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Influence of a novel calcium-phosphate coating on the mechanical properties of highly porous collagen scaffolds for bone repair

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Abstract

Lyophilised collagen scaffolds have shown enormous potential in tissue engineering in a number of areas due to their excellent biological performance. However, they are limited for use in bone tissue engineering due to poor mechanical properties. This paper discusses the development of a calcium-phosphate coating for collagen scaffolds in order to improve their mechanical properties for bone tissue engineering.

Pure collagen scaffolds produced in a lyophilisation process were coated by immersing them in sodium ammonium hydrogen phosphate ($\text{NaNH}_4\text{HPO}_4$) followed by calcium chloride (CaCl_2). The optimal immersing sequence, duration, as well as the optimal solution concentration which facilitated improved mechanical properties of the scaffolds was investigated. The influence of the coating on composition, structural and material properties was analysed.

This investigation successfully developed a novel collagen/calcium-phosphate composite scaffold. An almost 70-folded increase in the mechanical properties of the scaffolds was found relative to a pure collagen scaffold, while the porosity was maintained as high as 95%, indicating the potential of the scaffold for bone tissue engineering or as a bone graft substitute.

Key words:

Bone repair

Bone tissue engineering

Scaffold

Calcium-phosphate coating

Introduction

Cortical and cancellous bone is a combination of two materials, hydroxyapatite (HA) and collagen, each material possessing distinct limitations. However, together as a composite, they form an excellent material in terms of the overall mechanical properties (Currey 2002). HA, which represents approximately 65 % of the bone, has a very high stiffness, but shows a brittle behaviour. Collagen fibrils possess a two-phase, viscoelastic material behaviour with a high tensile strength (Currey 2002, Dendorfer *et al.* 2007) but low compressive modulus. The combination of the stiff mineral and the high rupture strength of the fibres builds up an efficient composite, similar to technical composite materials and reinforced concrete (Dendorfer *et al.* 2007).

As a material for scaffolds in tissue engineering (TE), collagen provides excellent biological performance, as it improves cell attachment, growth and proliferation (Angele *et al.* 2004, Farrell *et al.* 2006, O'Brien *et al.* 2005). Consequently, collagen scaffolds have been used for many years in various *in vitro* and *in vivo* studies in skin regeneration (Yannas *et al.* 1989), cartilage repair (Sellers *et al.* 2000) and many other tissues (Frenkel *et al.* 2004). However, pure collagen scaffolds possess insufficient mechanical properties for bone TE (Angele *et al.* 2004, Harley *et al.* 2007). Various methods of cross-linking the triple helical structure of collagen can improve the mechanical strength of the scaffolds. However, the stiffness of crosslinked collagen scaffolds still remains an order of a magnitude lower than bone.

HA and calcium-phosphate (CP) are used extensively as scaffold materials for bone TE and as bone graft substitutes due to their high mechanical stiffness and good biocompatibility (El-Ghannam 2005, Rezwan *et al.* 2006). In particular CP has an excellent biocompatibility due to its chemical and crystal resemblance to bone mineral and it has been shown to bind directly to bone (Hammerle *et al.* 1997, Jarcho *et al.* 1977). However, HA shows a much slower biodegradability

compared to CP (Rezwan *et al.* 2006) and the rigidity, brittleness and poor resorbability of pure ceramics have limited their use in this area (Karageorgiou *et al.* 2005, Russias *et al.* 2007).

Recently, many investigations have begun to focus on composite scaffolds by combining the advantages of different materials (Rezwan *et al.* 2006). Composite scaffolds using synthetic polymers and ceramic phases have been produced in the recent years (Kim *et al.* 2006, Maeda *et al.* 2006). However, as each scaffold consists of some phase which is not found naturally in the human body, they have all exhibited drawbacks with biocompatibility, biodegradability or osteoconductivity (Rezwan *et al.* 2006). Natural collagen scaffolds coated with HA or CP have been investigated using a bi-phasic immersion process with promising results in terms of their biological performance (Du *et al.* 2000, Yaylaoglu *et al.* 1999). However, this study used commercially available collagen sheets with initial porosities of 50-60% which is very low compared to collagen sheets developed by O'Brien *et al.* (2004) with a porosity of 99.5%. Such low porosities reduce cell migration into the scaffold and limit nutrient perfusion to, and diffusion of waste products from the cells reducing the potential of such materials for tissue engineering applications.

