Abstract: Although calcium phosphate cements (CPCs) are used for bone regeneration in a wide range of clinical applications, various physicochemical phenomena are known to hinder their potential use under challenging conditions, such as in minimally invasive surgery or in the case of highly vascularized surgical sites, mainly because of their lack of injectability or their low washout resistance. The present work shows that the combination of CPCs with a thermoresponsive hydrogel is a good strategy for finely tuning the cohesive and rheological properties of CPCs to meet clinical requirements. The thermoresponsive CPC used combines alpha-tricalcium phosphate with an aqueous solution of poloxamer 407, which exhibits an inverse thermoresponsive behaviour, with a gelling transformation at around body temperature. These novel CPCs exhibited temperature-dependent properties. Addition of the polymer enhanced the injectability of the paste, even at a low liquid-to-powder ratio, and allowed the rheological properties of the cement to be tuned, with the injection force decreasing with the temperature of the paste. Moreover, the cohesion of the paste was also temperature-dependent and increased as the temperature of the host medium increased due to gelling induced in the paste. The thermoresponsive cement exhibited excellent cohesion and clinically acceptable setting times at 37°C, irrespective of the initial temperature of the paste. The addition of poloxamer 407 slightly delayed the setting reaction in the early stages but did not hinder the full transformation to calcium-deficient hydroxyapatite. Moreover, the frozen storage of premixed thermoresponsive cement pastes was explored, the main physicochemical properties of the cements being maintained upon thawing, even after 18 months of frozen storage. This avoids the need to mix the cement in the operating theater and allows its use off-the-shelf. The reverse thermoresponsive cements studied herein open up new perspectives in the surgical field, where the sequential gelling/hardening of these novel cements allows for a better and safer application, and in the development of self-setting ceramic inks for 3D inkjet printing applications.
Thermoresponsive calcium phosphate cements

Yassine Maazouz¹, Edgar B. Montufar¹,§, Julien Malbert¹, Montserrat Espanol¹, Maria-Pau Ginebra¹,²*

¹Biomaterials, Biomechanics and Tissue Engineering Group. Department of Materials Science and Metallurgical Engineering, Universitat Politècnica de Catalunya. BarcelonaTech (UPC), Av. Diagonal 647, 08028 Barcelona, Spain

²Institute for Bioengineering of Catalonia, C. Baldiri Reixach 10, 08028 Barcelona, Spain

*Corresponding author:
Maria-Pau Ginebra
Biomaterials, Biomechanics and Tissue Engineering Group
Department of Materials Science and Metallurgical Engineering
Universitat Politècnica de Catalunya. BarcelonaTech (UPC)
Av. Diagonal 647, 08028 Barcelona, Spain
Telephone: +34 934017706, Fax: +34 934016706
E-mail: maria.pau.ginebra@upc.edu

§Current address: CEITEC - Central European Institute of Technology, Brno University of Technology, Purkyňova 656/123, 612 00 Brno, Czech Republic.

Abstract

Although calcium phosphate cements (CPCs) are used for bone regeneration in a wide range of clinical applications, various physicochemical phenomena are known to hinder their potential
use under challenging conditions, such as in minimally invasive surgery or in the case of highly vascularized surgical sites, mainly because of their lack of injectability or their low washout resistance. The present work shows that the combination of CPCs with a thermoresponsive hydrogel is a good strategy for finely tuning the cohesive and rheological properties of CPCs to meet clinical requirements. The thermoresponsive CPC used combines alpha-tricalcium phosphate with an aqueous solution of poloxamer 407, which exhibits an inverse thermoresponsive behaviour, with a gelling transformation at around body temperature. These novel CPCs exhibited temperature-dependent properties. Addition of the polymer enhanced the injectability of the paste, even at a low liquid-to-powder ratio, and allowed the rheological properties of the cement to be tuned, with the injection force decreasing with the temperature of the paste. Moreover, the cohesion of the paste was also temperature-dependent and increased as the temperature of the host medium increased due to gelling induced in the paste. The thermoresponsive cement exhibited excellent cohesion and clinically acceptable setting times at 37ºC, irrespective of the initial temperature of the paste. The addition of poloxamer 407 slightly delayed the setting reaction in the early stages but did not hinder the full transformation to calcium-deficient hydroxyapatite. Moreover, the frozen storage of premixed thermoresponsive cement pastes was explored, the main physicochemical properties of the cements being maintained upon thawing, even after 18 months of frozen storage. This avoids the need to mix the cement in the operating theater and allows its use off-the-shelf. The reverse thermoresponsive cements studied herein open up new perspectives in the surgical field, where the sequential gelling/hardening of these novel cements allows for a better and safer application, and in the development of self-setting ceramic inks for 3D inkjet printing applications.

Keywords: calcium phosphate cement;
1 Introduction

Minimally invasive surgical (MIS) techniques reduce the risk, damage and pain caused to the patient, as well as healthcare costs, compared to traditional surgical procedures [1][2]. In the field of orthopaedic surgery, some interventions such as vertebroplasty, kyphoplasty or the treatment of osteonecrosis of the hip, are performed via MIS [3]. In order to perform these procedures safely, however, the properties of the material used should be adapted to each step of the process in order to overcome key problems found during material injection [4][5].

