Materials Letters 157 (2015) 176–179

Contents lists available at ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/matlet

Collagen scaffolds as a tool for understanding the biological effect of silicates

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ARTICLE INFO

Article history:
Received 19 February 2015
Received in revised form
22 April 2015
Accepted 23 May 2015
Available online 27 May 2015

Keywords:
Silicon
Collagen
Mineralization
Cartilage
Osteoblast
Stem cell

ABSTRACT

Dietary silicon is essential in the maintenance of bone and cartilage. However, the mechanism by which silicon, in the form of silicates, triggers a biological response has never been uncovered. Here we demonstrate the incorporation of orthosilicic acid ($Si(OH)_4$), the form of silicon in the body, within collagen scaffolds for use as an *in vitro* platform to identify key genes affected by silicates. Ice-templated collagen–silicate scaffolds, containing 0.21 wt% silicon, were validated by examining the mRNA levels for an array of genes in human osteoblasts and mesenchymal stromal cells (MSC) after 48 h in culture. Several novel genes, such as tumor necrosis factor alpha (TNF), were identified as having potential links to orthosilicic acid, verifying that collagen–silicate scaffolds are a versatile platform for identifying novel mechanisms in which silicates regulate musculoskeletal tissue.

1. Introduction

Since work began on dietary silicates in the 1970s, there has been no definitive explanation for the biological effects of silicates either *in vivo* or *in vitro* [1,2]. The necessity of silicates has been widely acknowledged, especially in bone, where dietary silicates have been linked to increased bone mineral density [1,3]. However, the early studies have never been replicated, possibly due to a lack in vital co-factors, or differences in experimental study design [2].

Even *in vitro*, results are often contradictory and, depending on the study, silicates are reported to either increase or decrease the expression of bone markers and collagen synthesis [4,5]. In Bioglasses, release of silicate and calcium ions drives osteoblastic differentiation, but the effects of individual components remain unclear [4,6–8]. Many factors contribute to this variability including cell passage number and the silicate source [9–11].

The subtle effects of silicates have spurred efforts to use *in vitro* models to understand the mechanism of action. Due to the interactions of silicates with collagen fibrillogenesis, the creation of stable tissue engineering constructs is difficult to achieve [12]. It was hypothesized that soluble silicates could be introduced into a scaffold composed of mature collagen which had already undergone fibrillogenesis to create a stable platform. To validate the model, the change in mRNA levels of human osteoblasts and mesenchymal stromal cells (MSCs) in response to collagen–silicate scaffolds was tested. The results verified that the scaffolds were a viable tool for studying silicates and allowed for the identification of novel genes for further investigation.

2. Materials and methods

2.1. Scaffold production

Unless noted, all reagents are from Sigma Aldrich (UK). Scaffolds were prepared by hydrating 1 wt% insoluble, bovine Achilles collagen in 0.05 M acetic acid, at pH 2, and homogenizing. Slurry was frozen in stainless steel molds at −30 °C for 90 min, then lyophilized using a Virtis freeze drier (SP Industries, USA) at 0 °C for 20 h under a vacuum of less than 100 mTorr. Cross-linking was done using carbodiimides: a 5:2:1 ratio of N-(3-Dimethylamino propyl)-N′-ethylcarbodiimide hydrochloride (EDC): N-Hydroxysuccinimide (NHS): collagen COOH groups in 70% ethanol [13].

Within the current study, "silicate" will be used to refer to a silicon-containing molecule, such as orthosilicic acid, while "silicon" will be reserved for compositional analysis. Orthosilicic acid,

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http://dx.doi.org/10.1016/j.matlet.2015.05.113

