Fibroblast encapsulation in hybrid silica–collagen hydrogels†

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Silica–collagen scaffolds are synthesized by the simultaneous polymerization of aqueous silicates and self-assembly of protein triple helices in the presence of living human dermal fibroblasts.

The possible applications of sol–gel processes in biomedical science cover a wide range of devices from bone repair materials to artificial organs.[1] Indeed the sol–gel technology is well-adapted to the elaboration of acellularized bioceramics such as apatites or bioglasses.[2] In contrast, when considering cell-containing biomaterials, the sol–gel encapsulation in inorganic matrices appears suitable for bacteria, yeasts and photosynthetic microorganisms,[3] but more limited for mammalian cells of medical relevance.[4] In the latter case, hybrid materials, where living organisms are first entrapped in a biopolymer network before mineralization, is the most effective route.[3b,5] However, until now, the mineralization occurs at the polymer surface and not within the hydrogel.

In the present work, we have examined the possibility to prepare silica–collagen hybrid hydrogels in conditions compatible with the encapsulation of human dermal fibroblasts. Targeted applications are biological dressings, especially for chronic ulcer treatment, where fibroblast proliferation within a biocompatible matrix with controlled biodegradation is necessary to stimulate healing.[6] At this time, cellularized hydrated collagen gels are commonly obtained using the method of Bell *et al.*, consisting of the neutralization of diluted acid soluble collagen solutions.[7] When fibroblasts are entrapped in these detached free-floating collagen matrices, a strong contraction activity of the cells is observed, leading to collagen reorganization. This reorganization is a drawback not only because it reduces the volume of the materials but also because it induces cell phenotypic modifications, such as the loss of proliferation potential and apoptosis.[8] The induction of apoptosis in fibroblasts seems to be specific of contractile collagen gels, as it is not observed in anchored or high-density collagen gels.[9] Hence, it is important to develop new materials that would resist matrix contraction by cells, and therefore prolonged the viability of entrapped fibroblasts. In this context, many collagen-based composite materials have been designed by mixing with other polymers,[10] or incorporation/*in situ* growth of calcium phosphates, calcium carbonates and metal/oxide ceramics.[11] However, it would be desirable to obtain hybrid materials consisting of an interpenetrated network of collagen and inorganic polymers, enhancing the protein/mineral interface.

In this context, several strategies have been developed over the last ten years in order to obtain silicified collagen materials.[12] Most of them involve silicon alkoxide precursors in solution or in the gas phase.[3b,13] As an alternative, collagen–sodium silicate hybrid materials were prepared using a co-gelation process based on the exposure of the mixture to ammonia vapors.[14] However, none of these methods were expected to be suitable for the simultaneous formation of hybrid silica–collagen hydrogels and cell encapsulation due either to the presence of cytotoxic alcohol by-products or to unfavorable pH conditions.

Based on our previous studies on cell encapsulation,[15] we have studied the possible adaptation of the Bell procedure to the *in situ* polymerization of aqueous silicates. Shortly, cold diluted collagen solutions (1.2 mg mL^{-1}) in culture medium were mixed with acidified (pH 3), diluted sodium silicate solutions in polystyrene dishes. After neutralization with diluted NaOH, the human dermal fibroblast suspension in culture medium was added. The final gels were obtained as disks of 3.4 cm in diameter and 2 mm in thickness.

We found that only silicate solutions with concentrations of 5 mM and below could be added to collagen solutions, allowing both preservation of immobilized fibroblast viability after one day, as preliminarily checked by optical microscopy (not shown), and gel formation. This threshold value is in good agreement with previous data showing that poly-silicic acids, whose concentration relative to silicic acid $Si(OH)_4$ increases with increasing silica content, inhibit the self-assembly of collagen triple helices.[16]

During two weeks after encapsulation, the number of metabolically active fibroblasts, as obtained from tetrazolium assay (MTT),[17] increases in all samples, with the highest survival rate being obtained in the presence of 1 mM silicate (Fig. 1). Slightly lower or higher silica contents, 0.5 and 2.5 mM respectively, still lead to a higher number of immobilized active cells than with pure collagen gel whereas this number is lower for a 5 mM concentration. After 3 weeks, the survival rate is lower than after two weeks but significantly higher than after 1 day and almost equivalent for all samples.

