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Self-hardening calcium deficient hydroxyapatite/gelatine foams for bone regeneration

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Abstract In this work gelatine was used as multifunctional additive to obtain injectable self-setting hydroxyapatite/gelatine composite foams for bone regeneration. The foaming and colloidal stabilization properties of gelatine are well known in food and pharmaceutical applications. Solid foams were obtained by foaming liquid gelatine solutions at 50°C, followed by mixing them with a cement powder consisting of alpha tricalcium phosphate. Gelatine addition improved the cohesion and injectability of the cement paste. After setting the foamed paste transformed into a calcium deficient hydroxyapatite. The final porosity, pore interconnectivity and pore size were modulated by modifying the gelatine content in the liquid phase.

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1 Introduction

Calcium Phosphate Cements (CPCs) are used as synthetic bone grafts in several dental and orthopedic applications. Their low temperature consolidation process allows for the incorporation of drugs, biological signals or polymeric additives. They are versatile materials, in the sense that their formulation can be tailored to different clinical requirements. Nevertheless, CPCs have low macroporosity, this limitation hindering bone colonization and retarding material resorption. Different approaches have been proposed to increase CPCs macroporosity, foaming of the liquid phase being among the most promising [1–3]. This process requires the incorporation of surface active additives, able to act as foaming agents, to the cement liquid phase.

In this study we propose to use gelatine as foaming agent in an alpha-tricalcium phosphate (α-TCP) cement that sets through a hydrolysis reaction giving a calcium deficient hydroxyapatite (CDHA), according to Eq 1 [4]:

$$3Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5OH \quad (1)$$

In fact, gelatine is already well known in the pharmaceutical and food industries for its foaming and colloidal stabilizing properties [5]. Moreover, the feasibility of combining gelatine with CPCs has already been put forward by several authors, which have shown that the addition of gelatine into CPC formulations improves the cohesion of the cement paste [6] and increases the compressive strength of the cement [7–9]. Furthermore, it has been reported that gelatine incorporation does not delay the setting reaction of the CPCs [6–8]. Finally some authors

have proposed the incorporation of gelatine soluble particles as porogenic agents of CPCs [6, 10].

In this work we focus on the foaming capacity inherent to the amphiphilic character of the gelatine molecule, that makes it a surface active compound. In addition, other advantages derived from its origin and specific properties, make it not only a good candidate as foaming agent but also a multifunctional additive for CPCs, with the ability to achieve several objectives simultaneously, namely: (1) to improve cement paste injectability and cohesion; (2) to mimic the composite structure of bone, relying on the fact that gelatine is actually denaturated collagen, the main protein in the bone extracellular matrix; (3) to improve cell adhesion, through integrin recognition of the RGD peptide sequence contained in gelatine [11]; (4) to increase de resorption rate, since several studies have shown that gelatine is biocompatible and resorbs completely in vivo [12]. In summary, the aim of this work is to obtain injectable CDHA/gelatine foams for bone grafting applications, able to be implanted via minimally invasive surgical techniques.

2 Materials and methods

2.1 Cements and foams preparation

α-TCP was synthesized by solid state reaction at 1,400°C of a mixture of $CaCO_3$ (Sigma-Aldrich, UK) and $CaHPO_4$ (Sigma-Aldrich, UK) followed by quenching. After that, the α-TCP was milled (Fritsch Pulverisette 6, Germany) for 15 min to obtain a mean particle size of 5.43 ± 1.15 μm, as measured by laser diffraction (LS 13 320 Beckman Coulter, USA). Afterwards, 2 wt% precipitated hydroxyapatite (HA, Merck, Germany) was mixed with the α-TCP to obtain the cement powder. The liquid phase consisted in water solutions of 250 Bloom bovine gelatine type B (Rousselot, France), in different concentrations (1, 10, 15 and 20 wt% with respect to the liquid phase of the cement). The liquid phase was prepared by dissolving gelatine in distilled water at 50°C. In some cases 2.5 wt% of Na_2HPO_4 (Merck, Germany) was added to the liquid as an accelerant of the setting reaction [13]. Non-foamed cement pastes were prepared by mixing the cement powder and the gelatine solution with a spatula for 1 min at 50°C. Different liquid to powder (L/P) ratios were used (0.40, 0.47 and 0.80 ml/g). Finally, just after mixing, the pastes were moulded or placed into a syringe at room temperature.