When the fabrication methods used in these two studies are analysed, a number of areas of disagreements arise, indicating that the coating process is not investigated sufficiently. Both studies use different techniques (i.e. immersion sequences and rinsing steps between coating treatments), exposure times and concentrations of the solutions (Du *et al.* 2000, Yaylaoglu *et al.* 1999) and the optimal process for coating collagen scaffolds with CP is yet to be elucidated.

The objective of this study was to develop a novel collagen/calcium-phosphate composite scaffold by coating a highly porous collagen scaffold used in our laboratory (O'Brien *et al.* 2007, O'Brien *et al.*, O'Brien *et al.* 2005) with calcium-phosphate. This paper discusses (i) the optimisation of the coating treatments to

achieve the best mechanical properties of the scaffolds and (ii) the influence of the coatings on the structure and morphology of the scaffolds.

Material & Methods

Scaffold design

Fabrication of pure collagen scaffolds

Collagen scaffolds were fabricated from a collagen suspension using a freeze drying method that has been previously described (O'Brien *et al.* 2004, O'Brien *et al.* 2005, Yannas *et al.* 1989). The collagen suspension was produced from microfibrillar type I collagen, isolated from bovine tendon (Integra Life- Sciences, Plainsboro, NJ, USA) suspended in 0.05M acetic acid. The suspension was mixed at 15,000 rpm using an overhead blender (IKA Works, Inc., Wilmington, NC, USA) at a temperature of 4°C which was maintained using a cooling system (Lauda, Westbury, NY, USA). After the blending process was complete, the resultant collagen slurry was lyophilised in a freeze-dryer (VirTis Co., Gardiner, NY, USA) with a final freeze-drying temperature of -40°C to produce collagen sheets with a mean pore size of approximately 95 µm (O'Brien *et al.* 2005).

Calcium-phosphate coating

Calcium chloride (CaCl₂) and ammonium sodium hydrogen phosphate (NaNH₄HPO₄) (Sigma Aldrich, Germany) solutions were prepared by mixing in Tris buffer at a pH of 7.4 (50 mM Tris, 1% NaN₃) according to Yaylaoglu *et al.* (1999).

Samples with a diameter of 10mm and a height of 4mm were cut from collagen sheets with a punch. Scaffolds were pre-hydrated in PBS and then coated with calcium-phosphate using stepwise immersions in the chemical solutions, NaNH₄HPO₄ and CaCl₂. Four different experiments were carried in order to determine the optimal technique for coating the collagen scaffolds with CP.

In the first experiment, the effect of different coating sequences was analysed. 4 different treatments were investigated.

1. Scaffolds were immersed in CaCl_2 for 22 hours, followed by 22 hours in $\text{NaNH}_4\text{HPO}_4$ (C-P).
2. Scaffolds were immersed in $\text{NaNH}_4\text{HPO}_4$ for 22 hours, followed by 22 hours in CaCl_2 (P-C).
3. Scaffolds were immersed in CaCl_2 for 22 hours, followed by 22 hours in $\text{NaNH}_4\text{HPO}_4$. This procedure was then repeated (C-P-C-P).
4. Scaffolds were immersed in $\text{NaNH}_4\text{HPO}_4$ for 22 hours, followed by 22 hours in CaCl_2 . This procedure was then repeated (P-C-P-C).

In the second experiment, the effect of a rinsing step between the immersion steps was analysed. Yaylaoglu *et al.* (1999) used a rinsing step to wash out loose particles. 2 different coatings were investigated, one with rinsing steps between the treatments, one without, using the P-C-P-C sequence. As will be shown in the Results, the treatment without a rinsing step was determined to be optimal and was therefore used in Experiments 2 and 3.

In the third experiment, the effect of exposure time was analysed. The P-C-P-C sequence without rinsing steps was used, with exposure times varied from 10 minutes to 48 hours; specifically exposure times of 5, 10, 30 minutes, 1, 6, 12, 22 and 48 hours were used. As will be shown in the Results, no significant difference in modulus between 1 and 12 hours was seen and therefore 1 hour was used in Experiment 4 as a short term treatment, while 22 hours was used as a long term treatment due to its superior results.

In the fourth experiment, the effect of different concentrations of the C and P solutions was analysed using the P-C-P-C sequence without rinsing steps. Calcium chloride and ammonium sodium hydrogen phosphate in three different concentrations were prepared (0.1, 0.5 and 1.0 M). An exposure time of 1 hour and 22 hours was used, resulting in 6 different scaffold variants (Table 1).