Because these data suggested that fibroblasts could survive within the hybrid silica–collagen gels, we turned our attention to the structural evolution of the gel with time. From a macroscopic point of view, all the hydrogels showed a similar contraction profile characterized by a rapid shrinkage phase during the first 7 days, followed by a continuous slow decrease of the surface down to day 21 (Fig. 2). However, whereas the surface of collagen hydrogels was reduced to about 3% of the initial surface after 21 days, the contraction was lower in silicified collagen hydrogels which could present up to 3 times higher surfaces than the control at the end of the incubation

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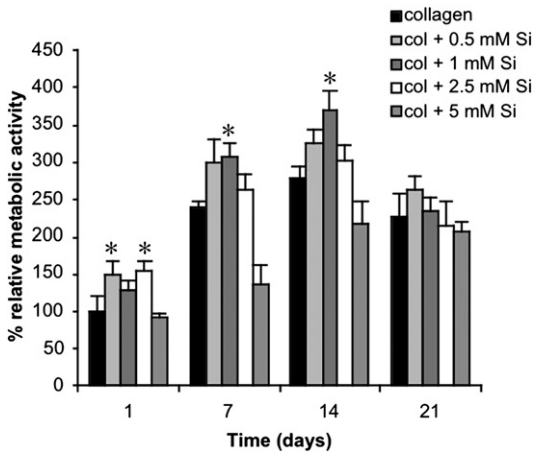

Fig. 1 Metabolic activity of entrapped fibroblasts (MTT assay). Control collagen hydrogels at day 1 were normalized to 100%. Results are expressed as mean ± SD from triplicates experiments. * indicates statistical significance ($p < 0.05$) from Student test.

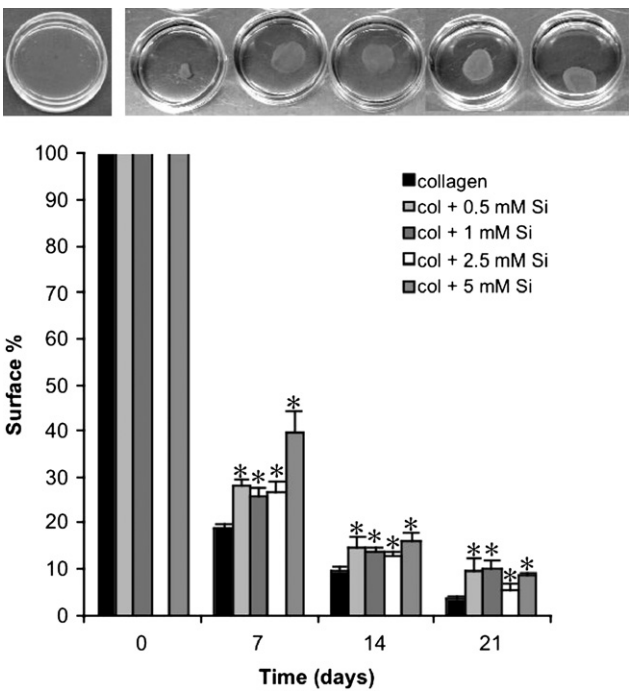

Fig. 2 Cell-mediated contraction of the hydrogels. Results are expressed as mean ± SD from triplicates experiments. * indicates statistical significance ($p < 0.05$) from Student test. Top: images of initial hydrogel and hydrogels after 21 days.

time. At the same time, due to the reported important dissolution of sol–gel silica near neutral pH,[18] the silicon content of gel supernatant was followed by ICP-AES (ESI†), indicating an important release of silica over the first week post-encapsulation followed by a slower dissolution until day 21. This may explain why the surface of the gels after this time has become almost independent of the initial silica content.

Scanning electron microscopy images indicate that the pure collagen hydrogels initially consist of a highly porous structure formed by thin homogeneous collagen fibrils that tend to aggregate upon gel contraction (Fig. 3A). At low silicification rates (<5 mM), the collagen fibrils form a more entangled network with overcrossing areas where they appear packed in parallel bundles, with the density of this network increasing with time (Fig. 3B and ESI†). At 5 mM, rope-like twisted bundles of collagen fibrils are observed, whose average diameter increases with time, from *ca.* 400 nm at day 1 to more than 700 nm at day 14 (Fig. 3C and ESI†). Noticeably, when blank samples consisting of collagen and silica were prepared in the absence of cells, the fiber diameter increased with silicate concentration after one day but it did not evolve further (ESI†). At 5 mM, twisted bundles were also observed but with a smaller diameter (*ca.* 200 nm) (ESI†). TEM further confirms the observations of SEM and moreover shows that the characteristic collagen cross-striations are visible in all conditions (Fig. 3D–F and ESI†). Moreover, co-aligned aggregated fibrils are identified in materials with low silica content (Fig. 3E). At 5 mM, the twisted arrangement of fibrils is clearly observable (Fig. 3F).