Self-setting calcium phosphate slurries, also referred to as calcium phosphate cements (CPCs), are well known bone-filler materials with more than 30 years of use in orthopaedics [6]. Unlike acrylic bone cements based on polymethyl methacrylate (PMMA), the exothermy of the CPC hardening reaction is very low, and the end product of the cementitious reaction is a calcium phosphate that is very similar to the mineral phase of bone. Although CPCs are considered to be injectable materials, balancing their characteristics to achieve injectable pastes with adequate cohesion, setting time and sufficient mechanical strength for clinical application remains a challenge in the field of injectable bone substitutes [7][8][9]. Specifically, the cohesion and stability of the paste is a crucial issue in terms of material performance and safety. Indeed, serious health risks were detected some years ago when a commercial CPC formulation was used off-label in vertebroplasty, with particle leakage into the blood stream after injection into vertebral bodies eventually being held responsible for causing blood clotting and subsequent embolism, which resulted in the death of several patients [10][11].

Several strategies have been explored to enhance the injectability of CPCs [12][13], either by reducing the permeability of the cement powder [14], modifying the particle surface properties [9][15] or increasing the viscosity of the liquid cement phase [16]. However, even though a high viscosity of the liquid cement phase can be beneficial to the cohesive and anti-washout properties, as well as for avoiding leakage and migration of the material after
implantation, it is detrimental to handling of the material by the surgeon [17][18]. Furthermore, most hydrogels used as liquid phase thickeners exhibit a decrease of viscosity with increasing temperature, as is the case for gelatine or agarose, which is not suitable for clinical applications since the temperature of the surgical site is higher than that of the operating theatre. This makes the cement more likely to undergo extravasation from the bone defect once injected. The preferred evolution would rather be the opposite: whereas a low viscosity would be convenient for mixing and injection, an instantaneous increase of viscosity once the material fills the bone defect would be beneficial [3], since this would ensure stability and washout resistance to the blood flow and/or to other surrounding fluids.

In light of the above, the use of polymeric additives featuring an inverse thermal gelling, like poloxamers, is of particular interest for controlling the properties of injectable biomaterials [19]. To the best of our knowledge there are no publications focusing on the combination of reverse thermoresponsive hydrogels with self-hardening calcium phosphate cements. Poloxamers are triblock (A-B-A) amphiphilic copolymers comprising two external blocks of polyoxoethylene (PEO) and a central block of polyoxpropylene (PPO), as shown in Figure 1 [20]. These copolymers present an inverse thermal gelling above the critical micelle concentration (CMC) driven by the formation of entangled micelles due to the difference in lower critical solution temperature (LCST) between PEO and PPO [20]. This induces self-assembly of the macromers above the LCST of the PPO blocks. A further increase in temperature leads to the formation of a gel [21]. The gelling temperature and the strength of the gel depend on the concentration (above the CMC) and the lengths of the PEO/PPO blocks. A prominent member of this family of polymers known as poloxamer 407 (Figure 1, marketed as Pluronic F127) has a gelling temperature close to physiological temperature at relatively low concentration (18–30 wt%). Some studies have reported that 20 wt.% solutions of poloxamer 407 in a phosphate buffer exhibited a gelling temperature of about 27°C, whereas 25wt% and 30wt% solutions exhibited this gelling at much lower temperatures of 19°C and 10°C,
respectively [22][23]. These findings highlight the high dependence of gelling temperature on the concentration of the aqueous poloxamer solution. Other poloxamers (i.e. poloxamer 188) require a high concentration (≈50 wt. %) in order to gel at physiological temperature, which can be accompanied by considerable swelling [20].

Poloxamer solutions exhibit a complex rheological behaviour, being liquid below the sol-gel transition temperature and experiencing a drastic change in shear moduli/viscosity around the sol-gel transition temperature. Nonetheless, the elastic shear modulus increases linearly after the onset of this transition and subsequently increases with a lower slope, thus resulting in an “S”-shaped viscosity versus temperature curve [23]. This curve is shifted towards lower temperature when electrolytes (i.e. phosphate buffer) are incorporated into the solution, producing lower gelling temperatures for the same poloxamer concentration [23]. These properties make them particularly interesting for a number of biomedical applications, especially wound dressings, local drug delivery, temporary embolization and cell encapsulation [24]. The objective of this work is to explore the potential of combining poloxamer 407 with self-setting calcium phosphate pastes, with the aim of conferring a reverse thermoresponsive behaviour on them.

2 Materials and methods

2.1 Solid phase preparation

Alpha- (α-Ca₃(PO₄)₂, α-TCP) and beta-tricalcium phosphate (β-Ca₃(PO₄)₂, β-TCP) were obtained by solid-state reaction from a 2:1 molar mixture of calcium carbonate (CaCO₃, Sigma Aldrich) and anhydrous dicalcium phosphate (CaHPO₄, Sigma Aldrich) at 1400 and 1100 °C respectively. Air quenching was performed for α-TCP after heat treatment to prevent the formation of β-TCP during cooling. Powders of these two phosphates were obtained by dry milling
(Pulverisette 6, Fritsch Gmbh) for 40 min at 450 rpm with 10 agate balls (30 mm diameter), followed by 60 min at 500 rpm (with the same balls) and finally 60 min at 500 rpm with 100 agate balls (10 mm diameter). The phase composition of the obtained powders was assessed by X-ray diffraction (XRD; Bruker D8 Advance), and the similarity of particle sizes between α-TCP and β-TCP powders was verified by laser diffraction (LS 13 320 Beckman Coulter) and nitrogen adsorption (ASAP 2020 Micromeritics) as described by Montufar et al. [9].