Scaffold analysis

Mechanical testing

Mechanical characterisation of the scaffolds was performed using a uniaxial testing system (Zwick Z005 with a 5 N load cell) in phosphate buffered saline (PBS) as previously described (Al-Munajjed *et al.* 2007). The diameter of each wet scaffold was measured using a digital camera and the image editing software ImageJ was used to calculate individual stresses and strains. Compressive tests were performed on submerged scaffolds at room temperature in a water bath filled with PBS. The scaffolds were fixed between two platens in the setup. After the initial contact between the scaffold and the platens a pre-strain of 5% was applied. The Young's Modulus of each scaffold was determined between strains of 2 and 5%.

Porosity analysis

The pure collagen scaffolds were weighed before and after the treatments using a digital scale (Mettler Toledo, PB 153-S, Switzerland; accuracy of 0.1 mg). The individual dimensions of the scaffolds were measured using a digital camera and the image editing software ImageJ. The weight of the composite scaffolds was compared to the weight of the pure collagen scaffolds to find the percentage of collagen and calcium-phosphate in the scaffolds (Equation 1 and 2). The density was calculated using the weight and volume of each individual scaffold, equation 3.

$$\%_{collagen} = \left(\frac{weight_{collagen}}{weight_{scaffold}} \right) \quad (1)$$

$$\%_{HA} = \left(\frac{\text{weight}_{HA}}{\text{weight}_{scaffold}} \right) \quad (2)$$

$$\rho_{scaffold} = \left(\frac{\text{weight}_{scaffold}}{\text{volume}_{scaffold}} \right) \quad (3)$$

The collagen/calcium-phosphate ratio was calculated using the percentage of collagen and calcium-phosphate of the scaffold and the densities of pure collagen (1.343 g/cm³) (I. Gordon Fels 1964) and the density of calcium-phosphate (3.14 g/cm³), equation 4.

$$\text{ratio}_{coll / HA} = \frac{\%_{collagen} * \rho_{coll}}{\%_{HA} * \rho_{HA}} \quad (4)$$

The relative density of the scaffolds was then calculated using the collagen/calcium-phosphate ratio of each scaffold (equation 5). The individual porosities of the scaffolds were calculated using equation 6 (Gibson *et al.* 1988).

$$\rho_{relative} = \left(\frac{\rho_{scaffold}}{\rho_{coll / HA}} \right) \quad (5)$$

$$\text{porosity} = 1 - \rho_{relative} \quad (6)$$

Scanning Electron Microscopy

Scanning electron microscopy (SEM) (LEO 1455VP) was used to qualitatively compare the pore structure of the different scaffolds produced by the CP coating techniques. Energy-Dispersive X-Ray (EDX) spectroscopy was performed to analyse the material compound of the scaffolds. Cylindrical scaffold samples,

10mm in diameter, were dehydrated after storing in phosphate buffered saline using a freeze-dryer (VirTis Co., Gardiner, NY, USA). The scaffolds were vertically bisected to analyse the inner pore structure. The samples were placed directly onto the sample holder of the SEM without the need of prior sputter coating. A carbon adhesive tape was used to fix the scaffolds to the SEM setup. 10 KeV was used as a setting to scan the scaffolds.

Statistical analysis

Paired t-tests were performed to compare individual sets of data to determine statistical significance. One-way analysis of variance (ANOVA) and pair-wise multiple comparison procedures (Tukey Tests) were used to compare data groups in SPSS. Error is reported in text and figures as the standard deviation. A probability value of 95% ($p < 0:05$) was used to determine significance.

Results

Mechanical testing

The effect of coating sequence (Experiment 1) on the compressive moduli of the scaffold variants is shown in Figure 1(a). All treated scaffolds showed a significantly increased stiffness relative to the pure collagen (control) scaffolds (0.28 kPa) due to the chemical formation of the CP phase. Superior results were achieved by using the process that began with an initial exposure to $\text{NaNH}_4\text{HPO}_4$ (P) followed by a second exposure to CaCl_2 (C) and repeating these steps a second time i.e. the P-C-P-C sequence. This process resulted in a compressive modulus of 1.3 kPa.

The effect of scaffold rinsing (Experiment 2) on the compressive moduli of the scaffold variants is shown in Figure 1(b). The scaffolds produced without rinsing during the fabrication process showed significantly higher values for the compressive modulus compared to the control group and the collagen/CP scaffolds with rinsing. This process resulted in a compressive modulus of 10.3 kPa.

