Spray-congealed solid lipid microparticles as a new tool for the controlled release of bisphosphonates from a calcium phosphate bone cement

Research paper

Spray-congealed solid lipid microparticles as a new tool for the controlled release of bisphosphonates from a calcium phosphate bone cement

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A B S T R A C T

The aim of this work was to develop an innovative drug delivery system potentially useful for the local delivery of Bisphosphonates to bone tissue. We propose the use of Solid Lipid Microparticles (MPs), up to now mainly used for oral and topical drug delivery, as carrier for bisphosphonates due to the favourable biocompatibility and lower toxicity of the lipids compared with many polymers. The delivery platform consisted of a biomimetic α-tricalcium phosphate-gelatin cement (CPC) enriched with alendronate loaded MPs (MPs-AL) produced by the spray congealing technology. Alendronate direct addition to cement composition is limited since Alendronate is able to sequester calcium from calcium phosphates, thus preventing the setting of the cements. At variance, this approach permitted to load a relatively high amount of the drug on the CPC and allowed the controlled release of the highly water soluble alendronate. A Design of Experiment (DoE) was employed for the screening of the effects of the formulation variables related to the presence of unloaded microparticle (MPs) on the cement most important mechanical properties. Then, MPs loaded with 10% w/w of alendronate were produced using five different carriers (Stearic Acid, Stearilic Alcohol, Cutina HR, Tristearin and Precirol ATO5). All MPs-AL exhibited a spherical shape, encapsulation efficiency higher than 90% and prevalent particle size ranging from 100 to 150 μm. Solid state characterization (DSC, HSM and X-ray powder diffraction) demonstrated that encapsulation of alendronate into MPs did not alter its crystal structure. MPs-AL addition to the cement provoked a modest lengthening of the setting times and of the hardening reaction leading to the complete transformation of α-tricalcium phosphate into calcium-deficient hydroxyapatite, without significantly affect the cement mechanical properties. Moreover, the results of in vitro AL release study performed on cements enriched with MPs-AL showed that the system allows a controlled release of the drug over time.

1. Introduction

Calcium phosphate cements (CPCs) are biocompatibile and bioactive materials, able to activate osteogenesis [1–4]. CPCs involve mixing a solid phase with a liquid phase, which provides a mouldable paste that stiffens during setting and hardening into a solid phase. As a consequence, they are ideal materials for orthopedic implants that can perfectly fit to a bone cavity. Bone healing process is promoted by CPCs resorption, which can occur via direct action of bone cells and/or through chemical dissolution/hydrolysis in the body fluids [5]. The solid phase of CPCs is constituted of one or several calcium phosphates, whereas the liquid phase is often water or a phosphate solution. Since the development of the first CPC formulation [6], a variety of different compositions has been proposed and the role of a number of parameters on the properties of the cements has been investigated [4,7–9]. Brushite, ($CaHPO_4{\cdot}2H_2O$, DCPD), and hydroxyapatite, both in the stoichiometric form ($Ca_{10}(PO_4)_6(OH)_2$, HA) or as calcium-deficient hydroxyapatite (CDHA) are the possible end members of the cementitious reaction [10].

Most apatitic CPCs relies on the hydrolysis of α-tricalcium

Abbreviations: MPs, microspheres; AL, sodium alendronate; MPs-Excipient, microspheres obtained from different excipients; Alc, stearylic alcohol; Aci, stearic acid; Cut, cutina HR; Pre, precirol ATO5; Ste, tristearin; MPs-AL, alendronate-loaded microspheres; MPs-Excipient-AL, alendronate-loaded microspheres obtained from different excipients; CPCs, calcium phosphate cements; CPCs-MPs, calcium phosphate cements enriched by microspheres; CPCs-MPs-AL, calcium phosphate cements enriched by alendronate loaded microspheres; CPCs-excipient-AL, calcium phosphate cements enriched by alendronate-loaded microspheres of a specific excipient