2.2 Liquid phase preparation

The liquid phase used to prepare the cement pastes comprised 2.5 wt% Na₂HPO₄ (Sigma Aldrich; P32869) aqueous solution (also called accelerant). To prepare thermoresponsive cements 20 wt% poloxamer 407 (Sigma Aldrich P2443) was added to the accelerant solution. These formulations are coded as CPC 0% PLU and CPC 20% PLU respectively.

2.3 Paste preparation

The pastes were prepared by mixing the α-TCP or β-TCP powder with the liquid phase previously cooled to 0 °C in a dual asymmetric centrifugal mixer (Speed-Mixer, DAC 150.1 FVZ-K), at liquid-to-powder (L/P) ratios of 0.35 and 0.65 g/g. After preparation, the paste was introduced into a commercial syringe with an aperture with an inner diameter of 2 mm (cartridge diameter of 13 mm with nominal capacity of 5 ml), unless otherwise specified. The syringes containing the pastes were subsequently introduced into a thermal bath at the desired temperature for 15 minutes. The temperature of the paste was monitored by inserting a type k thermocouple into the cement paste.

2.4 Influence of temperature on the injection behaviour of the paste

The injection test was performed as described previously [9]. Briefly, pastes at different temperatures (0, 7, 12, 15 or 20 °C) were extruded from the syringe at a constant velocity of 15 mm/min using a universal testing machine (MTS Bionix 858). The load was recorded as function of the plunger run, allowing a maximum force of 500 N. The yield load and injection
load were obtained from the extrusion curves (load–plunger run), with the plunger run being considered to be the percentage displacement of the syringe plunger with respect to its initial position. The percentage injectability was calculated using the following equation.

\[ \text{Inj} \, (\%) = \frac{(W_f - W_a)}{(W_f - W_e)} \times 100 \quad \text{(Eq. 1)} \]

Where Inj is the percentage injectability, \( W_e \) is the weight of the empty syringe, \( W_f \) is the weight of the syringe full of paste, and \( W_a \) is the weight of the syringe after the injection test. The test was performed in triplicate.

The apparent viscosity of the paste in the steady state flow region (considered as a constant load/pressure drop) of the injection curves was determined, assuming a laminar flow, by applying the Hagen–Poiseuille equation, which correlates the flow of the paste (\( Q \)) with the apparent viscosity (\( \eta_0 \)), the diameter and length of the cannula (\( D \) and \( l \)) and the pressure drop \( \Delta P \).

\[ Q = \frac{\pi \Delta P D^4}{128 \eta_0 l} \quad \text{(Eq. 2)} \]

2.5 Influence of temperature on the cohesion of the paste

The cohesion of the paste was determined by visual inspection after injecting the pastes in Ringer’s solution (0.9 wt% NaCl in distilled water) [25]. The paste was considered to exhibit cohesion if it retained the extruded shape during 24 h, which is sufficient time for the cement to harden. The ability of the paste to retain its shape was assessed in triplicate, as a function of the initial temperature of the paste (0, 7 or 18 °C) and the temperature of the Ringer’s solution bath (0, 10, 20, 25, 30 or 37 °C).
2.6 Setting and hardening of the thermoresponsive pastes

The setting times of cement pastes at different initial temperatures (0, 7, 18 and 37 °C) were measured at 37 °C, using the Gilmore needles test according to standard ASTM C266-99 [26]. Additionally, the effect of environmental temperature (7, 20 and 37 °C) was assessed using a cement paste that was initially at 18 °C.

To assess the effect of the hydrogel on the kinetics of the transformation of α-TCP, in situ XRD was performed on fresh CPC 0% PLU and CPC 20% PLU pastes. As-prepared pastes were introduced into the holder, covered with a Kapton polyimide film (Chempex Industries, Cat. No. 3022-5) and sealed with silicon vacuum grease to minimize liquid evaporation. Analyses were performed using a D8 Advance powder diffractometer (Bruker) with Bragg-Brentano geometry equipped with a germanium monochromator using Cu Kα radiation at 40 kV and 40 mA. Data sets were collected every 30 min for 18 h from 30-35° 2θ, with a step size of 0.019° 2θ and a counting time of 1 s per step. The temperature was recorded to be 28 °C. The diffraction patterns were compared with the Joint Committee on Powder Diffraction Standards for α-TCP (JCPDS No. 9-348), β-TCP (JCPDS No. 9-169) and hydroxyapatite (HA; JCPDS No. 9-432). Phase quantification was performed by comparing the ratios of the area for the most intense peak using the XRD analysis software EVA (Bruker).

