*RUNX-2*, *COL-I*, *OC* and *VEGF* mRNA) analyses were performed in hBMSCs cultured at collagen/chitosan materials selected based on the results obtained in our initial cell viability and ALP activity experiments. Furthermore, in order to verify whether the materials tested by us can enhance cell-mediated mineralization of ECM, we carried out SEM and EDS analyses.

The main novelty of our studies involves demonstration of the intrinsic osteoinductivity of composites developed without the use of any osteogenic inducers. To the best of our knowledge there has been no studies presented in literature on the *in vitro* osteogenic potential of that type of hybrid materials composed of biopolymeric injectable hydrogels and silica particles in hBMSCs cultures.

2. Materials and methods

Collagen type I rat tail (3.9 mg/ml solution, BD Biosciences), genipin (Challenge Bioproducts Co., 98%), chitosan (low molecular weight, Aldrich), tetraethoxysilane (TEOS, ≥98%, Fluka), ethanol (99.8%, spectroscopic grade), ammonium hydroxide (25%, pure p.a., Chempur), acetic acid (Chempur), MEM Alpha Medium (Gibco), Fetal Bovine Serum, qualified, US origin (FBS, Gibco), Penicillin-Streptomycin Solution 10,000 Units/ml Penicillin, 10,000 μg/ml Streptomycin (HyClone), Ficoll-Paque reagent (GE Healthcare, Fairfield, CT, USA),

Trypsin 0.25% (1×) Solution, with 2.5 g Porcine Trypsin (1:250)/l in HBSS with 0.1% EDTA (HyClone), Alamar Blue (Invitrogen), Cell Digestion Buffer and Cell Assay Buffer compounds: *tris* hydroxymethyl aminomethane, ($(HOCH_2)_3CNH_2$) (Tris) (Sigma Aldrich, 99.8%), zinc chloride, $ZnCl_2$ (POCh), magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$ (POCh), Triton X-100 (POCh), *p* nitrophenyl phosphate (pNPP) (Sigma-Aldrich), GeneMATRIX Universal RNA Purification Kit (EURx), glutaraldehyde (Sigma-Aldrich), hezamethyldisilizane (HMDS, Sigma-Aldrich).

2.1. Preparation of the silica particles, the hydrogels and the hybrid materials

2.1.1. Silica particles

The silica particles of two sizes (S1 and S2) were fabricated by the Stöber synthesis [22] employing the procedure, as described earlier [19]. The hydrodynamic diameter and zeta potential values of the resulted particles were assessed using a Malvern Nano ZS light-scattering apparatus. The mean hydrodynamic diameters for S1 and S2 particles were 210 ± 1 nm and 438 ± 5 nm, while the zeta potentials (ζ) were −56.9 ± −9.1 mV and −62.9 ± −6.8 mV for S1 and S2 particles, respectively.

2.1.2. Hydrogels

The pristine hydrogels were fabricated using the procedure developed by us and described earlier [23]. Collagen hydrogel (Col) synthesis was carried out as follows: 800 μl of the stock collagen solution was mixed with 200 μl of 20 mM genipin solution in 10× PBS buffer (pH = 7.4). Chitosan hydrogel (Ch) was obtained by adding 200 μl of 20 mM genipin solution to 800 μl of 2 wt% chitosan solution in 1% acetic. The collagen/chitosan hydrogel (ColCh) (with the Col/Ch weight ratio 50:50) was fabricated by mixing 690 μl of the stock collagen solution, 169 μl of 2 wt% chitosan solution in 1% acetic acid and 172 μl of 20 mM genipin solution. All the prepared polymeric sols were vigorously vortexed and incubated at 37 °C until gel formation.

2.1.3. Hybrid materials

Hybrid materials were prepared according to a similar procedure with the difference that before incubation the water dispersion of silica particles S1 or S2 (0.3 ml, 16.6 mg/ml) was added to 1 ml of the polymeric sols. Then, the procedure was continued analogously as for plain hydrogels. Final silica particles concentration in sols was 3.8 mg/ml.

Using collagen, chitosan and collagen/chitosan hydrogels and introducing silica particles of two sizes (S1 - 210 nm and S2 - 440 nm) the following hybrid materials were prepared (see Table 1): collagen-silica named ColS1 and ColS2; chitosan-silica named ChS1 and ChS2; collagen-chitosan-silica named ColChS1, ColChS2.

Table 1
Overview of materials developed (Col – collagen-based materials, Ch – chitosan-based materials, ColCh – materials based on collagen-chitosan matrix, silica particles sizes: S1 - 210 nm, S2 - 438 nm).

Group 1	Group 2	Group 3
Col	Ch	ColCh
ColS1	ChS1	ColChS1
ColS2	ChS2	ColChS2