The effect of exposure time (Experiment 3) on the compressive moduli of the scaffold variants is shown in Figure 1(c). Exposure times of 5, 10 and 30 minutes showed no significant improvements compared to the control group. The exposure times between 1 and 48 hour showed a significantly increased stiffness while a treatment with CP exposure time of 22 hour showed significantly superior result compared to the shorter exposure times. No significant difference could be seen between the 1 hour treatment which resulted in a modulus of 3.6 kPa and the 12 hour treatment which resulted in a modulus of 5.0 kPa. The 48 hour (8.1 kPa) variant exposure showed a lower mean value compared to the 22 hour exposure (10.3 kPa), however this decrease was not statistically significant.

Finally, the effect of calcium-phosphate concentration (Experiment 4) on the compressive moduli of the scaffold variants is shown in Figure 1(d). All coated scaffolds showed a significantly increased compressive modulus relative to the pure collagen scaffolds (0.28 kPa). The 0.5M scaffold variants showed significantly higher moduli compared to all other groups in terms of absolute magnitude. In particular, the scaffolds produced after 22 h exposure to the 0.5 M solutions showed the highest compressive moduli.

Porosity analysis

The effect of concentration on the porosity of the scaffold variants is shown in Figure 2. All coated scaffolds showed a reduced porosity after the treatment compared to the control (pure collagen scaffold) group. A trend of decreasing porosity could be seen with increasing concentrations of the chemical solutions. The same trend was seen by increasing the exposure times. Scaffolds treated with 0.5 m for 22 hours and both scaffolds treated with 1.0 M solutions showed significant reduced porosities compared to the pure collagen scaffolds. However, porosities as high as 92 % and 91 %, respectively, were still achieved.

Scanning electron microscopy

SEM analysis of the calcium-phosphate coated scaffolds showed a homogeneous distribution of the pores throughout the scaffolds. Figure 3 shows a representative SEM image of a scaffold coated with calcium chloride and phosphate solutions of 0.5 M concentration and exposure times of 1 hour. The white arrow shows a CP cluster within the scaffold structure. The coated scaffolds showed a highly porous, interconnected, homogenous pore structure demonstrating that the introduction of the CP phase had no negative effect on these parameters.

Energy-Dispersive X-ray (EDX) spectroscopy detected the presence of elements within the scaffolds after calcium-phosphate treatments. Figure 4 shows an EDX

diagram of the scaffold treated with 0.5 M calcium chloride and phosphate solutions and a 1 hour exposure time. All treated scaffolds showed carbon as part of the collagen structure, as well as calcium (Ca), phosphorus (P) and oxygen (O) as part of the calcium-phosphate coating. Chloride (Cl) and sodium (Na) were also found in all treated scaffolds.

Discussion

Bone tissue engineering has had limited clinical success to date and one of the reasons for this, is that an optimal scaffold for engineering bone in vitro remains to be established. In order for a scaffold to be successful in bone tissue engineering, a trade-off between sufficient mechanical properties and a porosity and permeability high enough to allow cell migration, tissue formation and angiogenesis is required (Frenkel *et al.* 2004, O'Brien *et al.* 2007). The objective of this study was to develop a collagen/calcium-phosphate composite scaffold with optimal properties for bone tissue engineering by coating a highly porous collagen scaffold with calcium-phosphate. This paper discusses (i) the optimisation of the coating treatments to achieve the best mechanical properties of the scaffolds and (ii) the influence of the coatings on the structure and morphology of the scaffolds.

The novel composite scaffolds which were developed in this study show a significant increase in mechanical properties in comparison to the pure collagen control scaffold. A small reduction in porosity relative to the control scaffold was seen, however the new scaffolds retained a highly porous, interconnected structure with a pore volume as high as 92%. A homogeneous, interconnected scaffold structure and calcium-phosphate distribution was observed using Scanning Electron Microscopy. CP was detected using EDX in all scaffolds after coating. The results from the first experiment indicated that stepwise immersions into the two chemical solutions showed significantly increased moduli relative to the control scaffold. Exposure to the phosphate solution before exposure to the calcium chloride showed better results while repeating this procedure a second time improved the mechanical properties further. This treatment was therefore used for all further experiments.

