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COMMUNICATION

Combined release of platelet-rich plasma and 3D-mesenchymal stem cell encapsulation in alginate hydrogels modified by the presence of silica†

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We report the modified release of platelet-rich plasma from alginate platelet-rich plasma hydrogels altered by the presence of silica. These PRP–alginate–silica compositions can be used as injectable carriers for viable mesenchymal stem cells.

Platelet-rich plasma (PRP) is an autologous source of growth factors (GF) derived from blood plasma. It holds great promise in various clinical applications such as periodontal and maxillofacial surgery, spinal fusion, bone augmentation and treatment of skin and soft tissue ulcers.[1] In a study comprising 88 patients Marx *et al.* have shown that bone autogenous bone grafts with PRP displayed a significantly higher bone trabecular density and maturity in the reconstruction of maxillofacial defects.[2] In another study, Margolis *et al.* showed on a large set of patients that PRP treatment for diabetic foot ulcers was more effective than the standard one.[3] However, other studies have shown that PRP does not accelerate or improve the healing process.[4,5] These contradictory results have been attributed to the low control of the spatial and temporal delivery of PRP[6] and the variability in the quantity of growth factors between different PRP preparations.[7]

In typical preparations, the release kinetics of pure PRP is very fast with more than 95% released in the first hour.[7] This can be slowed down by combining PRP with biomaterials (*e.g.* ceramic,[8] polymer scaffolds[9] or gelatin gels[10]) which is found to increase PRP efficiency. *In vitro*, Lu *et al.* have shown using PRP–alginate beads that by controlling the spatial and temporal release of PRP growth factors, the osteogenic differentiation of stem cells could be promoted.[6]

Mesenchymal stem cells (MSCs) are a cell type of choice for tissue engineering applications. As for PRP, MSCs are of autologous origin and possess the ability to differentiate to different cell types under appropriate stimuli.[11] They can be used in combination with a carrier (*e.g.* scaffold, hydrogel, *etc.*) for a variety of applications. For example several studies have investigated the combination of PRP with mesenchymal stem cells (MSCs), showing positive results in various *in vivo* models.[12,13] However, there are other experimental results that showed no significant improvement in the presence of PRP,[14] emphasising the need for a better control of PRP release.

Ideally, a PRP delivery system should be injectable, provide fine-tuning of PRP release and allow for cell encapsulation. Current two-component systems composed of alginate and PRP allow for injectability and cell encapsulation,[6,12] but the ability to finely tune PRP protein and growth factors release is limited.

In our study, we introduced a third component, silica nanoparticles, during alginate–PRP gel synthesis to generate novel particles with the end goal being to modulate the release of TGF-β1 from the beads. It is known that the size or curvature of silica nanoparticles influences protein adsorption and conformation[15,16] such that a synthesis method for the particles was adopted whereby silica particles of 200–250 nm could be rapidly produced under mild conditions (pH 7) compatible with alginates, PRP and other components such as cells.[17] The synthesis was performed by addition of pentaethylenehexamine (PEHA), itself a mimic of molecules found in many silicifying organisms.[18,19] PEHA was chosen as variation of the reaction conditions including concentration and modest changes in pH could lead to a wide variety of particle sizes being formed. The other main component, alginate is a well-known injectable carrier, often used for cell and growth factor encapsulation, due to its mild gelation and controlled release properties, respectively.[20] It is a linear unbranched polysaccharide containing varying amounts of 1,4′-linked β-D-mannuronic acid and α-L-guluronic acid that can be crosslinked by divalent ions (*e.g.* Ca^{2+}, Mg^{2+}). This gelation is sufficiently mild to preserve cells and growth factors viability.[20–22] The kinetics of diffusion of molecules from the alginate network can be modified by changing the sizes of its pores *via* changes to the ionic strength used during synthesis.[23] The combination of silica nanoparticles with alginate and PRP would offer thereby a powerful tool to tune the release of PRP growth factors.

