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# Developing scaffolds for tissue engineering using the $Ca^{2+}$-induced cold gelation by an experimental design approach

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Received 30 March 2012; revised 8 June 2012; accepted 11 July 2012
Published online 15 September 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.32797

**Abstract:** The $Ca^{2+}$-induced cold gelation technique was found suitable to prepare highly porous biodegradable scaffolds based on bovine serum albumin (BSA) and alpha-casein from bovine milk for tissue engineering. A $2^3$ full factorial design was used to study the influence and impact of each factor on the several responses of the scaffolds. *In vitro* degradation (ID), swelling ratio (SR), porosity (PO), and pore size (PS) as well cytotoxicity (CT) were evaluated and shown to be dependent on the pH of sample preparation and on the amount of BSA and casein present, making these scaffolds tunable structures. Under optimized working conditions (4.19% of BSA, 0.69% of Casein, pH 7.07), the ID attained was 37.97%, the SR observed was 11.87, the PO was 82.11%, the PS measured was 180.63 μm at surface, and 175.91 μm at fracture, whereas maximum cell viability was 84% in comparison to controls. Moreover, the scaffold supported cell adhesion and proliferation. These results, consistent with the prediction by the experimental design approach, support the use of this methodology to develop tunable scaffolds for tissue engineering using the $Ca^{2+}$-induced cold gelation. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 100B: 2269–2278, 2012.

**Key Words:** cold gelation, scaffolds, experimental design, porosity, tissue engineering

**How to cite this article**: Ribeiro Artur JAM., Gomes AC, Cavaco-Paulo AM. 2012. Developing scaffolds for tissue engineering using the $Ca^{2+}$-induced cold gelation by an experimental design approach. J Biomed Mater Res Part B 2012:100B:2269–2278.

## INTRODUCTION

Three-dimensional polymer scaffolds provide a suitable space in which transplanted cells can grow and generate their own extracellular matrix. This artificial scaffold should ideally degrade into nontoxic components that can be eliminated or reabsorbed from the implant site.[1] High porosity (PO) is required to offer sufficient space for tissue ingrowth and to promote invasion of surrounding tissues. A well-defined pore size (PS) and interconnecting pore network are also essential for vascularization and nutrient diffusion. It is also desirable that these scaffolds are biocompatible, biodegradable, and exhibit appropriate mechanical properties if so required for the application.[2,3]

Several methodologies are nowadays used to produce porous scaffolds from biomaterials such as solvent casting particulate leaching,[4] gas foaming with pressurized carbon dioxide,[5] fibre-meshes,[6] emulsion freeze-drying,[7] salt leaching/gas foaming,[8] 3D printing,[9] and even combinations of these methods.

The work presented here exploits the potentialities of salt-induced cold gelation as a new method for the production of scaffolds. This technique was first described using whey protein isolate (WPI) for the preparation of food gels with improved functional properties.[10] Briefly, a low ionic strength solution of WPI, at a pH removed from its isoelectric point is heat denatured for 5–60 min between 70 and 90°C. In such medium, strong electrostatic repulsions between proteins and negligible salting-out effects prevent gelation of the proteins. During this heat denaturation step, proteins undergo specific structural rearrangements, including unfolding, and the formation of stable soluble protein aggregates.[11–13]

Gelation of this heat-denatured WPI solution can be then be induced by slow salt addition, which screens and disperses charge, leading to a decrease in electrostatic repulsions. In the case of divalent cations such as calcium, salt addition may also aid in the formation of salt bridges between negatively charged groups on proteins. Electrostatic charge dispersion and crosslinking both enhance protein aggregation, and eventual gel formation. Different molecular interactions lead to gel formation and structure stabilization including hydrophobic interactions, hydrogen bonding, electrostatic interactions, and covalent bonds.[10,14]

Taking advantage of the salt-induced cold gelation, we were able to obtain scaffolds with controlled size and shape. Gel formation is not immediate after salt addition, which

Additional Supporting Information may be found in the online version of this article.
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Contract grant sponsor: FEDER through POFC—COMPETE and by national funds from FCT; contract grant number: PEst-C/BIA/UI4050/2011

opens the possibility to cast the protein solution into an appropriated mold with desirable characteristics that will ultimately reflect on the scaffolds properties.

The novelity of the work present here lays on the obtention of a set of scaffolds prepared by $Ca^{2+}$-induced cold gelation in the presence of DL-Dithiothreitol (DTT) using different protein formulations constituted by two very distinct model proteins: a medium-sized random coil protein, alpha-casein, and a large globular protein, bovine serum albumin (BSA). The ability of DTT to reduce the disulfide bridges, therefore destabilizing the protein aggregates formed during the pre-heating step enhances the role of $Ca^{2+}$ on electrostatic charge dispersion and formation of salt bridges leading to protein aggregation and gel formation.

The influence of pH on the preparation of the protein scaffolds was also studied because the protonated state of proteins could play a central role on the gelation process, affecting the scaffolds' properties. A two-level factorial design was adopted along this study for a comprehensive understanding of the factors' effect on the scaffolds responses. This method is an excellent tool for the identification of the most preponderant variables that are closely linked with the scaffolds performances in terms of *in vitro* degradation (ID), swelling ratio (SR), PO, PS, and cytotoxicity (CT). By combining the $Ca^{2+}$-induced cold gelation method and the experimental design, we aim to contribute toward the improved synthesis of moldable and tunable scaffolds for tissue engineering.

## EXPERIMENTAL

### Materials

BSA, Casein Sodium Salt from bovine milk were purchased from Sigma-Aldrich, Spain and used without further purification. All other reagents were analytical grade and purchased from Sigma-Aldrich, Spain.