The introduction of a rinsing step during the fabrication process used by Yaylaoglu *et al.* (1999) was analysed. Their study claimed that the introduction of a rinsing step helped to remove loose particles in the pores of the scaffolds by washing them out with distilled water. The results of our investigation indicated that rinsing during the fabrication process significantly decreases the stiffness of the scaffolds. Scaffolds made without rinsing showed a 500% increase in compressive modulus compared to scaffolds made with a rinsing step. The SEM images and final porosities as high as 91-98% demonstrate that no loose particles were found in the scaffolds, even without a rinsing step. As a result of this experiment, rinsing was not used in any subsequent experiments.

The third experiment in this study investigated the effect of varying calcium and phosphate exposure time on scaffold mechanical properties. In this investigation, exposure times between 5 minutes and 48 hours were investigated. Exposures of less than 1 hour showed no significant effect on the mechanical properties but exposure to the calcium and phosphate solutions for longer showed a significant increase in the mechanical stiffness compared to the pure collagen scaffolds. The highest stiffness values were achieved using exposure times of 22 hours. A non-significant decrease at 48 hours relative to 24 hours indicated that a saturation point may occur, limiting further increase in mechanical strength. As no significant increase could be seen between 1 hour and 12 hour exposures, the 1 hour exposure seems therefore, in terms of an economic aspect, a potential alternative coating treatment to the 22 hour treatment. Two exposure times were therefore used for our further experiments, 1 hour as a short term and 22 hours as a long term CP treatment.

The influence of the concentration of the chemical coating solutions was investigated at exposure times of 1 and 22 hours. The treatments using the lowest concentration (0.1 M) showed the lowest increase in mechanical stiffness. However, compared with pure collagen scaffolds, this increase was significant. The porosity was the highest in this embodiment compared to all other composite

scaffolds (98%). Scaffolds that were treated with 0.5 M solutions showed the highest compressive moduli, although the porosity was decreased. After 22 h of exposure to the 0.5 M solutions, a significant reduction in porosity was observed compared to the control group. However the porosity was still as high as 92 and 95%, respectively. Using the 1.0 M concentrations leads to higher compressive moduli compared to the pure collagen scaffold, but the increase was significantly lower compared to the 0.5 M results. During the 1.0M process CP particles started to grow in the solutions and were not attached to the scaffolds. Using this concentration showed similar results in terms of mechanical properties compared to the scaffolds fabricated with the 0.1 M solutions; however a reduction in porosity could be seen. This treatment therefore seems inadvisable for bone tissue engineering.

Scanning Electron Microscopy showed a homogenous distribution of pores within the scaffolds. Energy-Dispersive X-ray spectra showed the presence of Ca, P and O as part of the calcium-phosphate layer. In addition the elements Na and Cl have been found. The mapping analysis of the EDX indicates that Ca and P appear together demonstrating that calcium-phosphate crystals form in the coating layers using this immersion technique.

The most significant result from this study is that while we have managed to improve enormously, the mechanical properties of a collagen scaffold by coating it with CP and furthermore, the coating does not greatly reduce the porosity of the new scaffolds. The new scaffolds have porosities well in excess of 90% which give them a distinct advantage over many of the materials currently used in tissue engineering. Fabrication techniques such as rapid prototyping (Landers *et al.* 2002) and solid freeform fabrication (Porter *et al.* 2001, Weiss 2001) have been used to fabricate pure calcium-phosphate scaffolds. However, porosities of only 60-70% (Vance *et al.* 2005) and 80-85% (Kim *et al.* 2005) are reported. With a similar CP process and commercially available Gelfix scaffolds, Yaylaoglu *et al.*, Kose *et al.* and Du *et al.* claim similar material results to our study of

precipitated CP to a collagen scaffold (Du *et al.* 2000, Kose *et al.* 2004, Yaylaoglu *et al.* 1999). However no data on the mechanical properties of these scaffolds is available to support their assumption. Using scaffolds with an initial porosity of 50-60%, a final porosity of less than 50% was achieved after their CP treatment. The scaffolds from our investigation show an overall porosity between 92 and 98% indicating the significant potential of these collagen/calcium-phosphate composite scaffolds for bone tissue engineering applications and potentially as an off-the-shelf strategy for bone repair. The excellent porosity should facilitate cell migration into the scaffold while nutrient perfusion to, and diffusion of waste products from the cells is also assured. The high porosity should also allow for angiogenesis and vascularisation of the construct after implantation *in vivo*. The improved mechanical properties have the potential to allow implantation of the scaffold into load-bearing areas to heal critical-sized bone defects *in vivo* albeit with external fixation. This would not be possible with the pure collagen scaffolds.

