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Development of injectable organic/inorganic colloidal composite gels made of self-assembling gelatin nanospheres and calcium phosphate nanocrystals

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ABSTRACT

Colloidal gels are a particularly attractive class of hydrogels for applications in regenerative medicine, and allow for a "bottom-up" fabrication of multi-functional biomaterials by employing micro- or nanoscale particles as building blocks to assemble into shape-specific bulk scaffolds. So far, however, the synthesis of colloidal composite gels composed of both organic and inorganic particles has hardly been investigated. The current study has focused on the development of injectable colloidal organic–inorganic composite gels using calcium phosphate (CaP) nanoparticles and gelatin (Gel) nanospheres as building blocks. These novel Gel–CaP colloidal composite gels exhibited a strongly enhanced gel elasticity, shear-thinning and self-healing behavior, and gel stability at high ionic strengths, while chemical – potentially cytotoxic – functionalizations were not necessary to introduce sufficiently strong cohesive interactions. Moreover, it was shown in vitro that osteoconductive CaP nanoparticles can be used as an additional tool to reduce the degradation rate of otherwise fast-degradable gelatin nanospheres and fine-tune the control over the release of growth factors. Finally, it was shown that these colloidal composite gels support attachment, spreading and proliferation of cultured stem cells. Based on these results, it can be concluded that proof-of-principle has been obtained for the design of novel advanced composite materials made of nanoscale particulate building blocks which exhibit great potential for use in regenerative medicine.

1. Introduction

The complexity of biomaterials has increased tremendously over the past decades in terms of both structure and composition by implementing regenerative cues that stimulate regeneration of surrounding tissues [1–4]. Incorporation of therapeutic biomolecules using traditional carrier materials is still associated with major drawbacks due to the poor control over the release of growth factors at the target sites. A recent study on the clinical efficacy of the well-known osteogenic growth factor bone morphogenetic protein-2 (BMP-2) has confirmed that growth factor delivery from conventional collagen sponges results in serious clinical complications [5], emphasizing the need for novel carrier materials capable of delivering growth factors in a controlled and sustained manner. In that respect, colloidal gels are a particularly attractive class of hydrogels since these materials allow for "bottom-up" synthesis of functional, self-healing materials by employing micro- or nanoscale particles as building blocks to assemble into shape-specific bulk materials [6–13]. For applications in regenerative medicine, charged micro- or nanospheres made of biocompatible polymers are the most obvious candidates to serve as building blocks since the physicochemical properties of polymeric particles can be tailored to desire in terms of size, charge and chemical derivatization. Therefore, colloidal gels have been formed by self-assembly of oppositely charged microspheres (e.g. chemically functionalized dextran [14]) or nanospheres (e.g. chemically functionalized poly(lactic-co-glycolic acid (PLGA) nanospheres [10,12,13]) [8,10,12–17]. Recently, our group has developed a novel class of colloidal gels made of oppositely charged gelatin nanospheres which displayed superior mechanical and biological properties over microstructured colloidal gelatin gels [15,18]. On the other hand, although the general mechanism of organic/inorganic heteroaggregation has been studied recently [19,20], this knowledge has not been translated yet towards the development of colloidal composite gels made of biocompatible organic and inorganic nanoparticles for biomedical applications. For regeneration of hard tissues, the introduction of inorganic building blocks

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into colloidal gels could offer considerable advantages over purely organic colloidal gels by (i) increasing stiffness and mechanical strength of the resulting composite due to the reinforcement effect of ceramic phase, and (ii) improving the biological tissue response owing to the osteoconductive properties of inorganic nanoparticles made of, for example, calcium phosphate (CaP) [21–23]. Moreover, the addition of a ceramic phase to polymeric gels could lead to improved control over sustained delivery of growth factors, since CaP ceramics have a strong affinity to proteins [24]. This protein-binding capacity of CaP nanoparticles could directly affect the degradation of gelatin-based carrier materials and the release profile of growth factors [25–29].

Nevertheless, it is not yet known if interparticle forces between organic and inorganic nanoparticles can be sufficiently strong to allow for the formation of cohesive colloidal composite gels for application in regenerative medicine. Therefore, we have studied the feasibility of forming colloidal composite gels by introducing neutral or charged CaP nanoparticles into colloidal gelatin gels. CaP nanoparticles were selected in view of their osteoconductivity [21] as well as inherent affinity to proteins such as collagen, gelatin and growth factors [25–29], and charged CaP nanoparticles can be easily obtained by decorating CaP nanoparticle surfaces with, for example, negatively charged citrate anions that have a strong affinity for CaP surfaces [30]. Gelatin (Gel) was selected as a source for organic nanoparticles since both positively (type A) and negatively charged (type B) gelatins are commercially available. As such, self-assembly between oppositely charged organic and inorganic nanoparticles was studied without the need for additional chemical functionalizations which might compromise the biocompatibility of the final constructs, thereby opening a simple and promising route for the fabrication of nanostructured composite biomaterials of improved functionality. Compared to conventional organic/inorganic composite hydrogels composed of continuous, monolithic matrices, nanostructured colloidal composite gels can possess superior properties over bulk composites in view of their (i) enhanced control over the properties of macroscopic scaffolds by fine-tuning the characteristics of sub-populations of particulate building blocks [6,11,31,32]; (ii) injectability/moldability allowing for optimal filling of irregularly shaped defects using minimally invasive approaches [10,14,15]; (iii) in situ gel formation without using the potentially cytotoxic gelling/cross-linking agents to trigger gelation [6,11,31,32]; and (iv) ease of incorporation of therapeutic agents [18,33,34].