The compressive strength of the cements after hardening for 7 days at 37 °C in Ringer’s solution was determined in wet cylindrical specimens (6 mm diameter, 12 mm height), using a universal testing machine (BIONIX, MTS, MN) equipped with a load-cell of 2.5 kN, at a crosshead speed of 1 mm/min. Six specimens were tested for each condition. The samples were then quenched in acetone to stop the hydrolysis reaction, and the crystalline phases present were determined by XRD from 4° to 90° 2θ, with a 0.020° 2θ step size per second. The cement microstructure was observed by field emission scanning electron microscopy (FE-SEM; Zeiss) after coating with Au–Pd alloy, and the open porosity and pore-size distribution were
characterised by mercury intrusion porosimetry (MIP, Micromeritics AutoPore IV 9500, USA). Cement samples were also analysed by ATR-FTIR (Nicolet 6700, Thermo Scientific) before and after 7 days of reaction at 37°C in Ringer’s solution. The fresh paste was lyophilised before ATR-FTIR analysis.

2.7 Frozen storage and thawing

Syringes containing the cement paste were frozen in liquid nitrogen for 15 minutes. They were then stored at -80 °C for 3, 7, 14, 21, 49 days and 18 months. For thawing, the syringes were placed in a thermal bath at 7 °C for 15 minutes. The temperature of the paste was monitored as described in section 3.3. The phase composition of the paste after storage was determined by XRD as described above. Previously, the frozen paste was freeze dried and the result compared with the XRD pattern for the recently prepared paste. The injection test, cohesion test in Ringer’s solution at 37 °C, setting time measurements and compression test were performed after paste thawing as described above to determine the effect of frozen storage time on these properties.

2.8 Statistics

The statistical significance of the results was analysed by one-way ANOVA with Tukey’s post-hoc tests (injectability study), two-way ANOVA with Sidák post hoc test (initial and the final setting times independently, considering the type of cement, either CPC 0% PLU or CPC 20% PLU, and the temperature of the paste as independent factors), or T-student test (compressive strength), using Minitab 16 software (Minitab, Inc., USA). Statistical significance was considered when p > 0.05. Data are presented as mean ± standard deviation.
3 Results and discussion

3.1 Influence of temperature on the paste injection behaviour

Figure 2a shows representative load vs. plunger displacement curves for CPC pastes containing 0 or 20 wt% poloxamer 407 (CPC 0% PLU and CPC 20% PLU, respectively) with L/P = 0.35 g/g, at different temperatures. The incorporation of poloxamer 407 leads to an increase in the injectability of the cement from 60.5 ± 0.8% for CPC 0% PLU to 98.1 ± 1.2% for the thermoresponsive cement (CPC 20% PLU) irrespective of the temperature of the paste, which can be considered as a total injection due to a small quantity of paste remaining inside the syringe cannula, as revealed by the fact that the syringe plunger was able to run all the way down. In contrast, in the absence of poloxamer 407, the injection curves showed an exponential increase in injection force as consequence of the phase-separation phenomenon. This result is even more relevant since the pastes were prepared at a low L/P ratio (0.35 g/g). Such pastes with high solid content are extremely difficult to inject due to phase separation [27], which is caused by preferential filtration of the liquid between particles, thereby forming a plug of particles at the plunger [28]. The uncontrolled changes in the L/P ratio of the extruded paste make the cement’s final properties unpredictable. The addition of poloxamer 407 prevented phase separation even in the case of a high solid content paste, thus allowing better mechanical properties to be achieved [29]. Although several previous studies have employed hydrogels in order to increase the viscosity of the liquid phase to prevent phase separation [4] [16][29], most of the hydrogels used are sensitive to temperature in such a way that their viscosity decreases as the temperature increases, as is the case for sodium alginate, chitosan or gelatin [30]. This is a major drawback since, according to the United Kingdom National Health Service, the operating theatre where the cement is prepared is at 18 °C, whereas the temperature at the surgical site is between 30 and 37 °C. This temperature difference is expected to produce a decrease in the viscosity of the cement paste, in contrast to the situation with poloxamer 407.
The thermoresponsive character of the CPC containing poloxamer 407 was clearly observed when analysing the injection loads (Figure 2a and 2b). Thus, the addition of the hydrogel resulted in a decrease in the injection load when decreasing the temperature of the paste, as can be seen from Figure 2b. In order to distinguish between the effect of poloxamer 407 on the injection behaviour and the potential effect on cement setting, the same tests were performed using an equivalent paste made with the less reactive allotropic phase beta-tricalcium phosphate (β-TCP), which does not undergo a cementitious self-setting reaction. Similar yields and injection loads were obtained (Figure 2b), thus confirming that the thermoresponsive character of the paste can be attributed to a structuration/cross-linking of the aqueous solution of poloxamer 407 with temperature. When the temperature of the paste decreased from 20 to 12 °C, the injection load decreased significantly (p < 0.05) and then remained stable until 0 °C, with an average value of 37 N. According to the Hagen–Poiseuille equation (Eq. 2), this corresponds to a dynamic viscosity of around 29 Pa.s. The increase in injection load with increasing paste temperature can be attributed to the formation of micelles that physically crosslink the poloxamer 407 from a liquid state into a gel [31][32]. At higher temperatures (25 °C) the load needed to inject the paste was higher than the maximum load allowed in the injection test (500 N), which further supports this hypothesis. A closer look at the injection curves of Figure 2a allowed an increase in the yield load (the load needed to start the flow of the paste at the beginning of the injection test) corresponding to an increasing shear modulus, which indicates a structuration of the paste, to be identified. Note that the yield load was equal to or higher than the injection load. This is in accordance with the phenomenon reported by Franco et al. [33] using β-TCP slurries in a poloxamer 407 solution and with Noël et al. [34], who studied a Portland cement in a triblock copolymer with similar properties to those of poloxamer 407. It is important to note that, at 20 °C, the CPC 20% PLU paste reached a yield load of around 300 N, corresponding to a dynamic viscosity of around 250Pa.s⁻¹ as calculated using Eq. 2, which would provide sufficient consistency to prevent the
paste from leaking from the surgical site where it is injected, as described in the work of Wang et al.[16]. The change in the consistency of the paste is illustrated in videos V1 (CPC PLU20% at 7°C) and V2 (CPC PLU 20% at 20°C) provided as supplementary information.