The aim of this study was to validate for the first time the use of a novel PRP–alginate–silica composite gel for combined controllable PRP release and cell-based therapy. Following characterisation of the materials formed, the effect of the presence of silica nanoparticles within the new composite materials on the release of total proteins and the transforming growth factor β1 (TGF-β1, one of the most abundant growth factors in PRP) was investigated. Finally, a successful protocol for MSCs encapsulation was established and the

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effect of silica condensation in an alginate–PRP hydrogel on the viability of MSCs over three days was studied.

PRP–alginate beads with or without silica were prepared. A detailed description of the experimental procedure is available as ESI†. Briefly, sodium alginate was dissolved in Iscoves Modified Dulbecco's medium (IMDM), followed by the addition of appropriate amounts of sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$). This solution was then mixed with an equal volume of a PRP/thrombin solution. Macro-beads with an average diameter of 4 mm were prepared by adding the mixture dropwise into a $CaCl_2$ solution (pH 7) containing PEHA by use of an auto-injector. The presence of the amine promoted the rapid condensation and aggregation of silica.[17,18] Gel beads were formed immediately and left maturating for 10 min in the $CaCl_2$ solution at room temperature.

The novel gel beads were lyophilised before being characterised by infrared spectroscopy (Fig. 1A) and electron microscopy (Fig. 1B and C). Compared to the reactants and the PRP–alginate bead mid-IR profiles, the PRP–alginate–silica bead mid-IR spectrum showed additional vibrational bands at 1000 cm^{-1}, 950 cm^{-1} and 450 cm^{-1}, attributable to Si–O stretching and bending vibrations in silica, indicating the polycondensation of silicate (or silicon species) within the beads (Fig. 1A). This was confirmed by the use of a colorimetric assay for silicon,[24] with the total silicon content in the PRP–alginate–silica beads representing 22% of the initial silicon content added.

Fractured and calcined (700 °C) PRP–alginate–silica beads examined by SEM showed aggregated silica particles of size around 50–200 nm suggesting PEHA catalyzed condensation of silicon species within the PRP–alginate beads (Fig. 1B and C), similarly to previous studies on silica condensation in the presence of PEHA and polysaccharide/protein compositions.[18,25,26]

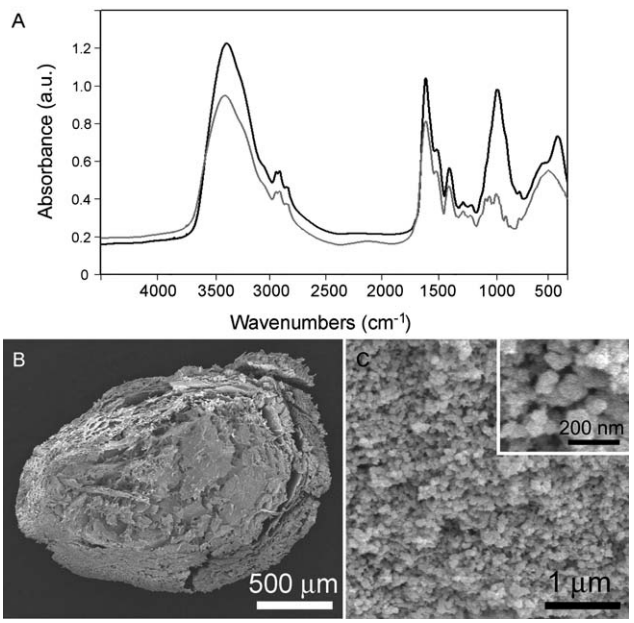

Fig. 1 Mid FT–IR spectra of lyophilized PRP–alginate and PRP–alginate–silica beads (A). SEM images of lyophilized PRP–alginate–silica bead 100 mM sodium metasilicate (B) and calcined PRP–alginate–silica bead 100 mM sodium metasilicate concentration with a higher magnification image in the inset (C).