At the same time, the remodeling activity of the cells, as monitored by the gelatin hydrolysis activity of the MMP-2 enzyme,[19] increases with time for all samples (Fig. 4). After 7 days, this activity is lower in silicified gels than in pure collagen gels, except for the 5 mM concentration. With time, the collagen degradation activity of the

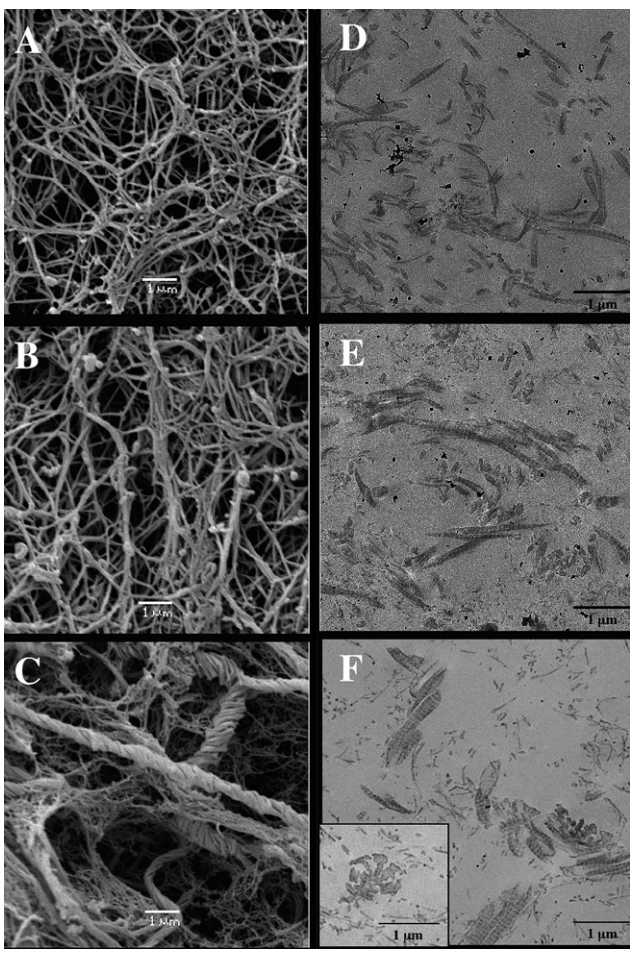

Fig. 3 Scanning and transmission electron microscopy images of collagen hydrogels (A and D respectively) and silicified collagen with 1 mM (B and E respectively) and 5 mM (C and F respectively) sodium silicates after incubation for 14 days.

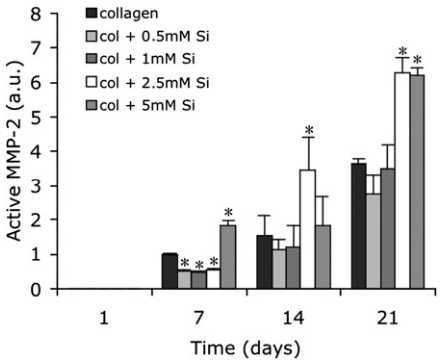

Fig. 4 Catabolic activity of entrapped fibroblasts (active form of the MMP-2 enzyme produced by the cells normalized to MTT assay). Results are expressed as mean ± SD from triplicates experiments. * indicates statistical significance ($p < 0.05$) from Student test.

entrapped cells is not significantly modified at low silica amounts but it progressively increases for the highest silica concentrations.

Interestingly, we have observed here that an optimal value, *i.e.* 1 mM, exists above which the cell survival rate decreases, while the production of the MMP-2 enzyme, involved in collagen degradation, is enhanced. This suggests that entrapped cells are not in a suitable environment and aim at remodeling the surrounding collagen network. This situation can be attributed to the formation of large silica–collagen fibril bundles which may not favor cellular adhesion.

The process driving the unprecedented formation of the rope-like twisted collagen fibers is difficult to understand at this time. In pure collagen gels, network contraction involves the aggregation of fibrils *via* a sliding process where fibrils are co-aligned and displaced one respective to the other until an optimized overlap is reached.[20] Such a co-alignment is also observed at low silicification rates, but seems to be hindered at 5 mM. From the works of De Gennes,[21] it is known that long polymer chains can form loops if they adopt a self-avoiding random walk. This occurs if strong repulsion exists between different fragments of the backbone. Here, it can be proposed that the coating of silica on the fibril results in negatively charged surfaces that hinder the co-lateral association of the hybrid fibrils and may therefore favor the formation of twisted bundles. In addition, the complex condensation/dissolution process of silicates may also be involved in the collagen gel morphological changes.

Until now, one of the main limitations of using collagen gels as biomedical materials is related to their strong contraction upon ageing. The here-described process allows limitation of this phenomenon in a simple manner by adding small amounts of aqueous inorganic species while maintaining the viability of entrapped fibroblasts. Indeed, the silica–collagen network represents an unusual environment for fibroblasts that must be studied further *in vitro*. In addition, *in vivo* studies of these materials are currently in progress to evaluate their suitability as implant devices.

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