The foaming process was carried out at 50°C by foaming 2 ml of the liquid phase for 1 min at 11,000 rpm with a domestic mini-mixer. In order to be able to transfer the procedure to the clinical situation, a removable stainless steel paddle was adapted to the rotating shaft, in order to be able to sterilize it. The foaming process requires low viscosity cement pastes to allow kneading the paste without breaking the bubbles in the liquid foam. For this reason the L/P ratio was increased to 0.80 ml/g. After adding the powder, the mixture was carefully homogenized with a spatula avoiding foam disruption. Finally, the foamed paste was introduced with a spatula in Teflon moulds and allowed to set.

2.2 Characterisation techniques

The effect of the accelerant incorporation and the gelatine content on the cement setting times were studied at a L/P ratio of 0.47 ml/g. Initial and final setting times were measured using the Gillmore needle method (ASTM C266-89). Cement pastes prepared at 50°C were immediately placed in ring moulds to obtain flat surfaces, and the test was performed at room temperature. The cohesion of the paste containing different amounts of gelatine was visually determined by injecting the paste in water at 37°C immediately after preparation, at a L/P = 0.80 ml/g and with accelerant.

The paste injectability was assessed as function of the gelatine content, either in non-foamed (L/P = 0.47 ml/g) or in foamed (L/P = 0.80 ml/g) cements. 3 ml of paste were transferred into a 5 ml syringe with a 2 mm aperture by means of a spatula. The paste was extruded at room temperature using a universal testing machine (MTS Bionix 858, USA) at a crosshead speed of 15 mm/min, up to a maximum load of 100 N. The starting of the extrusion was performed at a fixed time of 2.5 min since liquid and powder mixture. The applied load was registered as a function of the piston displacement, and the injectability (%) was defined as the percentage of the mass of extruded paste with respect to the original mass of paste in the syringe [14].

The effect of the environmental conditions on the setting reaction and the compressive strength was studied as a function of the gelatine content at L/P = 0.40 ml/g. The compressive strength was measured with a universal testing machine (MTS Bionix 858, USA) with a crosshead speed of 1 mm/min until fracture. The specimens (6 mm in diameter and 12 mm in height) were set for 7 days in different media, i.e. soaked in Ringer's solution (0.9 wt% NaCl in water) or in 100% humidity, both at 37°C. The phase evolution was determined by X-ray diffraction (INEL CPS-120, France; CuKα, 35 mA, 35 kV; step size of 0.029°, step time 50 s) according to a protocol previously described [4]. The samples were also analysed by Fourier transform infrared spectroscopy (FTIR, Bomem MB-120; 4,000–400 cm^{-1}, 2 mg of sample in 200 mg of KBr) to monitor the setting reaction and the presence of gelatine in the composites.

The total porosity of the samples was determined from the apparent density measured by mercury pycnometry. The macroporosity introduced during the foaming process was estimated assuming that in the non-foamed cement specimens the total porosity was only microporosity, and

therefore the macroporosity was entirely created by the foaming process. Consequently, the macroporosity was estimated from the apparent densities of the foamed and unfoamed cements (both containing gelatine in the liquid phase), coded as $d_{app\ foamed\text{-}CPC}$ and $d_{app\ CPC}$, respectively [15, 16], according to Eq. 2:

$$\text{Macroporosity} = \left[1 - \left(d_{appfoamed-CPC}/d_{appCPC}\right)\right] \times 100 \quad (2)$$

Gold-coated fracture surfaces were observed by scanning electron microscopy (SEM, Jeol JSM 6400). Open porosity was evaluated by mercury intrusion porosimetry (MIP, Micromeritics AutoPore IV 9500, USA).