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phosphate (α-TCP) powder that constitutes the main component of the solid phase and yields an apatitic phase similar to the biological one as result of the hardening process in an aqueous environment at 37 °C [11]. The not exhothermic setting reaction of CPCs has several ad-vantages, including the possibility to load biological molecules and drugs. The possibility to use CPCs as drug delivery systems has been tested through combination of the drugs with the solid or liquid phase, or through introduction in a particulate carrier added to cement for-mulation [13]. Antibiotics, anti-inflammatory agents, anti-osteoporotic and anticancer drugs, growth factors and proteins are the main cate-gories of substances which have been included in the composition of CPCs [12–14]. In particular, calcium phosphate cements have been proposed to deliver bisphosphonates (BPs) to the target sites in order to overcome the problem of poor availability of these drugs, as well as to minimize the side effects provoked by their systemic administration [14,15]. BPs are potent drugs for the treatment of pathologies char-acterized by excessive osteoclast mediated bone resorption, such as osteoporosis, Paget's disease and bone metastases, and have also been suggested as potential anticancer agents [16,17]. BPs inclusion in CPCs was obtained through addition to the liquid phase or chemisorption on one of the component of the solid phase [18–21]. The presence of BPs was generally found to lengthen the setting time and decrease com-pressive strength of the cement [18,21–24].

The use of biodegradable microspheres has been proposed to load other substances, such as growth factors and antibiotics, and also to increase the porosity of the CPCs [25–30]. The most used materials for this purpose are poly(DL-lactic-co-glycolic acid) (PLGA) [26–28,30,31] and gelatin [32,33]. In particular, it was shown that encapsulation of vancomycin in PLGA microspheres provided a longer sustained release with respect to loading the drug in the cement powder [31]. It is conceivable to hypothesize that the use of microparticles (MPs) as drug carriers inside CPCs should avoid/limit the lengthening of setting times caused by substances like bisphosphonates and also provide a tailored sustained release. Herein, we investigate the possibility to use solid lipid microparticles as carrier for bisphosphonates due to the favourable biocompatibility and lower toxicity of the lipids compared with many polymers [34]. Solid lipid MPs can be produced using different technologies such as melt dispersion technique, solvent evaporation, hot and cold homogenization, spray drying and spray congealing [34]. In this work, the spray congealing technique has been selected as it does not require the use of organic or aqueous solvents and hence it is environmentally friendly and less time consuming than other methods [35]. In addition, the spray congealing technology avoids the use of surfactants and it is easily scaled-up. The aim of the work is to design and develop an efficient drug delivery system able to load relatively high amount of sodium alendronate into the calcium phosphate ce-ments, while maintaining suitable cement mechanical properties. In particular the delivery platform consists of CPC enriched with Solid Lipid MPs. As numerous formulation parameters related to the MPs can (negatively) influence the CPCs characteristics, we firstly applied a Design of Experiment (DoE) for the evaluation of the effect of types, dimensions and amount of unloaded MPs on the CPCs most important mechanical properties. Then the study was performed using five dif-ferent types of excipients to prepare MPs loaded with alendronate and added to the composition of a biomimetic gelatin-α-TCP cement. The setting and hardening properties of the cements were investigated as a function of the presence of alendronate. In addition, in vitro sodium alendronate release study were performed on AL-loaded Solid Lipid MPs and on cement enriched with AL-loaded MPs.