### Preparation of BSA and BSA/casein scaffolds

The scaffolds were obtained by salt-induced cold gelation in the presence of calcium chloride ($CaCl_2$) and DTT at pH 7 and 9. Shortly, protein solutions of BSA 3 and 6% (w/v) in water were used for BSA scaffolds. Scaffolds of BSA/Casein were produced using the same amounts of BSA and 0.5 and 2% (w/v) of Casein in Tris50 mM pH 7.4. For the experimental design, central points were used with concentrations of 4.5% (w/v) of BSA, 1% (w/v) casein at a pH 8. The solutions were heated at 70°C for 30 min. After cooling, the protein solutions at room temperature for 1 h, $CaCl_2$ (25 mM) and DTT (25 mM) were added and the mixtures were stirred to homogenize the samples. The resulting solutions were cast on 24-well plates, left gel overnight and frozen at −20°C for 2 days and freeze dried for 2 days to remove the solvent completely. Resulting scaffolds with a diameter of 14 mm and a height of 9 mm were macroscopically opaque white and robust enough to be easily handle without breaking during experiments. Scaffolds were kept at room temperature until further use.

### *In vitro* degradation

BSA and BSA/Casein scaffolds were incubated, for 15 days, at 37°C in PBS at pH 5, 7.4, and 9. Solutions were changed every 24 h. At designated time points, samples were washed thoroughly with distilled water, dried in a desiccator, and weighed to estimate the extent of degradation by the following equation

$$\text{weight loss } (\%) = \left(\frac{m_i - m_f}{m_i}\right) \times 100$$

where $m_i$ is the initial dry mass of the sample and $m_f$ is the final dry mass.

### Swelling ratio

Dry scaffolds were immersed in PBS buffer at pH 5, 7.4, and 9 at 37°C for 24 h. The excess of buffer was removed, and the wet weight of the scaffolds was determined. The SR of the scaffolds was calculated as follows:

$$\text{swelling ratio} = \frac{W_s - W_d}{W_d}$$

where $W_s$ is the mass of the swollen material, and $W_d$ is the initial dry mass.

### Porosity

The PO of the scaffolds was determined in the swollen state in water using the following equation

$$\text{porosity } (\%) = \left(\frac{W_s - W_d}{d_{water}}\right) \times \frac{100}{V}$$

where $W_s$ and $W_d$ are the mass of the swollen and lyophilized scaffold, respectively, $d_{water}$ is the density of pure water, and $V=(\pi r^2 h)$ is the measured volume of the scaffold in the swollen state. All measurements were taken 24 h after soaking the scaffolds in water at 37°C using a paquimeter. Excess surface water was removed with a filter paper before each measurement.

### Microstructural morphology

Freeze-dried BSA and BSA/Casein scaffolds were fractured after immersion in liquid nitrogen. The samples were coated with 80% Au and 20% Pd before SEM (NOVA NanoSEM 200 FEI) observation at 5.0 kV. The mean PS was estimated using Image J software with at least 10 pores in three different spots for triplicate samples.

### Cell culture

Murine embryonic fibroblasts (MEF) isolated from E13.5 mouse embryos as described[15] were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 15% (v/v) of fetal bovine serum (FBS, Lonza) and 1% (v/v) of penicillin/streptomycin solution (PS, Sigma). The cells were maintained aseptically at 37°C in a 5% $CO_2$ humidified incubator and medium was refreshed every 2 to 3 days.

The BJ5ta cell line (telomerase-immortalized human normal skin fibroblasts) was purchased from ATCC through LGC Standards and was maintained according to ATCC

recommendations (4 parts DMEM) containing 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 1 part of Medium 199, supplemented with 10% (v/v) of FBS, 1% (v/v) of penicillin/streptomycin solution and 10 μg/ml hygromycin.

### Cytotoxicity evaluation

MEFs were used as model of general CT. CT induced by degradation products and leachables from BSA and BSA/Casein scaffolds was evaluated by exposing cells to cultured medium conditioned by contact with the scaffolds for 72 h. BJ5ta cells were later used to confirm results obtained with MEFs.

Scaffolds were disinfected by immersion in PBS supplemented with 1% (v/v) PS for 20 min. This procedure was repeated three times. To ensure that this procedure was sufficient for a contamination-free culture, disinfected scaffolds were incubated with culture medium at 37°C in a 5% $CO_2$ humidified incubator for 72 h without any signs of microbial contamination. The conditioned media were obtained by incubating the disinfected scaffolds in 5 mL of DMEM supplemented with 15% (v/v) of FBS and 1% (v/v) PS at 37°C in a humidified atmosphere of 5% $CO_2$ for 72 h.

Cells were seeded at a density of $10 \times 10^3$ cells/100 μl/well on 96-well tissue culture polystyrene (TCPS) plates (TPP, Switzerland) the day before experiments and then exposed to the previously obtained conditioned medium and further incubated at 37°C under a humidified atmosphere of 5% $CO_2$. At the end of 24, 48, and 72 h of contact, the cell viability was assessed using AlamarBlue assay (alamarBlue® Cell Viability Reagent, Invitrogen) in end-point measurements.