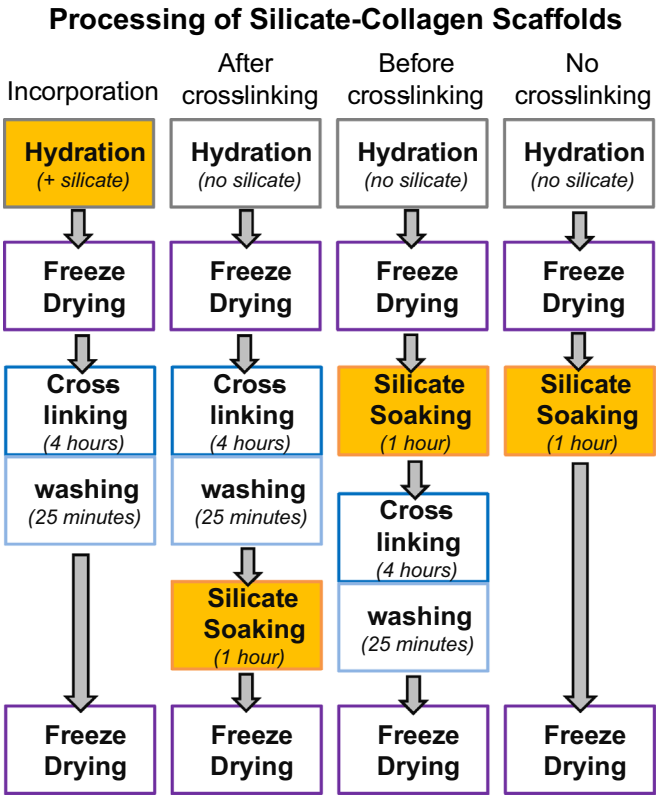

Fig. 1. Silicates were introduced into collagen scaffolds at different phases of production.

$Si(OH)_4$, was prepared at 2 mM from alkaline sodium silicate solution and introduced to scaffolds at various stages, Fig. 1. During collagen hydration, orthosilic acid was added to solutions of 0.05 M acetic acid at 10, 20, 30, and 100 vol%. To introduce silicates after freeze drying, scaffolds were soaked for 1 h in orthosilicic acid (pH 7), before or after cross-linking, with an additional control without cross-linking.

Scaffold structure was characterized using scanning electron microscopy (JEOL 820), and micro-computed tomography (μCT, Skyskan 1172). Chemical composition was investigated using energy dispersive x-ray spectroscopy (EDX) on a JEOL-5800LV under low vacuum (25–30 Pa) without platinum coating, and using inductively coupled plasma optical emission spectroscopy (ICP-OES) on acid digested samples.

2.2. Scaffold characterization

2.2.1. Cell culture

Two types of scaffolds were tested: 1 wt% collagen scaffold and 1 wt% collagen–silicate scaffold (orthosilicic acid introduced after cross-linking). Human osteoblasts and MSCs were isolated from tissue removed during joint replacement following informed consent. Ethical approval was obtained from Cambridgeshire Local Research Ethics Committee (LREC no. 06/Q0108/213). Both cell types were obtained from the same patient and used before passage 5. Osteoblasts were isolated by a method described by Meyer et al. and cultured in osteoblast medium (10% fetal bovine serum (FBS, **Invitrogen**), 30 μg/ml ascorbic acid, 1% penicillin/streptomycin in McCoys 5 A (Invitrogen)) [14].

Early MSCs were isolated from trabecular bone and enzymatically digested in the same way as osteoblasts. MSCs were sorted from the cell population using magnetic beads (Miltenyi Biotech) conjugated to an antibody against CD271, using the same principle developed by Jones et al. [15]. Cells were cultured in MSC medium (10% FBS, 30 ug/ml ascorbic acid, 1% penicillin/streptomycin in Minimal Essential Medium (Invitrogen)) and analyzed by flow cytometry (Beckman Coulter) prior to use.

Scaffolds ($5 \times 5 \times 2$ mm^3) were sterilized in 70% ethanol and seeded with 1.5×10^5 cells in 10 μl medium, and incubated 2 h (37 °C) before flooding with complete medium.