3 Results and discussion

The effect of gelatine addition and of the accelerant solution on the setting time of the cements is shown in Table 1. The setting time of the foams could not be measured by the Gillmore needles due to the low strength of the porous structure. Without the addition of the accelerant both times were longer than with accelerant, leading to final setting times not adequate for bone grafting requirements. For this reason all the subsequent experiments were carried out with 2.5 wt% accelerant. On the other hand, increasing the gelatine content reduced the initial setting time, this effect being clearer in the cements without accelerant. In contrast, higher gelatine amounts increased the final setting time. This can be attributed to the gelling process of gelatine that takes place at room temperature, which is able to avoid the light Gillmore needle penetration (initial setting time), but not that of the heavy one (final setting time). Moreover, the increase in viscosity with growing gelatine content can have a retarding effect of the α-TCP dissolution and re-precipitation reaction.

Table 1 Initial and final setting times measured for cement samples with different amounts of gelatine, with (+) or without (−) 2.5 wt% accelerant

Cement	Accelerant	Initial setting time (min)	Final setting time (min)
0 Gel	−	60	>100
10 Gel	−	52	>100
15 Gel	−	46	>100
20 Gel	−	27	>100
0 Gel	+	20	36
10 Gel	+	15	38
15 Gel	+	14	48
20 Gel	+	14	52

L/P = 0.47 ml/g

The results of the injectability test are shown in Fig. 1. The applied force versus piston displacement curves (Fig. 1a) showed that the applied force increased at the beginning of the injection, reaching soon a constant level close to 20 N for all formulations studied. In the cement paste without gelatine the injection force started to increase again after about 50% of the total piston run, reaching the maximum force when some paste was still in the syringe, indicating a limited injectability. When gelatine was added the maximum piston displacement increased in a direct relation to the content of gelatine in the paste. The strong increase in the applied force for the 15 wt% gelatine sample at the end of the curve was due to the fact that no further paste remained in the syringe. Figure 1b confirms that the percentage of injectability increased with the gelatine content, being nearly total for 15 wt% gelatine. When the pastes were foamed, the injectability was further increased, due to the fact that, in addition to the effect of the porosity introduced, a higher L/P ratio was used. Thus, as shown in Fig. 1, even for an amount of gelatine as low as 1 wt% the injectability of the foamed paste was nearly 100% and the force–displacement profile was similar to that of the non-foamed paste prepared with 15 wt% of gelatine.

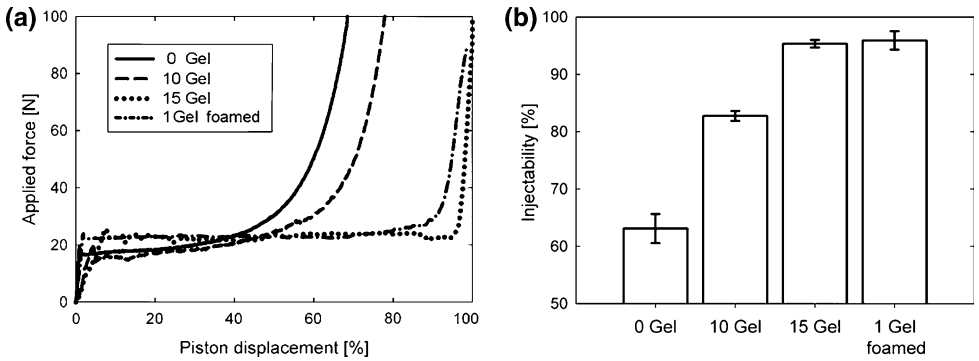

Fig. 1 **a** Evolution of the applied force versus piston displacement during the cement or foam pastes extrusion, and **b** percentage of injectability of the cement or foam pastes, as function of the gelatine content. 0 Gel, 1 Gel, 10 Gel and 15 Gel stand for the pastes containing 0, 1, 10 and 15 wt% of gelatine with respect to the liquid phase. L/P ratio = 0.47 and 0.80 ml/g for the cements and the foam, respectively. Injection performed 2.5 min after mixing at a crosshead speed of 15 mm/min