### Cell seeding and culture in the porous polymer scaffolds

To monitor the effect of the scaffolds on skin fibroblasts, cell proliferation was evaluated by quantifying DNA content. For seeding of BJ5ta into the scaffolds, sterilized scaffolds were gently placed in 24-well (TCPS) plates (TPP, Switzerland), then 250 μl of cell suspension ($2 \times 10^5$ cells/ml) was loaded onto the upper side of each scaffold and allowed to infiltrate into the scaffold. The scaffolds were then incubated at 37°C under 5% $CO_2$ conditions for 20 min to allow for initial cell attachment. An additional 250 μl of the cell suspension was pipette onto the seeded scaffolds. After a new incubation, 1 ml of medium was added and the plate was placed into a cell culture incubator and maintained at 37°C with 5% $CO_2$ for either 72 or 120 h. Culture media was renewed every 2 days. After each indicated time interval, cells/scaffold constructs were collected, rinsed with PBS and cell proliferation was determined in terms of DNA content measured with Hoechst 33258 (Invitrogen, CA). Briefly, cells were harvested from cell-scaffold constructs by incubating with a 0.25% solution of trypsin. Cells were then collected by centrifugation and lysed in a Tris 15 mM pH 7.4 buffer with consecutive frozen-thawing cycles. Cell lysates were incubated with equal volume of 5 μg/mL Hoechest 33258 solution for 40 min at room temperature in 96-well black plates (Nunc). Fluorescence was detected using a FLUOROSKAN ASCENT FL plate reader (ThermoScientific) at 350 nm excitation and 445 nm emission. The relative fluorescence unit value obtained from samples was interpolated against a DNA standard curve constructed using known number of cells, to determine the DNA content/number of cells in each sample.

**TABLE I. Factors Levels Used According to the $2^3$ Factorial Design**

| | Level | | |
|---|---|---|---|
| Variable | −1 | 0 | 1 |
| A—BSA concentration (%) | 3 | 4.5 | 6 |
| B—Casein concentration (%) | 0 | 1 | 2 |
| C—pH | 7 | 8 | 9 |

### Statistical analysis

Data were presented as average standard deviation (SD), n = 3. Statistical comparisons were performed by one-way ANOVA using Bonferroni corrected *t*-test with Graphpad Prism 4.0 software. Dunnett test was used to compare the results with a specific control. A $p$ value of less than 0.05 was considered to be statistically significant (expressed in the figures with asterisks [*]: $*p < 0.05$).

### Experimental design

The influence of BSA concentration (A), casein concentration (B), and pH at which scaffolds were prepared (C) on the scaffolds' properties such as ID, SR, PO, surface and fracture PS, and CT were studied using a $2^3$ full factorial design with three repetitions at the central point (Table I). The range and the levels of the variables investigated in this study are given in Table I and were chosen based on preliminary studies (data not show).

The Design-expert version 7.0—Free evaluation version (Stat-Ease, Minneapolis, MN) was used for regression and graphical analysis of the data. The ID (%), the SR, PO (%), the PS at surface (μm), the PS at the fracture (PF, μm) and CT (%) were taken as the responses of the design experiments. The model equation was determined by Fischer's test. The proportion of variance explained by the model obtained was given by the multiple coefficient of determination, $R^2$. The optimum conditions were obtained by the numerical and graphical analysis using the Design-expert program.

## RESULTS

In Table II, the ID, SR, PO, PS at surface, PF, and CT responses according to the variation of the factors imposed by the design were presented. These results are also depicted on Figures 1–4 with exception of the factors level 0.

### Effect of parameters on ID

ID of BSA and BSA/Casein scaffolds was evaluated by incubating the scaffolds for several days in PBS at pH 5, 7.4, and 9. This pH range suggests the possibility to use the systems

**TABLE II. Values for ID, SR, PO, PS at Surface, PF, and CT According to the $2^3$ Factorial Design**

| | Variables | | | Responses | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay | A | B | C | ID (%) | SR | PO (%) | PS (μm) | PF (μm) | CT (%) |
| 1 | −1 | −1 | −1 | 37.33 | 14.63 | 90.24 | 206.91 | 193.95 | 92.44 |
| 2 | 1 | −1 | −1 | 27.00 | 11.99 | 85.83 | 190.81 | 181.27 | 71.36 |
| 3 | −1 | 1 | −1 | 58.03 | 11.31 | 74.54 | 199.21 | 188.01 | 95.29 |
| 4 | 1 | 1 | −1 | 44.86 | 7.98 | 68.41 | 188.83 | 185.24 | 82.57 |
| 5 | −1 | −1 | 1 | 29.58 | 11.76 | 85.61 | 224.07 | 207.18 | 89.68 |
| 6 | 1 | −1 | 1 | 16.02 | 9.90 | 71.34 | 187.62 | 188.27 | 69.57 |
| 7 | −1 | 1 | 1 | 50.52 | 10.04 | 70.68 | 204.44 | 199.86 | 79.06 |
| 8 | 1 | 1 | 1 | 31.86 | 11.55 | 63.88 | 186.29 | 179.25 | 75.50 |
| 9 | 0 | 0 | 0 | 42.22 | 12.87 | 73.19 | 201.01 | 196.23 | 86.34 |
| 10 | 0 | 0 | 0 | 45.23 | 13.42 | 70.55 | 197.69 | 190.13 | 80.23 |
| 11 | 0 | 0 | 0 | 44.77 | 13.01 | 71.54 | 205.28 | 199.49 | 77.67 |

in acid, neutral, and basic conditions. The pH of the medium, within this range, does not influence the degradation rate of the scaffolds (Figure 1), and there are no significant differences when the same sample is incubated at pH 5, 7.4, or 9. The pH at which the scaffolds are prepared does, however, influence the degradation of the protein systems.

It is also evident that increasing the BSA concentration reduces the degradation rate of the scaffolds. In turn, increasing casein amount in the formulation leads to a decrease of stability translated into higher degradation rates. A greater amount of BSA in the same sample volume reflects an increase of the number of possible cross-links because more groups are available to interact with the calcium, which has direct influence on scaffold stability. We can speculate that casein somehow chelate the $Ca^{2+}$ ions from the medium or is interacting with BSA, reducing the amount of ions and groups available for the $Ca^{2+}$ mediated cross-link.