2.3. Quantitative polymerase chain reaction (qPCR)

RNA was harvested after 48 h with Qiazol (Qiagen) and purified with RNeasy® Mini Kit columns (Qiagen) and on-column DNase digestion. RNA from three replicates was pooled to make cDNA with RT^2 First Strand Kit (Qiagen). PCR arrays were used for MSCs (RT^2 Profiler PCR array, #330231 PAHS-082ZA, Qiagen) and osteoblasts (RT^2 Profiler PCR Array, #330231 PAHS-026ZA, Qiagen). Any dissociation curves which were not single peaks were excluded from the final analysis. Results are presented as fold regulations. Fold regulation is equal to the fold change for values greater than 1.0; otherwise fold regulation is the negative inverse of the fold change.

3. Results and discussion

3.1. Collagen–silicate Scaffolds

The amount of silicon present within the scaffolds was dependent on when silicates were introduced into the scaffold, Fig. 2. Incorporation of silicates during initial hydration was successful only with 100% orthosilicic acid. The key features of the scaffolds were not significantly altered by incorporation of silicates; the percentage porosity remained at 86%, with a mean pore diameter of 38 μm in the dry state. All scaffolds were interconnected, porous structures, suitable for tissue engineering, Fig. 2.

Incorporation of the silicate after freeze drying was only successful after cross-linking or when the cross-linking step was omitted. While the precise interactions between orthosilicic acid and collagen fibrils is undetermined, silicates can bind collagen fibrils, and, in hydrogels, they can affect mechanical stability [10,12]. As it is unlikely that all of the silicate could diffuse from the scaffold after 5 hours, the disappearance of silicon after cross-linking might be due to competition with the cross-linking agent for the same binding site along the collagen chain. When the scaffold was hydrated in 100% orthosilicic acid, the silicates may have saturated the collagen chains so that it could not be completely removed by the carbodiimide reaction. While some interaction with the carbodiimide cross-linking agent is suggested, with no statistical change in the percentage porosity, or the scaffold wall thickness (7.5–8 μm), it is not believed that large differences in the mechanical properties would be observed in this system. However, further studies on the cross-linking mechanism will require an in-depth study of the mechanical properties.

Using ICP, it was found that scaffolds soaked in silicates after cross-linking contained 0.21 wt% silicon, compared with untreated scaffolds (less than 0.004 wt%). Low levels of silicon in the control was expected due to the prevalence of silicates in the environment (e.g. glassware and water) [2]. In addition to silicon, several trace elements were present including sulfur, in the di-sulfide bonds, and chlorine, from the hydrochloric acid used to adjust the pH of collagen suspensions, Fig. 2. Due to the constrained fluid flow in the scaffold, the micro-environment around the cell would be difficult to predict. It is likely that the local silicate concentration is higher than if the same amount of silicate were added to cell culture medium. Further, it is not known if the form of silicate is altered during scaffold production, or in what form the silicate interacts with cells, making comparison to systems based on soluble silicates difficult [11].