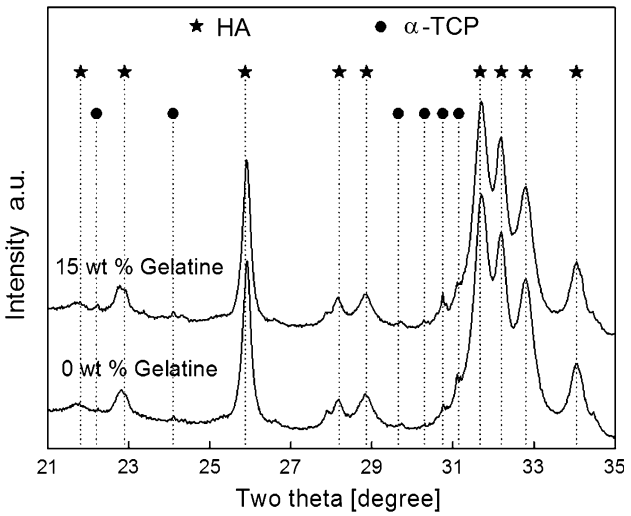

Fig. 2 X-ray diffraction patterns of the cements containing 0 and 15 wt% of gelatine in the liquid phase. Patterns after 7 days of immersion in Ringer's solution. L/P = 0.47 ml/g

Figure 2 shows the XRD patterns of the cements containing 0 and 15 wt% of gelatine in the liquid phase after 7 days of immersion in Ringer's solution. The main phase was in both cases hydroxyapatite, and only residual amounts of α-TCP were detected. No significant differences were observed in the conversion to CDHA with the introduction of gelatine in the cement. Although the crystal size was not calculated from the XRD data, it is interesting to note that the width at half maximum height of the peaks are very similar, indicating that the crystallinity of the two samples was comparable. In fact, the microstructure of the two samples, presented in Fig. 3, was very similar. Gelatine was not visible as a separate phase. In fact it has to be considered that the amount of gelatine incorporated in the cement represented a 7.07 wt% with respect to the total mass of cement, and moreover partial leaching during setting in Ringer's solution can also have occurred. The effect of the setting environment on the hydrolysis of α-TCP to CDHA and the compressive strength of the set cements with different gelatine contents is shown in Table 2. No significant differences were observed in the extent of conversion to CDHA. However, the setting environment significantly affected the compressive strength after 7 days of the gelatine containing cements, whereas for cements without gelatine (0 Gel), no difference was observed. When the samples were stored in 100% humidity, the compressive strength increased with the addition of gelatine, whereas when immersed in Ringer's solution the effect was the opposite, and the compressive strength decreased when the amount of gelatine increased (see Table 2). This can be attributed to the fact that, when set in humid atmosphere there is no gelatine leaching and consequently gelatine acts as agglutinant of the CDHA crystals, improving the mechanical behaviour. On the contrary, when immersed in Ringer's solution at 37°C, since gelatine has not been crosslinked it can dissolve in

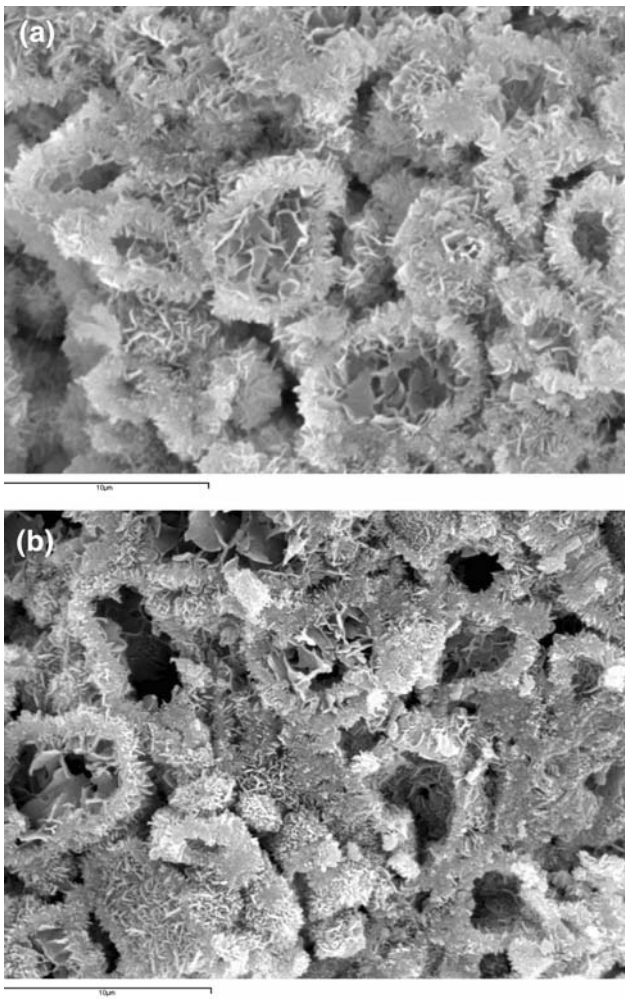

Fig. 3 SEM microstructures of the cements containing **a** 0 and **b** 15 wt% of gelatine in the liquid phase after 7 days of immersion in Ringer's solution. L/P = 0.47 ml/g. Scalebar corresponds to 10 μm