It was important to determine, using the experimental design, the contribution of each factor and how the different parameters interact to influence the ID of these scaffolds. Because this response is independent of the pH incubation buffer in the range 5–9, the experimental design assays were carried out at physiological pH 7.4.

The model expressed by Eq. (1), where the variables take their coded values represents the ID of the scaffolds as a function of the amount of BSA (A), casein (B), and pH at which samples were prepared (C).

$$\mathrm{ID}(\%) = 36.90 - 6.97\,A + 9.42\,B - 4.91\,C \qquad (1)$$

The statistical significance of the linear model equation (Table S1 of Supporting Information) was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant ($p < 0.0001$) at a 99% confidence level. The model did not show lack of fit and presented a determination coefficient of $R^2 = 0.98$, that explains 98% of the variability in the response.

The model confirms and quantifies the influence of each parameter. Both the concentration of BSA and the pH at which the scaffolds were prepared had a negative effect on the ID. These negative effects (−6.97 for BSA concentration

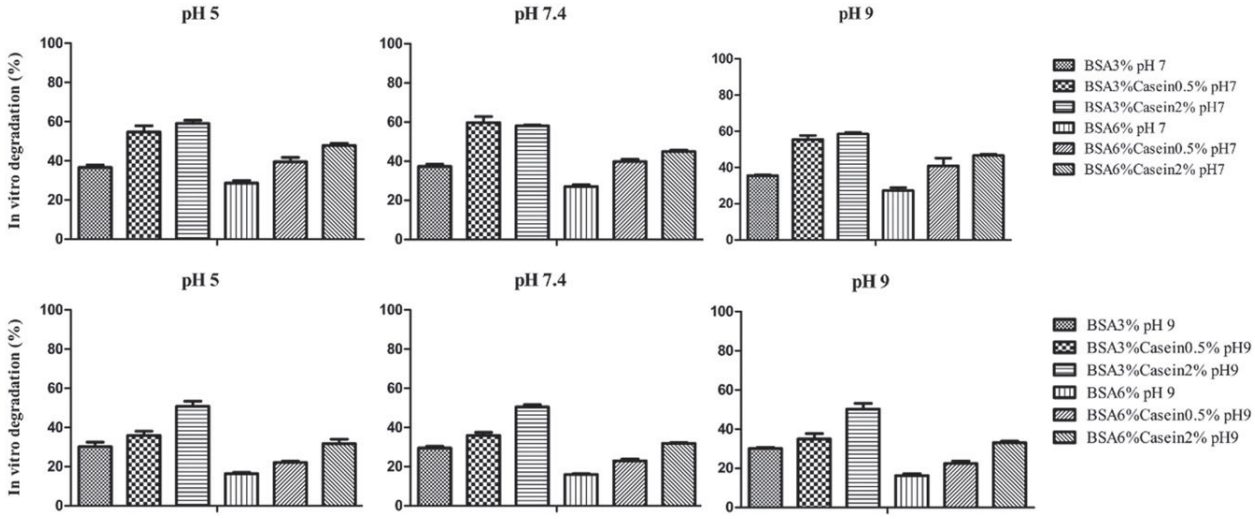


**FIGURE 1.** ID of BSA and BSA/Casein-based scaffolds incubated in PBS at pH 5, 7.4, and 9 for 15 days at 37°C. Samples prepared at pH 7 (A) and prepared at pH 9 (B). Data were reported as mean ± SD of three independent experiments, each with triplicates.

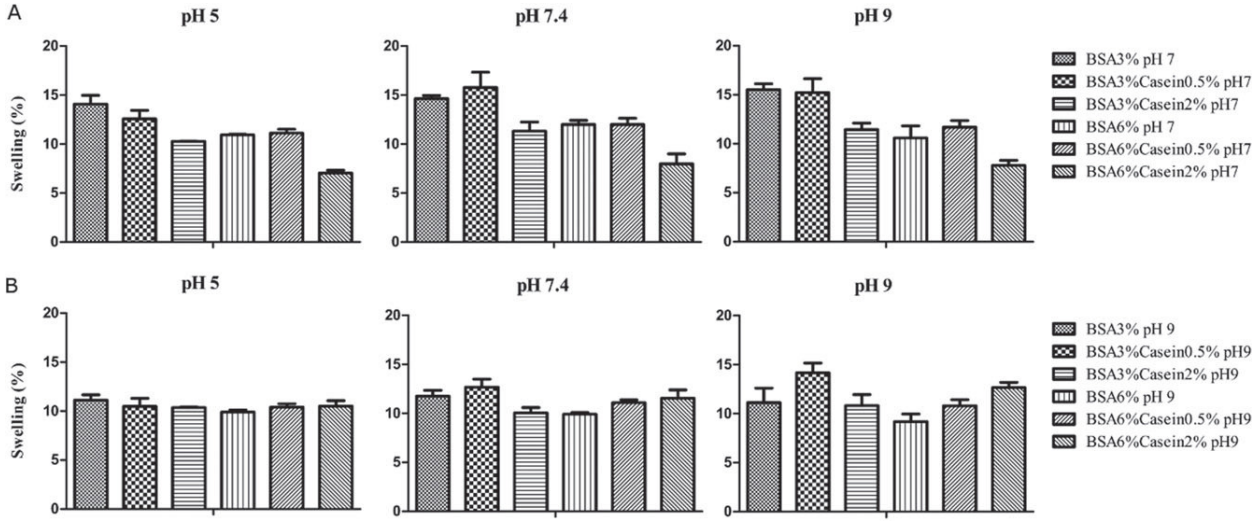


**FIGURE 2.** SR of the scaffolds calculated after incubation in PBS at pH 5, 7.4, and 9 for 24 h at 37°C. Samples prepared at pH 7 (A) and prepared at pH 9 (B). Data were reported as mean ± SD of three independent experiments, each with triplicates.

and −4.91 for pH value) result in a reduction of ID with the increase of both variables. Meanwhile an increase of casein concentrations increases in a factor of 9.42 the degradation of the scaffolds.