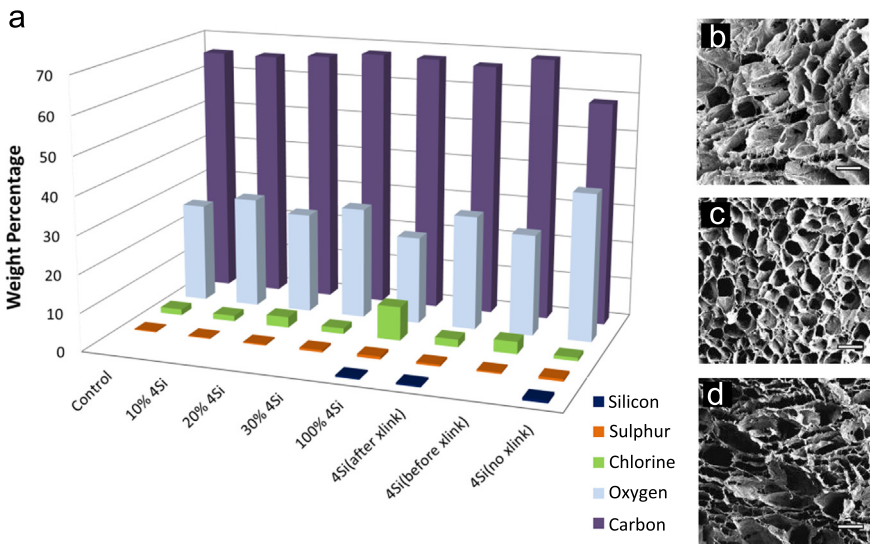

Fig. 2. The chemical composition of collagen–silicate scaffolds was dependent on when silicates were introduced. (a) Composition from EDX; all measurements are an average of three repeats, standard deviations were $<20\%$ of the mean in every case. All scaffolds had interconnected porosity: (b) without silicates, (c) hydrated in 100% ortho-silicic acid, and (d) soaked in silicates after cross-linking. Scale bar 200 μm. (xlink=cross-linking).

However, we have shown that incorporation of 0.21 wt% silicate within scaffolds was sufficient to influence mRNA levels, without affecting the scaffold stability.

3.2. Biological activity of collagen–silicate scaffolds

The collagen–silicate scaffold chosen for cell testing was a scaffold made with orthosilicic acid introduced after cross-linking. The composition varied from the control scaffold only by the presence of silicon, Fig. 2. Given the link between silicon and calcification, osteoblasts are often used to study silicates, but connective tissues also contain high amounts of silicates [1]. Thus MSCs, which have the potential to differentiate into both bone and connective tissue lineages, were also tested. Both cell types came from the same individual, ensuring that differences in cell sensitivity were due to cell phenotype and not individual donor variation.

The biological activity of both cell types was altered by the incorporation of silicates into the collagen scaffold, although osteoblasts showed greater sensitivity to silicates than MSCs, Fig. 3. The largest down-regulation in gene expression in osteoblasts was observed for tumor necrosis factor alpha (TNF, −5.23 fold) and bone morphogenetic protein 4 (BMP4, −3.41 fold). In contrast, the greatest up-regulation was in expression of CD36 (4.06 fold) and matrix metallopeptidase 10 (MMP10, 2.24 fold). Interestingly, none of the genes strongly up-regulated are currently linked to mineralization or calcification, the major research area of interest for silicates [16]. Also, surprisingly, collagen genes were unaffected. However, like the current study, there are reports in literature that the presence of silicates and calcium ions can greatly affect the cell cycle and proliferation of osteoblast progenitors [6].

The MSCs were not greatly affected by incorporation of silicate into collagen scaffolds, which hasbeen previously noted in another study [8]. In the presence of silicates, integrin-beta 1 (ITGB1, 1.91) and bone morphogenetic protein 6 (BMP6, 1.71) showed the greatest up-regulation in mRNA levels, while the largest down-regulation was observed in the expression of TNF (−2.24) and platelet-derived growth factor receptor beta (PDGFRB, −1.58). Interestingly, despite differences in sensitivity, the mRNA level of TNF was down-regulated by both osteoblasts and MSCs. This verified that the constructs were able to influence biological activity, possibly due to silicates modifying cell adhesion to the