2. Materials and methods

2.1. Microspheres

2.1.1. Preparation of microparticles (MPs) and of alendronate-loaded microparticles (MPs-AL)

For the production of the microparticles, five different excipients were tested: Stearic Acid, Stearilic Alcohol, Cutina HR (hydrogenated castor oil) and Tristearin were purchased from Farmalabor S.R.L., Italy, while Precirol ATO5 (Glyceryl palmitostearate) was kindly supplied by Gattefossè, France. The microparticles were produced by the spray-congealing process using the wide pneumatic nozzle (WPN), which is an external-mix two-fluid atomiser already described in detail in previous work [36]. For the preparation of the MPs and MPS-AL, the excipient was heated at a temperature of 10 °C above its melting point. Alendronate sodium trihydrate (AL) (ChemOs GmbH Germany), when present, was added to the molten excipient and stirred to obtain a homogeneous suspension which was then loaded into the feeding chamber of the WPN. The batch size was 20 g. Two WPN operating parameters can be set: the pressure of the inlet air and the temperature of the device. In the preliminary studies for the production of MPs, the atomisation was carried out setting the air pressure at 3.5 bar and heating the WPN at three different temperature (50, 60 and 70 °C). Then, MPs-AL were obtained with the air pressure at 3.5 bar and the nozzle temperature at 70 °C. The atomisation of the molten fluid led to the formation of melted droplets which then solidified during the fall in the cooling chamber held at room temperature. The final MPs were collected, size separated and stored in polyethylene closed bottles at 4 ± 2 °C.

2.1.2. MPs characterization

2.1.2.1. Particle size and morphological analysis. The size distribution of MPs was evaluated by sieve analysis, using a vibrating shaker (Octagon Digital, Endecotts, London, UK) and 6 standard sieves (Scientific Instruments s.r.l., Milano, Italy) of 50, 75, 100, 150, 250 and 500 μm.

The MPs were observed by using a scanning electron microscope ESEM Quanta 200 (FEI, Cambridge, UK) at 10.0 kV accelerating voltage without any coating.

2.1.2.2. Determination of drug content. 40 mg of MPs-AL were accurately weighted and added to 10 mL of PBS pH 7.2. The suspensions were heated 10 °C above each excipient melting point to melt the carrier and gently shaken for 60 min. The solution was filtered and the drug content was assayed by a spectrophotometric method [37], that requires the use of a derivatizing agent. Briefly, a working solution of the derivatizing reagent was prepared by dissolving 12.5 mg of anhydrous ortho-phthalaldehyde (OPA, Sigma-Aldrich) in 2 mL of PBS, then 62.5 mL di 2-Mercaptoethanol (ME, Sigma-Aldrich) solution were added and the final volume of 25 mL obtained by adding PBS. This solution was freshly prepared before each experiment. For the calibration curve, proper aliquots of AL standard solution (1mg/mL in PBS) were mixed with 2 mL of derivatizing solution and 0.25 mL of NaOH 5M and the volume filled up to 25 with PBS in order to obtain a concentration of AL ranging from 5 to 100 mg/mL; the absorption was measured at 330 nm (R^2 = 0.9978).

Each formulation was analyzed in duplicate and the mean ± SD was reported. Finally, the encapsulation efficiencies (EE) were calculated as follows:

$$EE\ (\%) = (Wa/Wt) \times 100$$

where Wa is the actual drug content and Wt is the theoretical drug content.

2.1.2.3. MPs-AL dissolution studies. In vitro dissolution tests of MPs-AL were performed in PBS pH 7.2 at a temperature of 37 °C. For each sample 40 mg of MPs were poured into 10 mL of PBS under continuous stirring (50 rpm). As the AL is very soluble in water (10 mg/mL, Sigma

Aldrich data), sink conditions (concentration C < 0.2 solubility Cs) were assured. The solution was withdrawn at pre-determined time intervals up to 4 h using a 0.40 μm poroplast filter in order to separate the microparticles from the buffer and a fresh buffer solution (10 mL) was added. The amount of drug dissolved was analyzed via spectrophotometer at 333 nm (UV2 Spectrometer, Unicam, Cambridge, UK) using the method reported in the *Determination of drug content* section [37]. The dissolution tests were performed at least in triplicate and the mean ± SD was reported.