### Effect of parameters on SR

It is believed that the swelling behavior of physically cross-linked proteins significantly affects the ability to establish bioadhesion interactions,[16] mechanical strength,[17] drug release, and degradation rate[18] of the scaffolds. Thus, the hydrophilicity of the scaffold is one of the critical features in the evaluation of biomaterials for tissue engineering.

To determine how the pH affects the swelling behavior, the BSA and BSA/Casein scaffolds were immersed in three different pH PBS solutions for 24 h at 37°C. The SR was found to be independent from the pH of the medium, for the tested pH values (Figure 2).

Although the SR was not affected by the pH of the incubation buffer, it was significant dependent on the pH at which the scaffolds were prepared. For the 3% BSA scaffolds, samples obtained at pH 7 show significant higher SRs than those prepared at pH 9; at this pH value, the improved stability of the structure given by the presence of more $Ca^{2+}$-mediated cross-links reduces its ability to swell. Even though there is a tendency to obtain higher SRs in samples prepared at pH 7, those differences were not so evident in the scaffolds with 6% of BSA.

The isolated effect of the variables on the SR is not as clear as for the ID. Attending to the model determined by the experimental design expressed by Eq. (2), increasing each of the factors results in a decrease on the SR. However, these isolated effects are clearly compensated by their mutual influence.

$$\mathrm{SR} = 11.15 - 0.79A - 0.93B - 0.33C + 0.34AB + 0.70AC + 0.91BC + 0.51ABC \quad (2)$$

An increase on BSA (A) concentration and pH (C) did not result on a decrease on scaffolds swelling as expected by the isolated impact of each (−0.79 A and −0.33 C) because their combination (0.70AC) compensates their negative effect. Thus attending to the combination among the three factors, the model explains the slight variation between scaffolds. According to the analysis of variance for the response SR (Table S2 of Supporting Information), the considered model is significant, although there is a higher chance (2.15%) that the model could occur due to noise compared to the considered model for the ID response (0.01%).

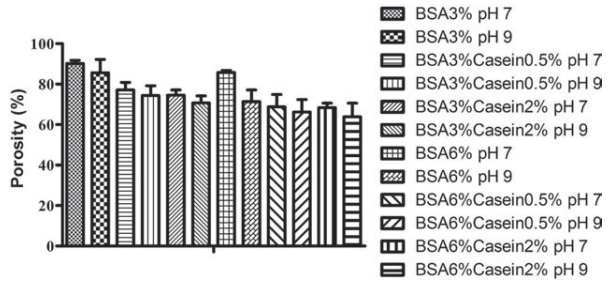


**FIGURE 3.** PO of the protein-based systems determined after incubation in water for 24 h at 37°C. Data were reported as mean ± SD of three independent experiments, each with triplicates.

### Effect of parameters on PO and PS

Scaffolds should have a porous architecture with high surface area to allow for maximum cell loading and cell-matrix interactions, as well to improve transport into and out of the matrix for nutrients and oxygen.[19] Pore morphology also affects the scaffold degradation kinetics and the mechanical properties. The PO of the BSA and BSA/Casein scaffolds was quantified in the swollen state after soaking the samples in water at 37°C for 24 h (Figure 3). The protein-

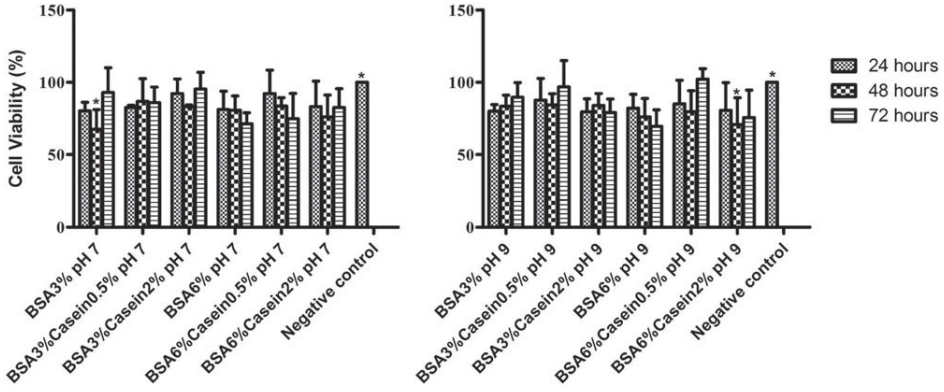


**FIGURE 4.** MEF cell viability measured by Alamar Blue assay at 24, 48, and 72 h of culture with undiluted conditioned medium that contacted with BSA or BSA/Casein scaffolds for 72 h. Cell viability (%) is presented for each material and for each time point. The data represent mean ± SD of three independent experiments, each with triplicates. The results were compared with the control: * = significantly different from the control ($p < 0.05$).

based scaffolds showed PO in the range 64–90% for the amount of proteins and tested pH values.

Generally, the samples obtained at pH 7 present slightly higher values than the samples obtained at pH 9, although there is not a marked influence on PO of the production pH. The most evident feature is the reduction in PO with increasing total protein content. Regarding this result, the direct relation between total protein concentration on the system and PO was expected to be evident and with a negative contribution on the model determined by the experimental design and expressed by Eq. (3).

$$\mathrm{PO} = 76.32 - 3.95A - 6.94B - 3.44C + 0.72AB - 1.32AC + 1.34BC \quad (3)$$

Surprisingly, the mutual contribution of BSA (A) and Casein (B) yield a positive effect (0.72AB) in PO. The individual contributions of BSA and casein content, on the contrary, negatively correlate with scaffolds' PO, which was confirmed by the BSA6% Casein2% produced at both pH values.