Table 2 Effect of environmental conditions (immersed in Ringer's solution or kept in saturated humidity environment) on the conversion to CDHA and the compressive strength of gelatine containing cements, after 7 days of reaction

Cement	Reaction medium	% of reaction	Compressive strength (MPa)
0 Gel	Ringer's	95.5	29.0 ± 4.2
10 Gel	Ringer's	95.9	15.5 ± 2.4
15 Gel	Ringer's	95.2	11.2 ± 2.2
0 Gel	100% humidity	94.4	29.8 ± 2.8
10 Gel	100% humidity	93.4	35.3 ± 3.4
15 Gel	100% humidity	93.4	32.5 ± 5.9

L/P = 0.40 ml/g

the liquid medium, leading to greater microporosity and reducing the strength. In fact, previous studies pointed out the same trend, reporting an initial increase of the compressive strength of CPCs with the incorporation of gelatine when setting was performed in simulated body fluid, followed by a decrease attributed to the gelatine leaching to the liquid media [7–9]. Partial leaching of the gelatine was evidenced by FTIR spectra as shown Fig. 4, where the IR spectra of the gelatine containing cements set for 12 days either in 100% humidity or in Ringer's solution are compared. The band at 1,527 cm^{-1} corresponding to the amide II band of the gelatine, is the only band that does not overlap with the bands of the CDHA, and a slight decrease in its intensity is observed for the sample that has been immersed for 12 days in Ringer's solution. However, it is important to note that although gelatine was not-cross-linked, only a part of it was leached, another part remaining trapped in the cement structure.

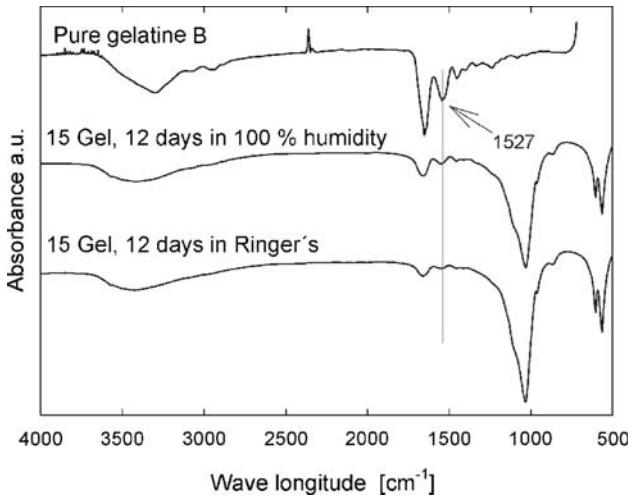

Fig. 4 FTIR spectra of cements containing 15 wt% gelatine in the liquid phase, after setting for 12 days in Ringer's solution or in 100% humidity. L/P = 0.80 ml/g

Figure 5 shows SEM images of the foamed (F) and non-foamed (NF) cement samples for two gelatine concentrations. It is evidenced that the foaming process incorporated spherical macropores into the cement that are maintained after setting. Moreover, a higher gelatine concentration resulted in smaller macropores and in a decrease of the total macroporosity, as shown in Table 3. This can be explained by the increase in viscosity of the liquid phase that resulted in higher foam stability, preventing the foam maturation mechanisms like interbubble gas diffusion and bubble coalescence. MIP results provided a more detailed characterisation of the open pore size distribution, as shown in Fig. 6. For the 10 Gel foamed sample at L/P = 0.80 ml/g (dot line), only 3% of open pores were larger than 10 μm. Taking into account that, according to mercury pycnometry this sample had a 37% macroporosity, this indicates that the macroporosity mainly consists of closed pores, or pores interconnected by submicronic throats. The fact that gelatine gelifies below 45–50°C increases foam stability and makes more difficult to obtain