2.1.2.4. Differential scanning calorimetry (DSC) studies. DSC measurements were performed using a PerkinElmer DSC 6 (PerkinElmer, Beaconsfield, UK). The samples, weighing 8–12 mg, were placed into the DSC under a nitrogen flux (20 mL/min) and heated from 25 °C to 300 °C at a scanning rate of 10 °C/min. For comparison, the same procedure was followed for the raw materials. Each analysis was carried out in duplicate.

2.1.2.5. X-rays diffraction studies. MPs and MPs-Al were packed into recessed silicon slides and subjected to X-ray diffraction analysis by means of a Philips X'Celerator powder diffractometer equipped with a graphite monochromator in the diffracted beam. CuKα radiation was used (40 mA, 40 kV). The spectra were obtained in the 4–40° 2θ range using a 0.067° step and a 3°/min speed.

2.1.2.6. Hot stage microscopy (HSM) analysis. Hot-stage microscopy (HSM) was carried out on hot stage apparatus (Mettler-Toledo S.p.A.) mounted on Nikon Eclipse E400 optical microscope connected to a Nikon Digital Net Camera DN100 for the image acquisition. The samples were heated at 25 °C for 1 min for equilibration and then heated from 25 °C to 20 °C above the melting point of each excipient at a scanning rate of 10 C/min.

2.2. Cements

2.2.1. Preparation of cements (CPCs)

Starting cement powders, made of α-TCP and 15 wt% of gelatin (type A from pig skin, Sigma Aldrich) were obtained as previously described [8], crushed in an electric mortar and sieved (< 40 μm).

Weighted amounts of the starting powders were mixed with DCPD ($CaHPO_4{\cdot}2H_2O$), following the procedure reported in [8], and the cement pastes were obtained inside Teflon moulds after addition of Double Distilled Water (DDW), using a liquid to powder ratio of 0.22 mL/g. Just after mixing CPCs were compacted by using a 4465 Instron dynamometer set at 80 N for 1 min, demoulded and then immersed in Phosphate Buffer (PB), pH = 7.4, at 37 °C for different periods of time up to 21 days.

These cements are used as a control. Some samples containing a suitable amount of Alendronate, dispersed as powder into the starting materials, were also prepared.

2.2.2. Preparation of CPCs containing MPs and MPs-AL

In order to obtain the microspheres-containing CPC, weighted amount of MPs were added to the cement powders before mixing with the liquid phase. Cements containing 1, 5 and 7 wt% of MPs were prepared following the same procedure described above and stored in PBS until 21 days. The cements were labeled CPCs-MPs.

The MPs-AL-containing cements were synthesized by using Alendronate loaded microspheres of selected dimensions, ranging between 100 and 150 μm. The cements were prepared as described above and labeled CPCs-MPs-AL.

2.2.3. Characterization

2.2.3.1. Setting times determination. Initial and final setting times were determined by the Gillmore method (ASTM, American Society for Testing and Materials: C 266-89. Standard test method for time of setting of hydraulic cement pasteby Gillmore needles). Initial and final setting times were determined by the Gillmore method (ASTM, American Society for Testing and Materials: C 266-89. Standard test method for time of setting of hydraulic cement pasteby Gillmore needles). The apparatus consists of two horizontal arms, which carry two weighted steel needles: the needle used to determine initial setting time (t_i) weighs 113 g and is 2.12 mm of diameter, while that used for the determination of final setting time (t_f) weighs 453.6 g and is 1.06 mm of diameter. The values of t_i and t_f are expressed in minutes measured from the beginning of mixing. The paste was considered set when the needle doesn't provoke any visible indentation on the cement surface.

2.2.3.2. Mechanical properties. Mechanical characterization was performed on the cylindrical specimens (6 mm in diameter and 12 mm high) after 1, 3, 7 and 21 days of soaking in PBS. A 4465 Instron testing machine, equipped with a 1 kN load cell, was used. At least six specimens for each incubation time were tested at a crosshead speed of 1 mm/min.