Taken together, the results from this investigation indicate that the recommended treatment for CP coatings of collagen scaffolds is a 1 hour exposure to the 0.5 M phosphate followed by 1 hour exposure to the 0.5 M calcium chloride solution and this process should be repeated once. This results in an optimal balance between mechanical strength, porosity and calcium-phosphate distribution. An almost 70-fold increase in the mechanical properties of the scaffolds was found relative to a pure collagen control scaffold which led to a compressive modulus of almost 31 kPa, while the porosity was maintained as high as 95%. Despite this increase, the construct does not exhibit the mechanical properties necessary for implantation into load bearing defects without external fixation. However, the handling properties of the scaffolds have been improved significantly. The novel collagen-hydroxyapatite scaffolds show sufficient mechanical properties for maxillofacial, cranial or other non-load bearing defects. In addition, the improved mechanical properties may facilitate increased cellular penetration to the centre of the scaffolds by helping to maintain the interconnected pore structure of the

scaffolds during hydration. Furthermore, if the scaffolds are used for tissue engineering, mineralisation of the scaffolds *in vitro* following extra cellular matrix deposition by osteoblasts would lead to a further increase in the mechanical properties.

In conclusion, this investigation successfully developed a novel collagen/calcium-phosphate composite scaffold, indicating its high potential for bone tissue engineering and bone repair.