3.2 Influence of temperature on the cohesion of the paste

The cohesion of the paste was first determined by injecting the pastes (CPC 0% PLU and CPC 20% PLU), which were at an initial temperature of 18 °C, directly into Ringer’s solution at 37 °C. The images presented in Figure 3 shows that, in the absence of poloxamer 407, at an L/P ratio of 0.65 g/g the paste was washed out immediately when in contact with Ringer’s solution. At an L/P ratio of 0.35 g/g, although the cement hardened after 24 h, the CPC 0% PLU paste was partially fragmented, thus revealing that the bare cement had limited cohesion, with the risk of particle leakage. The samples containing 20 wt% poloxamer 407 presented total cohesion, i.e. complete retention of their extruded shape during 24 h when injected directly into Ringer’s solution at 37 °C, even at high L/P ratios such as 0.65 g/g. CPC pastes containing 30 wt% poloxamer 407 were also analysed and were found to present particle leaching at L/P 0.35 g/g and an extensive swelling in both L/P 0.35 and 0.65 g/g. As such, they were discarded for the rest of the study.

The reverse thermoresponsive properties of the CPC were further confirmed when analysing its cohesion when injected into a liquid medium at different temperatures, as shown in Figure 4. The temperature of the Ringer’s solution drastically influenced the ability of the paste to retain its extruded shape. While a general tendency towards better shape retention was observed upon increasing the temperature, the CPC 20% PLU pastes injected into Ringer’s solution below 30 °C disintegrated and leached particles. In the case of Ringer’s solution above 30 °C, the poloxamer 407 contained inside the paste gelled instantaneously when entering into contact with the fluid, thus binding the particles together until setting occurred. In the other cases, the temperature of the Ringer’s solution was not sufficiently high to form a gel.
that could bind the particles together, thus meaning that the hydrogel dissolved in the aqueous immersion medium before setting could take place. This was attributed to the fact that poloxamer 407 gelling strength depends on temperature and, as described previously, aqueous solutions of poloxamer 407 at a concentration of 20 wt% in phosphate buffer were found to exhibit a gelling temperature of around 27°C [23], which is close to the change of behaviour at 30°C found in the present work [35]. Thus, the gelling of poloxamer 407 provided initial cohesion of the paste before the α-TCP particles were able to hydrolyse into calcium-deficient hydroxyapatite (CDHA) according to Eq. 3. After gelling, although poloxamer 407 was still slowly dissolved into the aqueous environment due to the fact that poloxamer gels contain low-energy bonds that can be broken by the action of water [36], this happens at a slower rate, thus allowing for the simultaneous advancement of the setting reaction that guarantees the stability and progressive hardening to a solid body. Therefore, the thermoresponsive cement was able to maintain its shape under these conditions owing to a sequential gelling / setting process.

$$3\alpha\text{-Ca}_3(\text{PO}_4)_2 + \text{H}_2\text{O} \rightarrow \text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5\text{OH} \quad \text{(Eq. 3)}$$

Interestingly, the initial temperature of the CPC 20% PLU paste did not affect its cohesive behaviour when injected into Ringer’s solution at 37°C (Figure 5). Thus, even if the paste is originally at a lower temperature, which has clear advantages in terms of injectability as previously shown, when it is brought into contact with a liquid of equivalent osmolarity to blood at 37°C, it instantaneously gains cohesion due to gelling of the polymer.

### 3.3 Influence of paste temperature on setting times

Although with no direct clinical relevance, measurement of the setting times at different environmental temperatures (between 7 and 37°C), fixing the initial temperature of the paste at 18°C (Figures 6 a) and b), shed light on the thermal dependence of the two mechanisms that play a role in this kind of materials. Thus, it was observed that both the initial and final
setting times of the pristine cements increased drastically when the temperature of the environment was reduced from 37 to 7 °C, which is due to the fact that dissolution/precipitation of the α-TCP into CDHA is a thermally activated process [37,38]. This was even more marked in the case of the CPC 20% PLU cement, which can be explained by the concomitant effect of gelling of the hydrogel as the temperature increased.