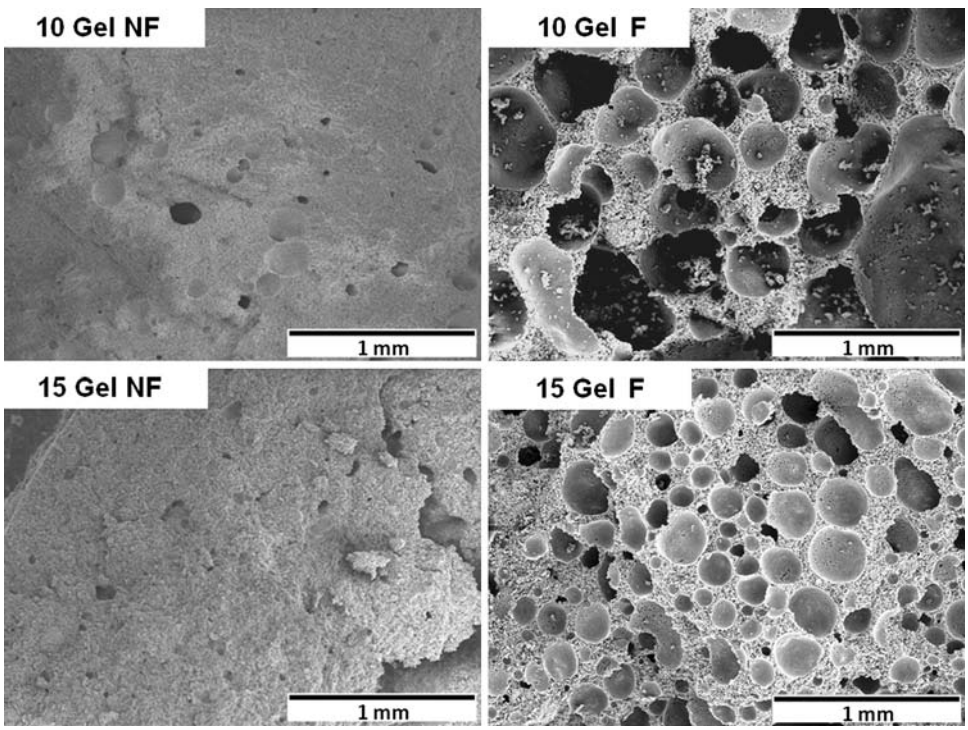

Fig. 5 Microstructure of samples containing 10 or 15 wt% of gelatine in the liquid phase, either non-foamed (NF) or foamed (F). L/P = 0.80 ml/g. All were set during 7 days in 100% humidity

Table 3 Total porosity and macroporosity measured by mercury pycnometry for samples containing 10 or 15 wt% of gelatine in the liquid phase, foamed (F) or non-foamed (NF)

Cement	Condition	Total porosity (%)	Macroporosity (%)
10 Gel	NF	57.6 ± 5.5	–
10 Gel	F	73.2 ± 1.3	36.8 ± 3.1
15 Gel	NF	57.2 ± 4.7	–
15 Gel	F	63.9 ± 5.1	15.5 ± 2.1

L/P = 0.80 ml/g. All samples were set during 7 days in 100% humidity

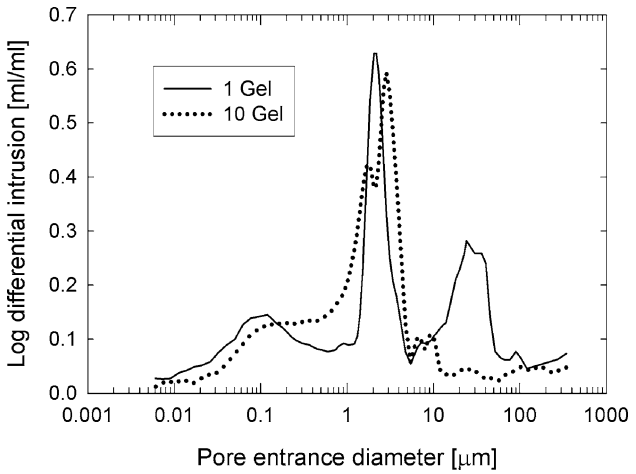

Fig. 6 Entrance pore size distribution measured by MIP for foamed cement samples containing 1 or 10 wt% of gelatine in the liquid phase. Prepared at L/P = 0.80 ml/g and set during 7 days in 100% humidity