Scaffolds porous sizes were measured analyzing SEM micrographs (Table S3 of Supporting Information) that confirmed the high PO of the samples. Regarding the average PS, it varies between 186.29 ± 19.06 to 224.07 ± 22.35 μm for the surface and 179.25 ± 18.75 to 208.47 ± 39.54 μm for the cross-sections.

The most evident feature is the negative effect of BSA and Casein concentrations that are confirmed by the models determined by the experimental design [Eqs. (4) and (5)].

$$\mathrm{PS} = 198.52 - 10.13A - 3.83B + 2.08C + 3.00AB - 3.52AC - 1.41BC \quad (4)$$

$$\mathrm{PF} = 190.38 - 6.87A - 2.29B + 3.26B - 3.01AC - 1.80BC \quad (5)$$

The presence of higher amounts of protein on the scaffolds formulations results in smaller pores. Despite the positive effect of pH determined by the models, the contribution of this parameter is not clear. There is a significant variance between the samples and the positive effect determined by the models is overcome by the negative mutual influence of pH together with the proteins. Besides, there are higher chances—3.37% for PS and 4.29% for PF—that the model could occur due to noise which can explain some deviation on the results.

### Effect of parameters on CT

Mouse embryonic fibroblast cultures were used to assess the BSA and BSA/Casein scaffolds biocompatibility (Figure 4). Some variations and trends in cell viability along time are observed although no major differences, with statistical significance, can be singled out. An increase on BSA concentration seems to result in a decrease on cell viability. However, an increase on casein content leads to an increase on cell viability, though this relation between casein concentration and cell viability is not linear. These findings are in accordance with the model determined by the experimental design expressed by Eq. (6). The model shows no lack of fit and the regression is statistically significant with a chance of 2.94% that it could occur due to noise.

$$\mathrm{CT} = 82 - 7.52A + 1.11B - 3.54C + 3.18AB - 2.28BC \quad (6)$$

The effect of casein concentration by itself is contraposed by its influence together with the effect of pH at which samples were prepared. Increasing the pH to values above the physiological pH leads to an increase on CT. Moreover, the positive effect of casein content on cell viability is overcome by the negative effect of BSA concentration. It is patent in Figure 4 and in Eq. (6) that the positive effect of casein is only valid for medium concentration values. These findings over the influence of the several studied parameters on CT were further confirmed with human normal skin fibroblasts (BJ5ta cell line) later used for cell seeding in the optimal polymeric scaffold.

### Optimization of scaffolds design

The reviewed literature suggests that there is no "ideal" scaffold for all tissue types,[20] because correct conditions for cell survival, differentiation, and growth must be provided

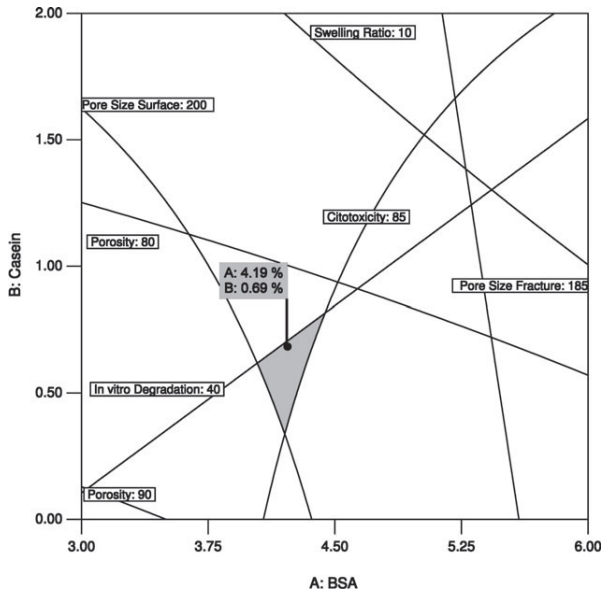


**FIGURE 5.** Optimum region by overlay of the six responses evaluated (ID, SR, PO, PS at surface, PS at fracture, and CT) as a function of BSA and casein concentration. Factor pH was kept at 7.07.

for each cell type. However, it was our intention to optimize the scaffold formulation to maximize all the analyzed response. For that a numerical and graphical optimization of the statistical program "Design-expert" was performed. The criteria imposed for the preparation of the scaffold formulation were (a) ID between 20 and 40%, (b) SR between 10 and 15, (c) PO between 80% and 90%, (d) PS both at surface and fracture between 185 and 200 μm, and (e) cell viability higher than 85%. The overlay plot attained (Figure 5) shows a shaded area where all these criteria are simultaneously satisfied.

The optimum point corresponding to 4.19% of BSA and 0.69% of casein for a pH of 7.07 is marked by a circle in Figure 5. Under the optimized conditions, the models predicted an ID of 39.97% (variation possible between 37.61 and 39.97%), an SR of 12.30 (variation between 11.87 and 12.68), a PO of 82.69% (variation between 80.60 and 84.47%), a PS at surface of 198.99 μm (variation between 198.31 and 199.68 μm), a PS at fracture of 188.38 μm (variation between 188.21 and 189.21 μm), and a cell viability of 85.99% (possible variation between 85.00 and 85.99%) in the confidence range of 95%. Although there are no optimal properties of a scaffold, research has shown that a scaffold PO of at least 90% is ideal for specific scaffold-cell interactions, nutrient and waste diffusion, and sufficient space for ECM regeneration within the scaffold.[21] The values for the BSA/Casein scaffolds are slightly below the ideal 90% but still are remarkable because any porogenic agent was used. To confirm these results, a validation assay was performed using the conditions imposed as optimum for the scaffold formulation. In Table III are presented the results for the studied six responses. The values reached in the validation assay for ID, SR, PO, PS, PF, and CT are in good agreement with the predicted values for the analyzed responses, validating the mathematical linear models attained in the studied region.