2-ways analysis of variance (ANOVA) was employed to assess statistical significance of the results. The significance was performed with a Bonferroni posttests. The difference was considered statistically significant with a p-value < .05.

2.2.3.3. X-rays powders diffraction. For X-ray diffraction analysis the cements were extracted from PBS, at selected times, and immediately immersed in liquid nitrogen for 10 min, in order to stop the hardening reaction. The samples were then ground in a mortar, packed into recessed silicon slides and subjected to X-ray diffraction analysis by means of a Philips X'Celerator powder diffractometer equipped with a graphite monochromator in the diffracted beam. CuKα radiation was used (40 mA, 40 kV). The spectra were obtained in the 4–45° 2θ range using a 0.067° step and a 3°/min speed.

The relative amount of α-TCP conversion into calcium deficient hydroxyapatite (CDHA) was determined through the measurement of the integrated intensities of the reflections at 25.9_ and 24.3_ of 2θ, corresponding to the 0 0 2 reflection of hydroxyapatite and to the 1 3 1 reflection of α-TCP, respectively. The amount of CDHA in the samples was determined from the ratio of the integrated peak areas expressed as $A_{002}/(A_{002} + A_{131})$, using a calibration curve obtained from weighted mechanical mixtures of hydroxyapatite and α-TCP.

2.2.3.4. Morphological analyses. Morphological investigation of the fractured surfaces of the cement samples was performed using a Philips XL-20 Scanning Electron Microscope. The samples were sputter-coated with gold prior to examination.

2.2.3.5. Alendronate release from CPCs-MPs-AL studies. In vitro dissolution studies were performed for CPCs made with MPs-Cut-AL and MPs-Pre-AL-using PBS pH 7.2 at a temperature of 37 °C. For each test, two weighted cements were immersed into 5 mL of PBS. The solution was withdrawn at pre-determined time intervals up to 21 days and fresh buffer solution (5 mL) was added. The buffer solution was filtered (0.2μm) and its drug content was determined via spectrophotometric analysis at 333 nm (UV2 Spectrometer, Unicam, Cambridge, UK). The dissolution tests were performed at least in triplicate and the mean ± SD was reported.

2.2.3.6. Experimental design. The screening of the effects of the MPs loading inside the CPCs was planned using a Design of Experiments (DoE). In particular, an asymmetrical factorial design was considered to evaluate the influence of three MPs characteristics (called independent variables) on the most important CPCs mechanical properties (dependent variables or experimental responses). For the runs of the DoE, unloaded MPs were employed. Table 1 shows the levels of the three selected independent variables (X_i). As dependent variables, the

Table 1
Experimental domain.

Factor	Associated variable	No. of levels	Levels
MPs percentage	X_1	3	1% 5% 7%
MPs size	X_2	3	50 μm 75–100 μm 100–150 μm
Type of excipient	X_3	5	Stearylic alcohol Stearic acid Tristearin Precirol® ATO5 Cutina® HR

Table 2
Experimental plan.

No. of Experiments	Random order	% MPs	Dimension (μm)	Type of excipient
1	2	1	50	Stearylic alcohol
2	1	5	75–100	
3	16	7	100–150	
4	17	1	50	Stearic acid
5	15	5	75–100	
6	18	7	100–150	
7	6	1	75–100	Tristearin
8	3	5	100–150	
9	5	7	50	
10	8	1	100–150	Precirol ATO5
11	11	5	50	
12	12	7	75–100	
13	7	1	75–100	Cutina HR
14	14	5	100–150	
15	13	7	50	

following CPCs properties were selected: the compressive strength (Y_1), the elastic modulus (Y_2) and the conversion of a-TCP into CDHA (Y_3). Each dependent variable was measured at four different soaking times (1, 3, 7 and 21 days).

The experimental plan (Table 2) was then designed using NEMRODW software [38]. The standard run order of the 15 different experiments was completely randomised to reduce the effect of external (uncontrolled) factors.