To prove the first evidence that silica nano-particles modify protein release from PRP–alginate gels, a high concentration of silicate (100 mM) was initially added to the reaction medium and total protein release from the beads and appropriate controls without silica was monitored over 4 days (Fig. 2). For the PRP–alginate beads (no silica), there was an initial fast release (95% of protein released within the first 2 h), followed by a plateau with no further material being released over the remainder of the experiment. For the PRP–alginate–silica beads, the amount of protein initially released was lower (approximately 80% after 2 h) and slow protein release continued over the next 78 h. Although the protein encapsulation efficiencies have not been specifically addressed, a previous study of protein release from alginate–PRP beads showed that protein loading in such systems is close to ~100%.[6] Interactions between polycondensed silica, a protein (gelatin) and alginate[25] are reported to be low between alginate and silica, and strong between the protein studied and silica and we anticipate the yield from the alginate–silica–protein beads described in this contribution to be as high as from alginate–protein beads.

Once evidence for the ability of the gel beads containing silica nano-particles to modify the release profile of 'total protein' was obtained, the next step was to investigate the effect of silica nano-particles on PRP growth factors release and viability of encapsulated cells in the alginate–PRP–silica hydrogel. Therefore, the maximum silicate concentration explored was reduced to 50 mM to match physiological osmolarity (300–330 mOsm).

To assess the ability of silica nano-particles to moderate the release of growth factors, the release of TGF-β1 from PRP–alginate beads prepared with different concentrations of sodium metasilicate (0, 5, 25 and 50 mM) was measured (ESI†). The variation of TGF-β1 release by the PRP–alginate beads with different concentrations of sodium metasilicate was difficult to interpret due to the large standard deviation values associated with material inhomogeneities (*e.g.* aggregates) that are commonly found in this kind of preparation. Nonetheless, all the sodium metasilicate containing beads showed a slower and significantly higher release of TGF-β1 at 48 h than the PRP–alginate system alone.

Finally, the potential of this novel injectable composite system for cell encapsulation was tested. Human MSCs (hMSCs) were isolated

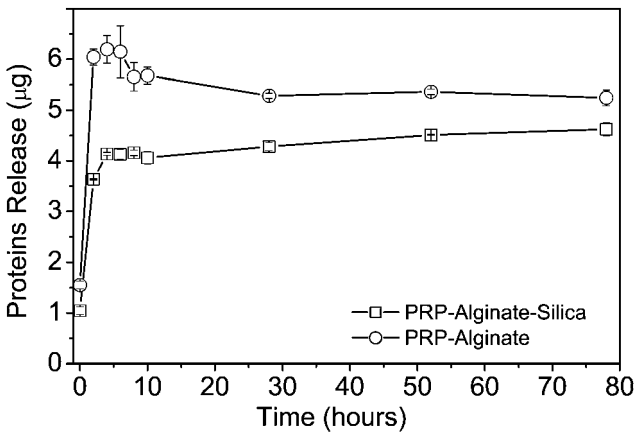

Fig. 2 Plots of total protein release from the PRP–alginate beads (○) and PRP–alginate–silica beads prepared with 100 mM silicon concentration (□) as a function of time in PBS solution at 37 °C; average and standard deviation are reported.

according to a reported procedure.[27] hMSCs were suspended in alginate, alginate–silica, PRP–alginate with thrombin, PRP–alginate–silica with thrombin (cell density of 3×10^6 cells.ml^{-1}). Three different concentrations of sodium metasilicate (5, 25 and 50 mM) were tested and the PRP–alginate volume ratio was set as 0.9. The pH and the ionic strength of the solutions were adjusted respectively to 7.4 and 330 mOsm or as close as possible in the case of the 50 mM silicate concentration. Once the pH and ionic strength had been adjusted for the different solutions, hMSCs could be encapsulated and were then cultured for 3 days. After dissolution of the beads,[28] the proportion of viable cells in the various silica-containing beads compared to the initial number of cells seeded was measured by the trypan blue exclusion assay (Fig. 3).