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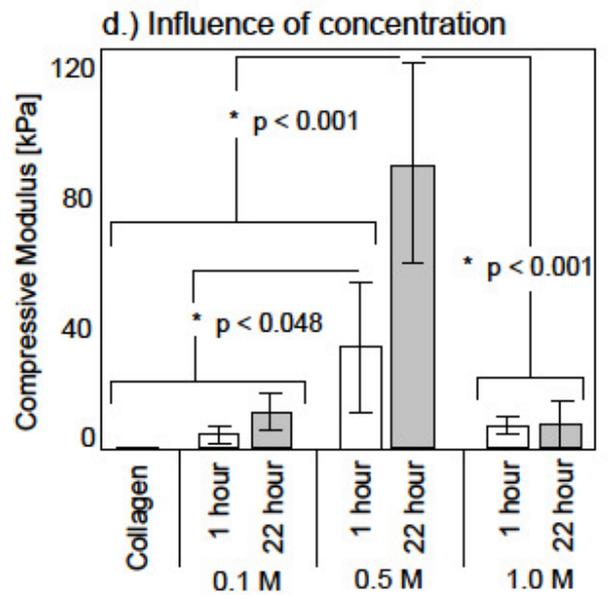
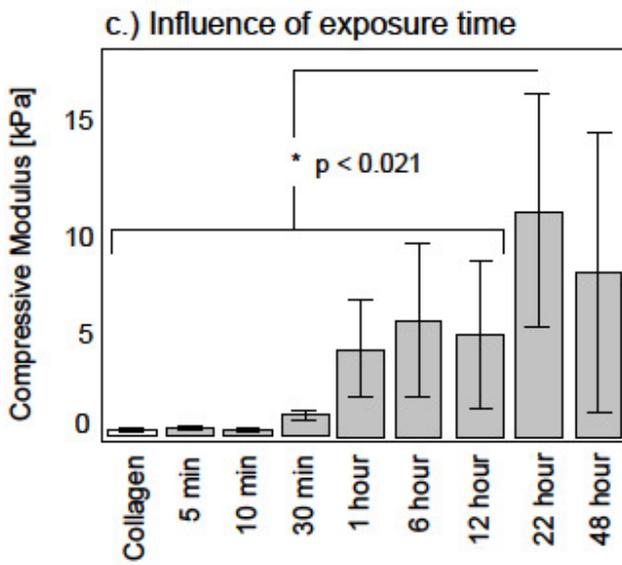
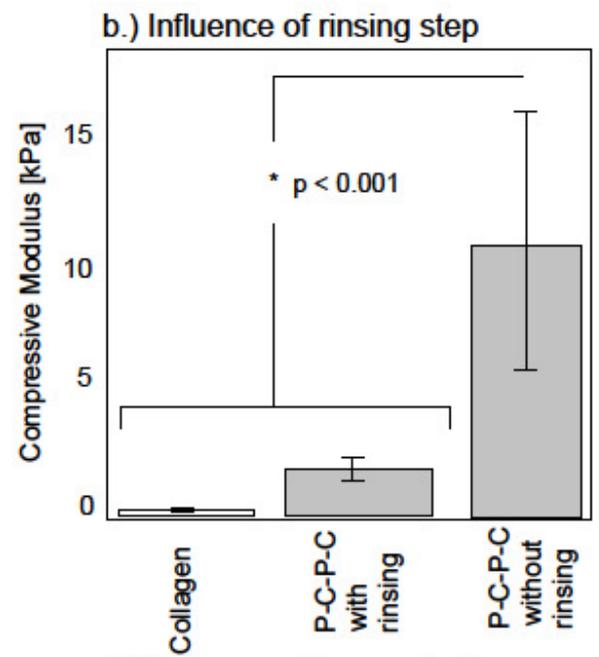
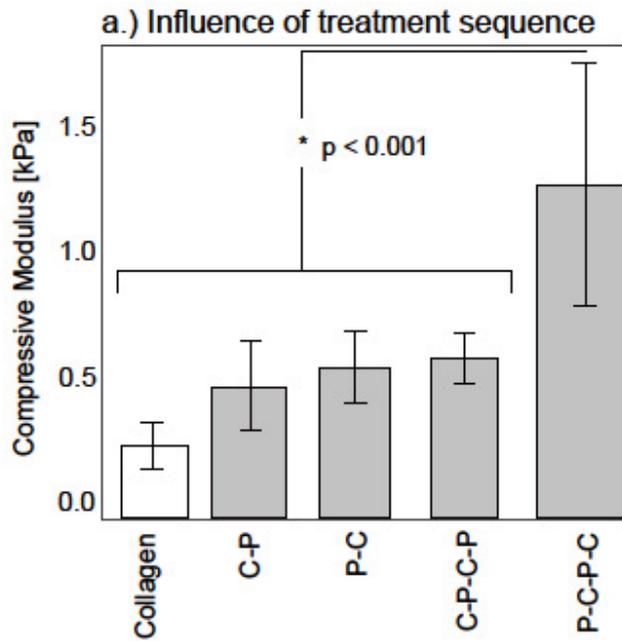
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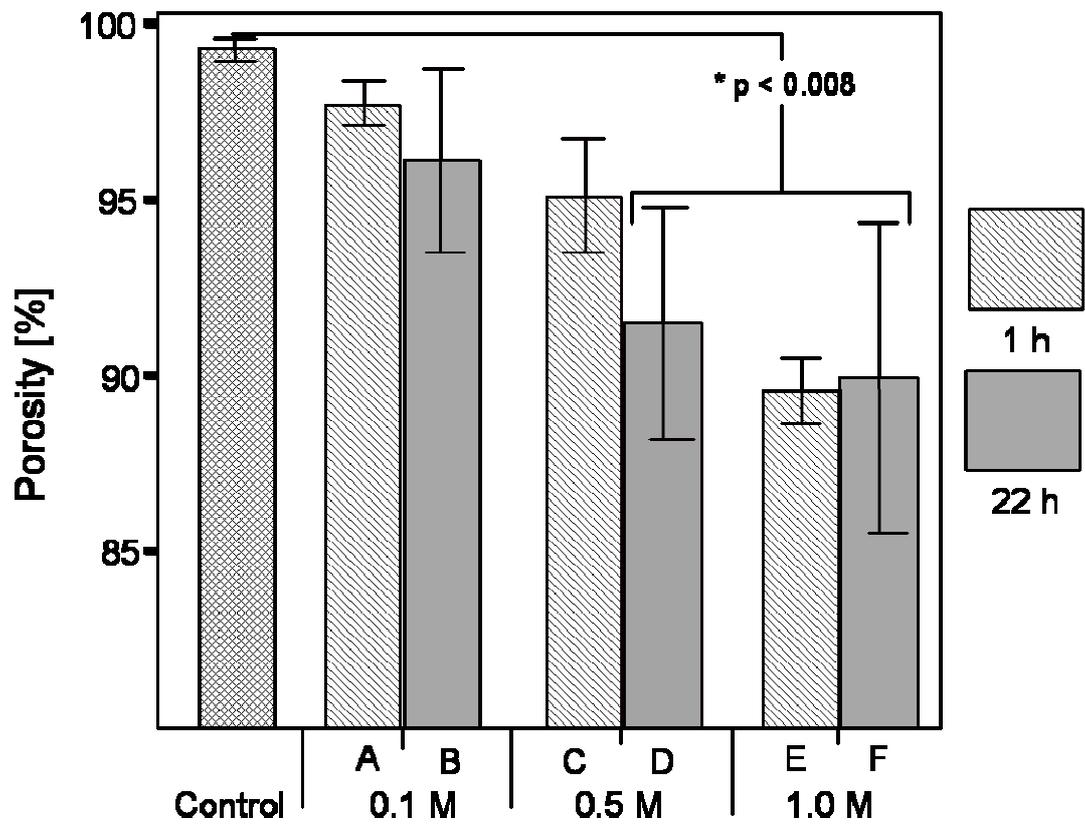
Figure 1: Compressive moduli of pure collagen scaffolds and collagen scaffolds immersed in calcium-phosphate solutions to investigate: (a) the influence of different treatment sequences (n=6); (b) the influence of using a rinsing step during the treatments (n=6); (c) the influence of different exposure times (n=6); (d) the influence of three different concentrations using two different exposure times (n=6).