The porous structure of the "ideal" scaffold shown on SEM micrographs in Figure 6 confirmed the high PO as well as homogeneity in PS, shape, and distribution both in the surface and in the cross-section. The gelation and the freeze-drying processes were uniform because no significant differences are detected between the surface and the cross-sections. It is also remarkable the good interconnectivity displayed in all samples and present in more detail in Figure 6(B,D).

For the validation of the BSA/Casein scaffolds obtained using the $Ca^{2+}$-induced cold gelation by an experimental design approach for their application in tissue engineering was essential, besides all the measured parameters, to check if cells were able to adhere and proliferate on the scaffolds. Cell proliferation was determined in terms of DNA content measured with Hoechst 33258, a fluorescent dye that binds to DNA. The number of cells in each sample was indirectly measured by interpolation of their relative fluorescence unit value against a DNA standard curve ($Y = 0.0003X + 1.5025$; $R^2 = 0.9874$) constructed using a known number of cells where Y is the relative fluorescence and X the number of cells.

At the end of 72 h of incubation, the number of cells growing in the scaffold was $1.18 \times 10^4 \pm 0.05$, yet at the end of 120 h, this number increased about nine times to $1.12 \times 10^5 \pm 0.01$ cells, clearly indicating that cells are able to adhere and proliferate on the porous BSA/Casein scaffold (Figures 7 and 8).

## DISCUSSION

Taking advantage of the principles of salt-induced cold gelation[11,14] in combination with freeze-drying, BSA and BSA/Casein scaffolds were obtained and characterized. The characterization of the BSA and BSA/Casein scaffolds is essential because several parameters like degradation into nontoxic components,[1] PO, PS, interconnecting pore network and biocompatibility,[2,3] determine the success or failure of their application in tissue engineering. By using the experimental design and in comparison with a first set of characterization experiments, we had a precise idea of the contribution of each parameter, predicting and controlling the scaffolds

**TABLE III. Validation Assay Results for the ID, SR, PO, PS at Surface, PF, and CT of the Optimal Scaffold Determined by "Design-Expert"**

| | ID (%) | SR | PO (%) | PS (μm) | PF (μm) | CT (%) |
|---|---|---|---|---|---|---|
| Validation assay | 37.97 ± 0.40 | 11.87 ± 0.68 | 82.11 ± 0.97 | 180.63 ± 20.98 | 175.91 ± 27.78 | 84.03 ± 5.99 |
| Optimal response | 39.97 | 12.30 | 82.69 | 198.99 | 188.38 | 85.99 |

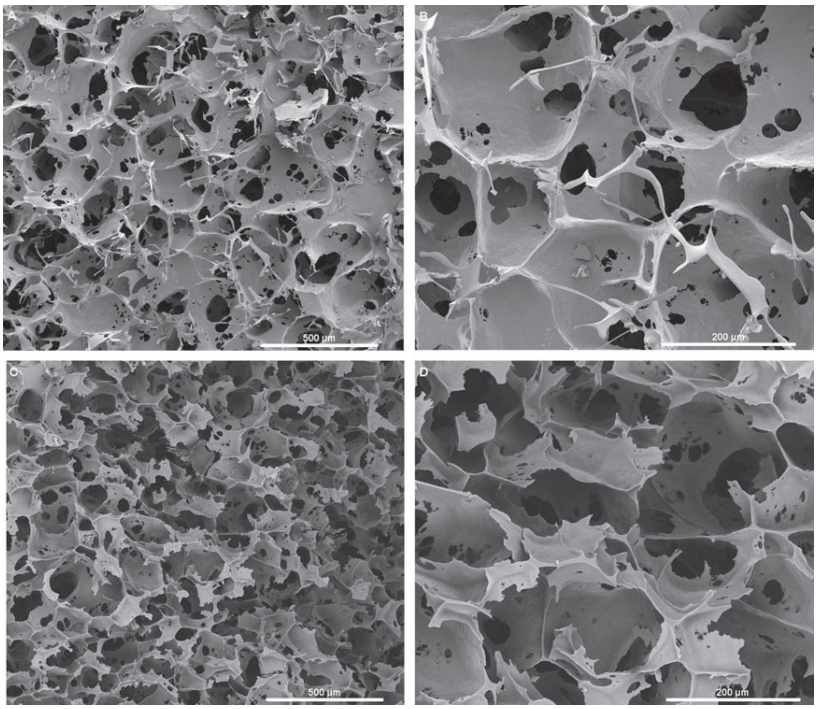


**FIGURE 6.** SEM micrographs of the "ideal" scaffold determined by "Design-expert": Surface (A, B) and cross-sectional views (C, D).

responses according to the formulations determined by the experimental design.

ID and SR as measures of scaffolds' stability showed to be independent of the pH values (pH 5, 7.4, and 9) at which scaffolds were incubated opening the opportunity for their application at several pH values, from slightly acid to faintly basic. Still the pH at which the scaffolds were prepared does influence their stability.

Higher degradation rates and SRs obtained when the scaffolds are prepared at pH 7 when compared with the scaffolds obtained at pH 9 could be related with the protonated state of BSA (pI 4.7) and Casein (pI 4.6). The two proteins, although not fully deprotonated at either pH values, present groups with negative charge that will interact with $Ca^{2+}$ and thus will be involved in the cross-linking process. The greater number of negative charged groups at pH 9 allows a higher number of cross-links between the groups, contributing to a more stable structure. The improved stability of the structure given by the presence of more $Ca^{2+}$-mediated cross-links reduces its ability to swell.