After one day of culture, the addition of silicate significantly increased the proportion of viable cells, both in alginate and PRP–alginate samples as compared to the controls (alginate and PRP–alginate without silicate) suggesting that the presence of the silicate in the preparation reduces any negative effect of the alginate gel formation on cell viability. The highest proportion of viable cells was achieved at a low silicate concentration (5 mM) which is probably due to the ability of low molecular weight silica species being most effective in disrupting the alginate network. With increasing amounts of silicate in the preparation (5 to 50 mM), the proportion of viable hMSCs in alginate–silica–PRP/no PRP (Fig. 3A and B) after 1 day of culture decreased in a linear fashion (Fig. 3B has $R^2 = 0.999$). It is likely that reactive silicon species are trapped in the alginate gel that rapidly forms following Ca^{2+} diffusion with silica particles continuing to form over time within the gel structure. The resulting physical changes in the gel matrix could possibly damage the immobilized cells in the hydrogel which could explain the reduced numbers of viable cells at higher silicate concentrations.

After three days of culture, the proportion of viable hMSC in the PRP–alginate–silica samples was high (0.91–0.93), independent of the silicate concentration. This is possibly due to the sustained release of growth factors (Fig. 2) in the presence of silicate compared to alginate–PRP samples without silicate (Fig. 3B) as hMSCs express the membrane receptors to the growth factors present in PRP and PRP is known to stimulate hMSCs proliferation.[2,29] This effect was maximal with the highest silicate concentration. It is known that gelatin–alginate interactions are weak while gelatin–silica are strong[25] and that alginate modulates the yield and release kinetics of the different growth factors contained in the PRP.[6] Therefore, we would expect that the yields and kinetics of the different proteins to be affected by the presence of silica in the PRP–alginate composition.

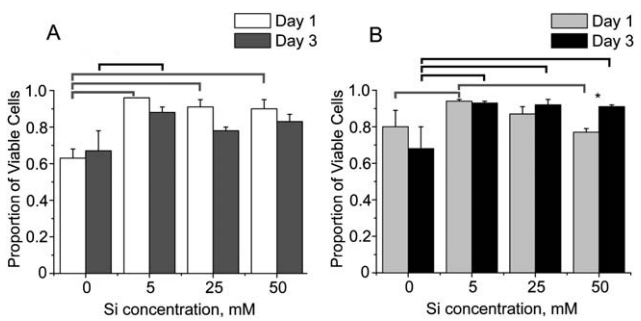

Fig. 3 Plots of the proportion of viable hMSCs relative to the initial viability of the seeded hMSC at 1 and 3 days of culture in alginate–silica beads with 0, 5, 25 and 50 mM silicate concentration (A) and in PRP–alginate–silica beads with 0, 5, 25 and 50 mM silicate concentration (B). (Data: average and standard deviation, lines $p < 0.05$ silicate concentrations, $*p < 0.05$ day 1 *versus* day 3.)

Without silica (see Fig. 3A), the proportion of viable hMSCs in alginate beads after 3 days of culture was lower (~0.63). The addition of PRP to alginate was beneficial at 24 h, although, after 3 days of culture, the proportion of viable cells in both alginate and PRP–alginate beads was still relatively low (~0.68) suggesting that the presence of PRP could only partially overcome the hMSCs initial adverse reaction to the alginate environment (lack of binding sites). The weak effect of PRP addition of later time points may be explained by rapid protein release (~4 h) in the absence of silica added to the preparation (Fig. 2). The exact mechanism of interaction between the composite materials and cells has not yet been elucidated. However, our hypothesis is that the small molecular weight oligomers and higher molecular weight nano-sized silica particles influence the physical and chemical characteristics of the polymer network as well as making possible the retention of proteins within the beads enabling an extended effect of PRP on encapsulated hMSCs.

In summary, we have reported a novel method for combined controlled PRP release and mesenchymal stem cell encapsulation. In recent years, it has been shown that combined growth factor delivery is more effective than delivery of a single factor[30] as growth factors each have a different role to play in a time dependent manner. Although delivery systems for sequential release of recombinant growth factors have already been proposed[30] using composites where different components are separately loaded with individual growth factors they are not suitable for an autologous source of growth factors such as PRP as it is itself a mixture of proteins and growth factors. The synthetic approach described in this contribution combining silica with alginate could offer for the first time the opportunity to tune PRP growth factor release. Furthermore, this composite system is injectable and preserves the viability of hMSCs, thereby opening the way for combined tissue engineering and drug delivery applications.

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