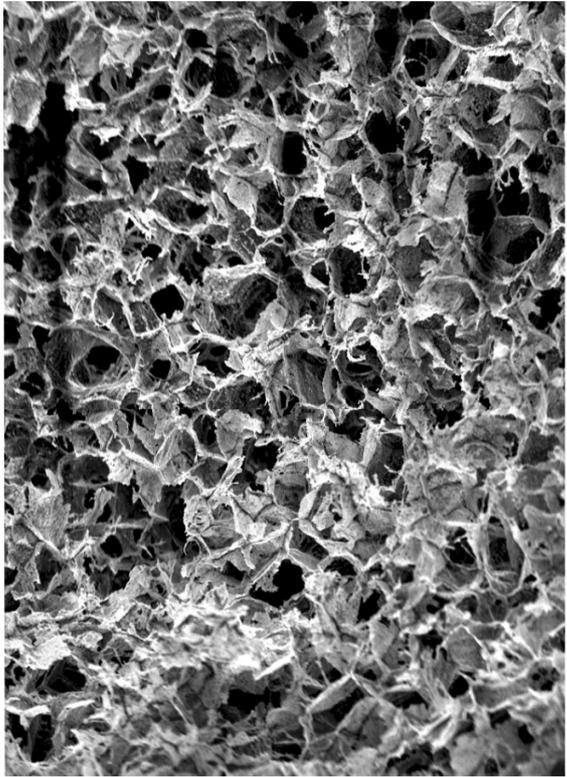
Figure 2: Porosity values of calcium-phosphate coated collagen scaffolds using three different concentrations and two different exposure times (n=10).

Figure 3: SEM image in a magnitude of 91x of a calcium-phosphate coated collagen scaffold using 0.5 M phosphate and calcium chloride solutions and exposure times of 1 hour.

Figure 4: Energy-dispersive X-ray spectroscopy of a calcium-phosphate coated collagen scaffold using 0.5 M calcium chloride and phosphate solutions and 1 hour exposure times.

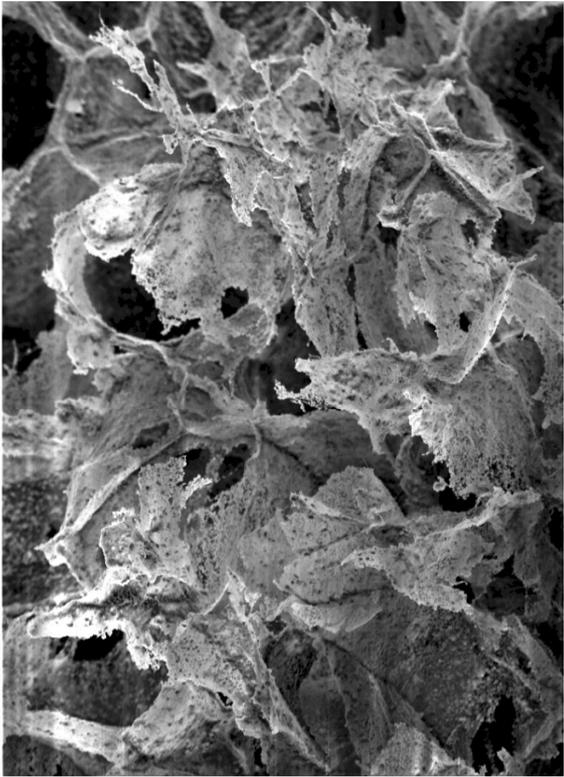






a.)

350 μm →



b.)

100 μm →