interconnected macroporosity. In this sense, a reduction in the gelatine content improves pore interconnectivity, as corroborated in Fig. 6, were the entrance pore size distribution corresponding to 1 Gel sample (solid line) shows 15% of interconnected macroporosity by throats larger than 10 μm. This result is especially relevant when comparing gelatine with other foaming agents such as albumen or synthetic surfactants like the polysorbates, where larger open macroporosity was evidenced [1–3].

Finally, a crucial requirement for injectable cements and foams is that the pastes do not disintegrate when in contact with the body fluids. Figure 7 shows that the addition of gelatine increased the cohesion of the cement and foam pastes when they were injected in water at 37°C, acting as a cohesion promoter. This result is in agreement with previous studies, where gelatine was incorporated in powder form in a CPC formulation [6].

4 Conclusions

B type bovine gelatine can be used as multifunctional foaming agent to obtain injectable, self-setting CDHA/gelatine foams. Since the liquid foam acts as a template for macroporosity, the liquid gelatine content can be adjusted to modulate final porosity, pore interconnectivity and pore diameter. Moreover, gelatine acts as a cohesion promoter of the paste, increases its injectability, and does not interfere in the CDHA formation.

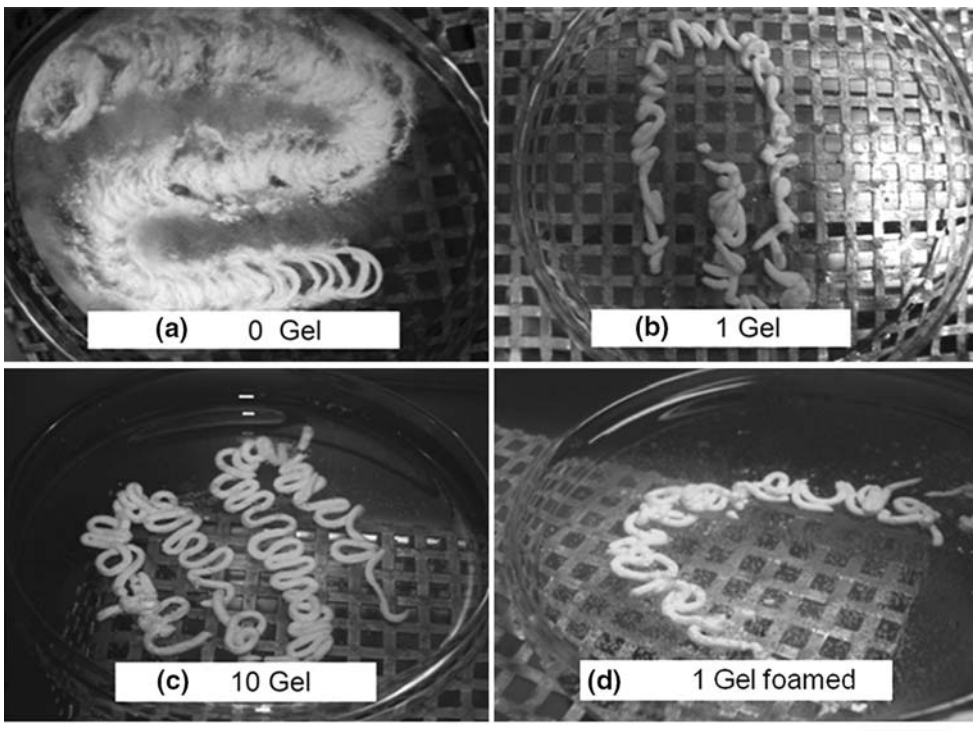

Fig. 7 Cement pastes containing 0, 1 or 10 wt% of gelatine in the liquid phase, either foamed or not, injected in distilled water at 37°C. Injection just after mixing (2.5 min). L/P = 0.80 ml/g. Scale bar corresponds to 2 cm approximately

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