Interestingly, as shown in Figure 6 c), when the paste of the pristine cement was initially at low temperatures and the setting time was measured at 37 °C, thus simulating physiological conditions, similar values were obtained irrespective of the initial temperature of the paste, which was indicative of a fast equilibration of the temperature of the paste with the environmental temperature. In contrast, longer setting times were recorded for the hydrogel-containing cements (Figure 6d) when the temperature of the paste was below the gelling temperature of the hydrogel. However, it must be stressed that the setting times at 37 °C were within the clinically acceptable range [39], even when the paste was kept at low temperature. The lengthening of the final setting times observed in the poloxamer-containing cements could suggest a slower setting kinetics of α-TCP, similarly to what was observed for CPC pastes prepared with gelatine [40].

3.4 Influence of poloxamer 407 on the setting reaction

As suggested by the setting times, in situ XRD measurements confirmed that the kinetics of transformation in the early stages was slowed by the presence of the hydrogel, as displayed in Figure 7. A closer look at the data sets acquired at early time points shows that the α-TCP peaks actually increased slightly and that hydroxyapatite began to form after 4 h in the case of the pristine cement and 8 hours in the case of CPC 20% PLU. Moreover, it is at this time point that α-TCP began to gradually disappear. This increase in the intensity of the α-TCP peaks has been ascribed to the presence of a film of water on the sample surface at the beginning of the reaction. This film would act by reducing the scale factor, and hence the amount of crystalline
phase, as it reduces the content of solid phase in the surface volume irradiated by the X-ray beam [41].

In spite of the delay observed in the initial stages, the XRD patterns of the cements after 7 days of hardening in Ringer’s solution at 37 °C showed that the incorporation of poloxamer 407 did not hinder hydrolysis of α-TCP and precipitation into CDHA (Figure 8a). The crystalline phase composition of CPC 20% PLU was 97 ± 3% CDHA and 3 ± 3% β-TCP, whereas that of CPC 0% PLU was 98 ± 2% CDHA and 2 ± 2% β-TCP. No statistically significant differences were found between them (p > 0.05). Another important finding was that poloxamer 407 was not detected by ATR-FTIR (Figure 8b) in the cement samples (CPC 20% PLU 7d) allowed to set for 7 days, whereas it was clearly detected in the fresh paste (CPC 20% PLU 0d), as indicated by the C-H stretching band at 2850 cm⁻¹ corresponding to the sp³ carbon hydrogen bond and the C-O stretching band at 1110.7 cm⁻¹ corresponding to the alkoxy bond characteristic of aliphatic ethers, both of which are also clearly visible in the spectrum of pure poloxamer 407 [42]. This suggests a progressive release of poloxamer 407 in the setting medium. However, it should be mentioned that the poloxamer 407 macromers produced during dissolution are chemically stable under these physicochemical conditions since ether and aliphatic alkyl bonds are stable in this range of temperatures (0–37 °C) and pH (7–9). This dissolution phenomenon, which is associated with the amphiphilic nature of poloxamer 407, has a potential use in local drug delivery applications, where it could be used as a carrier for a more efficient controlled release, especially in the case of drugs with different hydrophilicity/lipophilicity [43].

When observed by FE-SEM, set CPC 20% PLU samples exhibited the same microstructure as their CPC 0% PLU counterparts (Figure 9a and b), with both cements comprising an entangled network of nanosized needle-shaped CDHA crystals (100 nm in length and 20 nm in diameter approximately). This entanglement of CDHA crystals is responsible for the mechanical integrity of both cements [44]. Even though no clear differences between the cements’ microstructures
were observed by SEM, the pore entrance size distribution was shifted to larger values in the thermoresponsive cement, as confirmed by MIP (Figure 9c). Thus, while the value for the control cement was centred at around 15 nm, that for the thermoresponsive cement was centred at around 30 nm. In addition, the total open porosity increased from 33.8% to 37.4% upon adding poloxamer 407 to the cement. This can be attributed to the volume created by polymer leaching, in addition to the possibility of a slight swelling of poloxamer 407 [45], which could increase the space between α-TCP particles during setting. These differences in porosity and pore size distribution resulted in a 27.3% decrease in the compressive strength measured in wet specimens when poloxamer 407 was added to the liquid phase. (Figure 9d). In any case, the compressive strength of CPC 20% PLU (28.4 ± 5.1 MPa; L/P ratio = 0.35 g/g) was still higher than the compressive strength of commercial CPCs tested in vitro using similar testing conditions to those of the present study [46].

3.5 Frozen storage of premixed cements and properties upon thawing

The previous results showed that cements containing 20% poloxamer 407, with an L/P of 0.35, presented good cohesion at 37 °C and were fully injectable, with an injection force that could be tuned by controlling the temperature of the paste. This was not the case for the pristine cements. A further step to facilitate the surgical protocol involved exploring the viability of freezing the fresh paste, as a means of avoiding the preparation step in the operating room, which is known to be a cause of inconsistent performance of CPCs and increased risk of infection [47][48].