The reduction power of DTT, which is increased at alkaline pH values ($pK_a$ thiol groups in DTT is 9.2),[22] also affects the stability of the scaffolds. The ability of DTT to reduce the disulfide bridges, therefore destabilizing the protein aggregates formed during the pre-heating step, is dependent on the reactive thiolate form (S-) of the thiol group, more frequent at higher pHs. The presence of more protein aggregates in the scaffolds obtained at pH 7 decreases the number of possible cross-links contributing to less stable scaffolds reflected in higher weight loss rates and higher SRs.

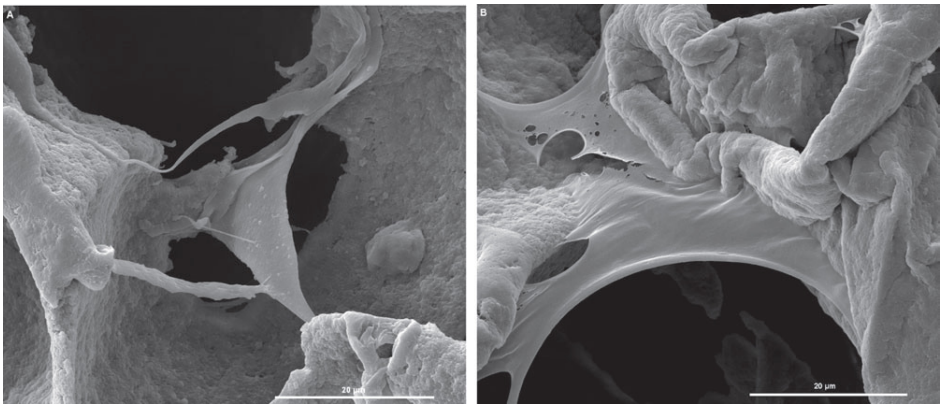


**FIGURE 7.** SEM micrographs of BJ5ta cells seeded in the "ideal" scaffold after 120 h of incubation: Surface (A) and cross-sectional view (B).

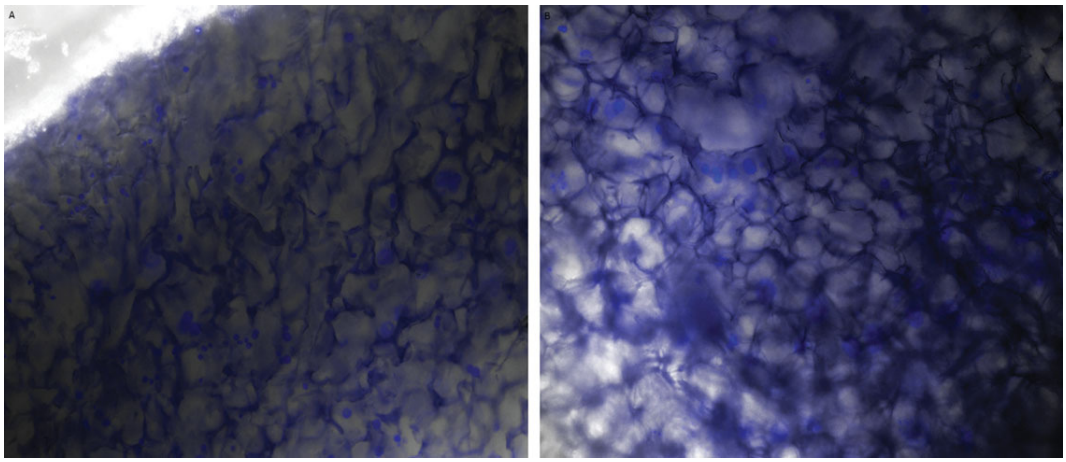

**FIGURE 8.** Fluorescence microscopy analyses of BJ5ta cells seeded in the "ideal" scaffold after 120 h of incubation. Nucleus stained with Hoechst 33258. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Independent of the pH at which samples are prepared, PO and PS vary with the amount of proteins present on the scaffolds formulation. During the freeze-drying process, inter/intra connected ice crystals formed inside the hydrogels, which then form pores during sublimation leading to a porous 3D polymeric scaffold.[23] As more protein is present in the same sample volume, smaller crystals could form that ultimately lead to structures with lower degrees of PO as well as smaller pores.

The clear, positive results obtained with seeding cells onto the scaffolds could nevertheless be greatly improved, especially after 3 days of culture, by using o-rings or precoating the wells with agarose gel to prevent cells from adhering outside the scaffolds onto the tissue culture plate, surface treated to favor cell adhesion.

## CONCLUSIONS

A simple method for obtaining macroporous protein-based scaffolds is reported in this work. The results of the study demonstrate that the $Ca^{2+}$-induced cold gelation technique can be used to create 3D scaffolds for tissue engineering applications. The systems here presented showed good degradation rates, SR, average PS, and interconnectivity, together with a uniform microstructure and good biocompatibility supporting cell adhesion and proliferation. The minimum and maximum values of each analyzed response are similar to those present in literature for other types of scaffolds especially designed for a specific tissue application, which makes these scaffolds suitable for the proposed applications using different cells types. Moreover, they could be used in a wide range of pH values because a variation of pH of the medium between 5 and 9 does not affect their performance.

In conclusion, the combination of the $Ca^{2+}$-induced cold gelation method together with the Experimental design is here put forward as a useful tool for the development of scaffolds for tissue engineering and tissue restoration, with an accurate control of several parameters essential for the success of the systems.

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