To investigate the interactions between CaP nanoparticles and gelatin nanospheres, the self-assembly process of colloidal mixtures containing organic and inorganic particles at diluted conditions (~0.02 w/v%) was monitored using dynamic light scattering (DLS) and transmission electron microscopy (TEM) as a function of parameters including particle charge, CaP-to-gelatin ratio (CaP/Gel ratio) and ionic strength, whereas the formation of colloidal composite gels at concentrated conditions was investigated using rheometry as a function of solid content (up to 20 w/v%), CaP/Gel ratio and ionic strength. Moreover, the biological performance of the resulting Gel–CaP colloidal composite gels composed of gelatin and CaP nanoparticles was preliminarily studied in vitro by evaluating (i) gel degradation, (ii) release kinetics of a (radiolabeled) osteogenic protein (BMP-2) and (iii) the cellular response of bone marrow stem cells cultured in contact with the colloidal composite gels.

2. Materials and methods

2.1. Materials

Gelatin A (GelA, from porcine skin, 300 Bloom, isoelectric point (IEP) ~9) and gelatin B (GelB from bovine skin, 225 Bloom, IEP ~5) were purchased from Sigma–Aldrich. Calcium hydroxide ($Ca(OH)_2$, 98+%, extra pure), sodium citrate tribasic dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), and glutaraldehyde (GA, 25 wt.% solution in water) were purchased from Acros Organics. Phosphoric acid (H_3PO_4, 85%) and acetone were from J.T. Baker. Recombinant human bone morphogenetic protein-2 (BMP-2, carrier-free, catalog no. 355BM/CF, molecular mass 13 kDa, IEP 8.5) was supplied by R&D systems. All other materials were purchased from Sigma–Aldrich.

2.2. Preparation of gelatin nanospheres

Gelatin nanospheres were prepared using a two-step desolvation method as reported previously [15]. Subsequently, GA was used to stabilize gelatin nanospheres with a molar ratio of GA relative to $[NH_2]_{gelatin}$ of 1:1 [15]. After cross-linking at room temperature for 16 h, glycine solution (100 mM) was added to the nanosphere suspension to block unreacted aldehyde groups. The nanosphere suspensions were then subjected to three cycles of centrifugation (16,100*g* for 5 min) and resuspension in deionized water by vortexing; thereafter the pH of nanosphere suspension was adjusted to 7.0. To investigate the self-assembly of nanoparticles at diluted conditions, gelatin nanosphere suspensions were stored at 4 °C until further use, while for preparation of composite colloidal gels, nanospheres were freeze-dried (Freezone 4.5, Labconco, USA) and stored at 4 °C. The particle size of gelatin nanoparticles dispersed in deionized water (solid content 0.01% w/v) was determined using dynamic light scattering (DLS, Zetasizer Nano-S, Malvern Instruments Ltd.), whereas the ζ-potential of gelatin nanospheres (dispersed in 5 mM HEPES buffer at pH 7.0) was measured by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments Ltd.).

2.3. Preparation and characterization of CaP nanoparticles

Apatitic CaP nanoparticles were prepared using an established wet-chemical precipitation method based on the following reaction between calcium hydroxide and ortho-phosphoric acid [35]:

$$5Ca(OH)_2 + 3H_3PO_4 \rightarrow Ca_5(PO_4)_3(OH) + 9H_2O$$

Briefly, 100 ml H_3PO_4 solution (75 mM) was added dropwise into an aqueous suspension of 100 ml $Ca(OH)_2$ (125 mM) under continuous stirring for 14–16 h at room temperature, followed by adjustment of the pH to 7.0. CaP nanoparticles were centrifuged (5000 rpm, 1 min) and resuspended in deionized water three times and finally stored as an aqueous suspension (40 mg ml^{-1}) after adjustment of the pH to 7.0.

Sodium citrate was used to render CaP nanoparticles negatively charged by surface adsorption as described before [36]. CaP nanoparticles were suspended in 10 mM aqueous solution of sodium citrate dihydrate at 10 mg ml^{-1} under continuous stirring at room temperature for 14–16 h, after which sodium-citrate-treated CaP nanoparticles (further abbreviated as C–CaP) were rinsed in deionized water (three cycles of centrifugation (13,200 rpm for 5 min) and re-suspension in deionized water by ultrasonication) and stored in aqueous suspension (10 mg ml^{-1}) after adjusting the pH value to 7.0. The lyophilized CaP powders were analyzed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR, Perkin Elmer), X-ray diffraction (XRD, Phillips X'Pert, PW3710) and ζ-potential measurement by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments Ltd.). The morphology of the particles was visualized by TEM (JEOL 1010) and scanning electron microscopy (SEM, JEOL 6301). The size of CaP and C–CaP nanocrystals was determined by averaging the length and width of at least 200 particles using TEM images by digital image analysis software (Image J, NIH).