CPC 20% PLU pastes were introduced in syringes and stored in a frozen state at -80 °C for different periods of time. After storage, syringes filled with the paste were thawed in a water bath at 7 °C for 15 min prior to the injection test. This condition was selected because, at this temperature, the CPC 20% PLU paste presented the lowest injection load (see Figure 2). As shown in Figure 10, the paste remained totally injectable for all storage periods tested (up to
18 months). Although longer storage times were not tested, the paste is expected to be stable for even longer storage periods. No statistically significant differences were found between the injection loads measured after different storage periods ($p < 0.05$), thus indicating that the cement does not react when stored at -80 °C. The cohesion of the pastes after thawing was confirmed by injecting the thawed paste into Ringer’s solution at 37 °C (results not shown). The XRD patterns for the freeze-dried paste immediately after preparation (storage time zero) and after 18 months of frozen storage clearly showed that the α-TCP within the paste did not react over this period (Figure 11). In addition, no statistically significant differences were found between the initial and final setting times or between the compressive strengths of the fresh and thawed cements (Figure 12). Other approaches previously used to prepare premixed CPCs available off-the-shelf generally consist in mixing the cement powder with a non-aqueous biocompatible solvent as liquid phase to prevent its setting during storage [13][49]. Once the cement is implanted, the solvent is exchanged by water contained in the physiological fluids, thus resulting in setting of the cement. However, the disadvantage of this approach is that the onset and setting of the cement is drastically delayed as it depends on the diffusion of water into the implant. Moreover, the final strength of the material is reduced [47]. In contrast, the present findings prove that thermoresponsive cements can be effectively stored at -80 °C and then thawed and used directly in the operating room with no reduction in their injectability and cohesion and without altering the setting parameters. Frozen storage may be especially relevant as regards the possibility of incorporating pharmacologically active molecules that are unstable at room temperature. This opens up possibilities, for example, in terms of combined local pharmacological treatment in oncology with bone augmentation [50] using a premixed, ready-to-use and injectable bone cement.
4 Conclusion

Totally injectable, low liquid-to-powder ratio, self-setting calcium phosphate pastes presenting washout resistance and clinically acceptable setting times and sensitive to temperature have been successfully obtained. Owing to their sequential gelling → setting property, these thermoresponsive cements may be suitable for minimally invasive procedures without the risk of particle leaching or expulsion from the surgical site. This represents an important development in order to improve the safety and efficacy of CPCs.

Furthermore, an effective frozen storage of thermoresponsive premixed cements is possible over long periods of time without affecting the physicochemical properties of the cement. The combination of the self-setting α-TCP with a thermoresponsive poloxamer hydrogel offers a versatile platform for bone regeneration performed by minimally invasive surgery, along with other potential applications such as the possibility to use similar thermoresponsive cements as inks for 3D inkjet printing techniques, owing to their sequential gelling and setting at physiological temperature, which allows biomimetic ceramic scaffolds with tailored properties to be printed in 3D layer by layer.

Acknowledgments

The authors acknowledge the Spanish Government for financial support through MAT2015-65601-R project, co-funded by the EU through European Regional Development Funds, and FPU scholarship of YM. MPG acknowledges the ICREA Academia award by the Generalitat de Catalunya.

References


Figure legends

Figure 1. Generic formula of the A-B-A copolymer molecule of poloxamer. For poloxamer 407 the number of units corresponding to polyethylene oxide blocks is a=101 and the number of units corresponding to the central block of polypropylene oxide is b=56.

Figure 2. a) Representative injection curves recorded for the reference cement at 7 and 20 °C, and for the cement containing 20 wt% poloxamer 407 at different temperatures (0, 7, 12, 15, 18 and 20 °C). L/P was 0.35 g/g in all formulations; b) Average injection loads of pastes CPC 20% PLU made with two polymorphs of tricalcium phosphate of comparable granulometry: α-TCP which is reactive and β-TCP which is not reactive in the tested conditions. Different symbols indicate statistically significant differences between groups (p < 0.05).

Figure 3. Optical images of the cement pastes with and without poloxamer 407 prepared at two different L/P ratios and two poloxamer concentrations after 24 h of contact with Ringer’s solution at 37 °C. In all cases the initial temperature of the paste was 18 °C.

Figure 4. Optical images of the thermoresponsive cement pastes injected in Ringer’s solution at different temperatures between 0 and 37 °C. Tm is the temperature of the Ringer’s solution. In all cases, the initial temperature of the paste was 18 °C.

Figure 5. Optical images of the thermoresponsive cement pastes at different initial temperatures, after injection to Ringer’s solution at 37 °C. Tp is the initial temperature of the paste.

Figure 6. Setting times of the cement pastes measured at different temperatures (7, 20 and 37°C), the pastes being initially at 18°C: a) CPC 0%PLU and b) CPC 20%PLU; setting times of the cement pastes measured at 37°C, where the pastes were initially at different temperatures (0, 7, 18 and 37°C): c) CPC 0%PLU and d) CPC 20%PLU. The L/P was 0.35 g/g in all formulations. Statistical significance of the results was analyzed independently for the initial and final setting
times by two-way ANOVA, considering the type of cement, either CPC 0% PLU or CPC 20% PLU, and the temperature of the paste as independent factors. Different symbols indicate statistically significant differences between groups (p < 0.05).