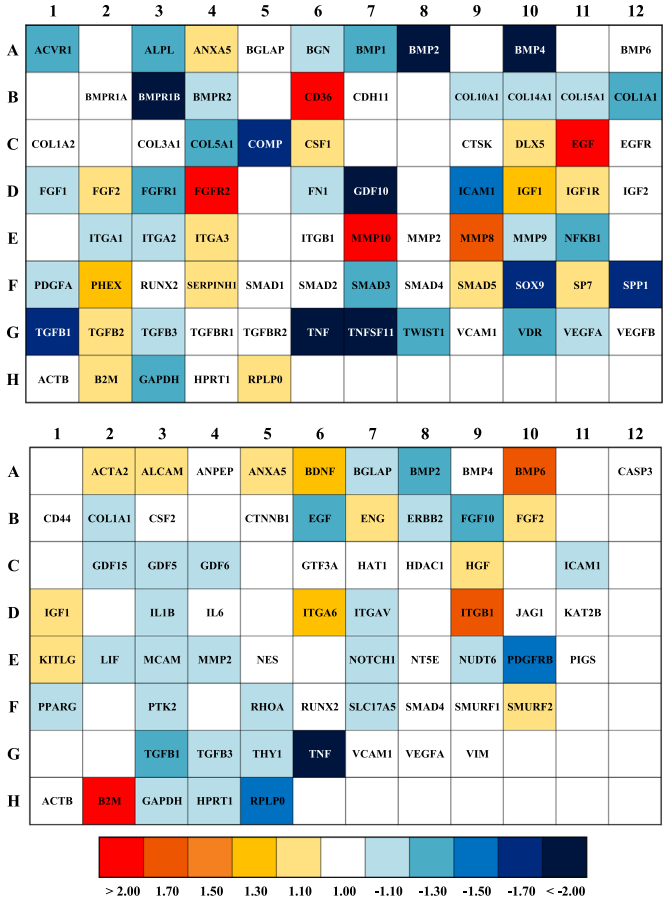

Fig. 3. Fold regulation of mRNA levels in (a) osteoblasts and (b) MSCs after culturing on collagen–silicate scaffolds (relative to untreated collagen scaffolds) after 48 hours.

scaffold through integrin binding, or interacting with cells in bound form (or following release) from the scaffold. Both of these effects could alter intracellular signaling, resulting in changes in mRNA levels, either through transcriptional or post-transcriptional mechanisms.

Cellular phenotype is a result of an interconnected network of

gene activity making it difficult to isolate only certain pathways in a complex chain of events. Many of the genes with the greatest change in mRNA levels are genes which can drive multiple cellular events, like growth factors. The most consistent down-regulation was TNF, a cytokine associated with osteoporosis and inflammation [17]. *In vitro*, TNF inhibits differentiation of osteoblasts from precursors and reduces matrix production while stimulating osteoclast resorption [18]. *In vivo*, TNF can delay early proliferation of osteoblasts and matrix formation, by interacting with other growth factors in the bone metabolic pathway [19]. As TNF has not been previously associated with the biological activity of silicates, it may offer a new gene of interest for further study.

One of the advantages of the collagen–silicate scaffolds was the absence of other ions, which might influence gene expression or modulate the behavior of the silicate. In materials, such as silicate substituted-hydroxyapatite (SiHA) or Bioglass, their dissolution releases, not only silicates, but calcium and phosphate ions as well. From such studies, it has been noted that significant biological effects are triggered by the presence of silicon, at levels around 12 μM, even when calcium and phosphate levels do not vary [20]. However, these results were dependent on co-culture systems and there is evidence that important interactions occur between MSCs and osteoblasts with endothelial cells, which promote the formation of vascular structures and the osteogenic activity of the MSCs [20–22]. Cell–cell interactions may thus provide a link between the effects of silicates on cells and increased bone formation *in vivo*. In summary, the study we have carried out is a step towards unifying the silicate literature by providing a platform for examining the influence of silicates on gene expression. The culture model we have developed would be an ideal system on which to further investigate endothelial–mesenchymal interactions with silicon.

4. Conclusion

Current *in vitro* models for studying the biological effects of dietary silicates on connective tissue cells are hampered by silicate interactions with processes such as collagen fibrillogenesis. Using orthosilicic acid and insoluble collagen, a method for producing stable collagen–silicate scaffolds, incorporating 0.21 wt% silicon, was presented. The collagen–silicate scaffold was validated as a model system for studying silicates by examining changes in mRNA levels of human osteoblasts and MSCs. In both cell types, the expression of powerful growth factors was affected, suggesting potential targets for the mechanism of action of silicates and offering researchers a new method to gain insight into the biological effects of silicates.

Acknowledgments

The authors gratefully acknowledge the financial support of the Gates Cambridge Trust , ERC Advanced Grant 320598 3D-E and from the National Institute for Health Research. RJ is supported by the Medical Research Council (Grant number MC_US_A090_0008/ Unit Programme number U1059).

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