Figure 7. In situ X-ray diffraction patterns collected over a period of 18h for a) CPC 0%PLU; b) CPC 20%PLU. For clarity purposes the most intense peaks for a-TCP and CDHA are labeled.

Figure 8. a) X-ray diffraction patterns after 7 days of setting of reference cement (CPC 0% PLU) and thermoresponsive cement (CPC 20% PLU). The position of the referenced peaks of CDHA (JCPDS No. 9-432) is indicated by small diamonds; b) ATR-FTIR spectrum of raw poloxamer 407 (Black), reference cement CPC 0% PLU after 7 days setting (red) and thermoresponsive cement CPC 20% PLU fresh paste (0d, green) and after 7 days of setting (7d, blue).

Figure 9. FE-SEM micrographs of the microstructure of fracture surfaces of the cements without a) and with b) Poloxamer 407 after 7 days of setting; c) Pore entrance size distribution determined by mercury intrusion porosimetry and d) compressive strength after 7 days of setting of reference cement (CPC 0%PLU) and thermoresponsive cement (CPC 20% PLU). Black star indicates statistically significant differences between groups (p < 0.05).

Figure 10. a) Representative injection curves recorded for CPC 20% PLU pastes after storage at -80 °C during different periods of time upon thawing at 7 °C; b) Injection load obtained from the aforementioned test. No statistically significant differences in the injection loads between the different freezing times were found (p > 0.05).

Figure 11. X-ray diffraction patterns of the CPC 20% PLU paste, either fresh (0d) or stored for 18 months at -80 °C and thawed, after freeze drying. The position of the α-TCP peaks (JCPDS No. 9-348) is indicated by small triangles.

Figure 12. a) Initial and final setting times obtained for CPC 20% PLU paste after different period of storage at -80 °C and thawed at room temperature; b) Compressive strength of CPC
20 PLU cement after different period of storage at -80 °C, thawing and immersed in Ringer’s solution at 37 °C during 7 days. No statistically significant differences were found between the different freezing times, neither in the setting times nor in the compressive strength (p > 0.005)
Figure 1
Click here to download high resolution image
Figure 2

(a) Load versus Displacement for different CPC and PLU compositions at various temperatures.

(b) Injection load at different temperatures for both α-TCP and β-TCP.

Temperature 0°C, 7°C, 12°C, 15°C, 20°C
Figure 3

CPC 0% PLU

L/P = 0.35 g.g⁻¹

CPC 20% PLU

L/P = 0.65 g.g⁻¹
Figure 4

- $T_m = 0 \, ^\circ C$
- $T_m = 10 \, ^\circ C$
- $T_m = 20 \, ^\circ C$
- $T_m = 25 \, ^\circ C$
- $T_m = 30 \, ^\circ C$
- $T_m = 37 \, ^\circ C$
Figure 5

$T_p = 0 \degree C$

$T_p = 7 \degree C$

$T_p = 18 \degree C$
Figure 8
Figure 11

The diagram shows the X-ray diffraction patterns of two different samples.

- **18 Months**: The blue line represents the sample after 18 months. It shows a variety of peaks at different 2θ values, indicating crystalline structure.

- **0 Days**: The red line represents the sample at 0 days. It also displays a range of peaks, with some peaks being more prominent than others.

The pattern indicates a transformation or change in the sample over time, possibly from a delta form (Δ) to an alpha-TCP form (α-TCP).

The intensity is measured in A.U. ( Arbitrary Units ). The peaks are marked with Δ symbols, indicating the specific 2θ values for each peak.
Figure 12

a) Frozen Time

<table>
<thead>
<tr>
<th>Setting time (min)</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0d</td>
<td>3d</td>
</tr>
<tr>
<td></td>
<td>7d</td>
<td>14d</td>
</tr>
<tr>
<td></td>
<td>21d</td>
<td>49d</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td></td>
</tr>
</tbody>
</table>

b) Compressive strength (Mpa)

<table>
<thead>
<tr>
<th>Compressive strength (Mpa)</th>
<th>0 Day</th>
<th>3 Days</th>
<th>7 Days</th>
<th>14 Days</th>
<th>21 Days</th>
<th>49 Days</th>
<th>18 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
<td>30</td>
<td>25</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>
Graphical Abstract
**Statement of significance**

Calcium phosphate cements are attractive bone substitutes due to their similarity to the bone mineral phase. Although they can be injectable, cohesion and stability of the paste are crucial in terms of performance and safety. A common strategy is the combination with hydrogels. However, this often results in a decrease of viscosity with increasing temperature, which can lead to extravasation and particle leakage from the bone defect. The preferred evolution would be the opposite: a low viscosity would enhance mixing and injection, and an instantaneous increase of viscosity after injection would ensure washout resistance to the blood flow. Here we develop for the first time a calcium phosphate cement exhibiting reverse thermoresponsive properties using a poloxamer featuring inverse thermal gelling.
Click here to download Supplementary Material: V1-CPC20%PLU 7C cut.mp4
Video V2
Click here to download Supplementary Material: V2-CPC20%PLU 20C cut.mp4