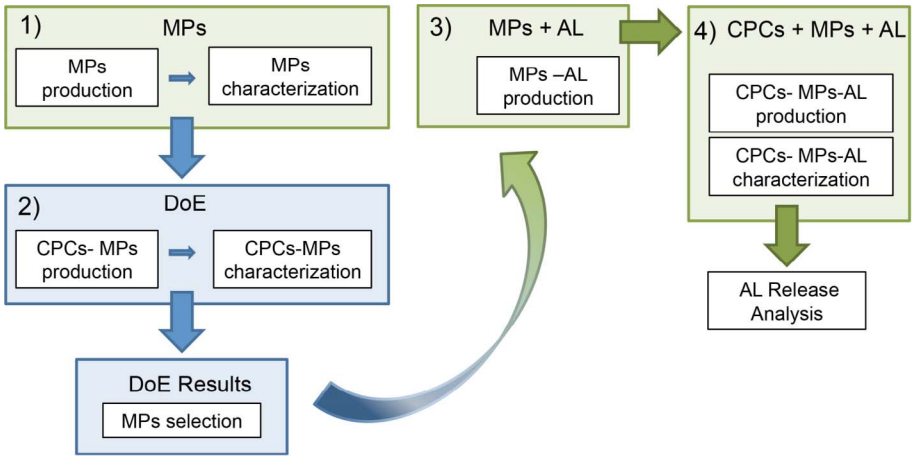
The effect of the variation of the independent variables *Xi* on the experimental responses Y_i was then studied and the data processing was carried out using NEMRODW software.

3. Results and discussion

Factors controlling Calcium Phosphates Bone Cements setting and hardening is negatively influenced by a lot of additives, including those able to improve biological performance of the cement itself. A successful method proposed to passivate additives and inhibit direct contact with the cement is the use of microspheres, usually made of polymeric materials, like PLGA and gelatin. In this work, we explore the possibility to enrich CPCs formulation with Solid Lipid Microparticles, up to now used mainly for oral and topical drug delivery, as vehicles for controlled release of API. In order to verify the capability of this composite system, we chose sodium alendronate as API, since it is known that the incorporation of bisphosphonates into the cement powders is hindered by their high affinity for calcium [18], which prevents the setting of the cements and the hardening reaction. In a previous work [19], we have demonstrated that sodium alendronate can be added as powder to the cement formulation up to a maximum amount of 0.2% wt. The results of our new study demonstrate that the multi-composite system permits to enhance this value, since it avoids the interaction between the drug and the cement and provides a controlled release of the high water soluble drug AL. A representation of the experimental approach followed in this work was illustrated in Scheme 1. Unloaded MPs produced from five different excipient were obtained by the spray congealing technique and fully characterized in terms of dimensional, morphological and structural properties, and added to the cement composition following the Experimental Plan reported in Table 2. On the basis of DoE results the type of excipient, dimensions and amount of MPs to be added to CPCs were selected and a new set of MPs, containing a proper amount of alendronate, was produced and added to the CPCs formulation.

3.1. MPs production

The MPs production was optimized as a function of nozzle temperature, as described in Section 2. All the obtained microspheres appeared round-shaped with a smooth surface and the distribution of their dimensions shows a Gaussian profile with slightly differences as a function of temperature. Fig. 1 reports, as an example, the SEM images recorded from MPs-Cut along with the particle size distribution: the microspheres appeared round-shaped with a smooth surface (figure a, b) with the prevalent particle size ranging from 100 to 150 μm (Fig. 1c). The range from 100 to 150 μm is that prevalent for all the formulations, except for Stearylic Alcohol where the prevalent fraction is that from 75 to 100 μm (see Fig. S1). A complete characterization of the produced MPs was carried out, and the results will be discussed shortly as a comparison with MPs-AL in Section 3.3.



Scheme 1. Schematic of the experimental approach.

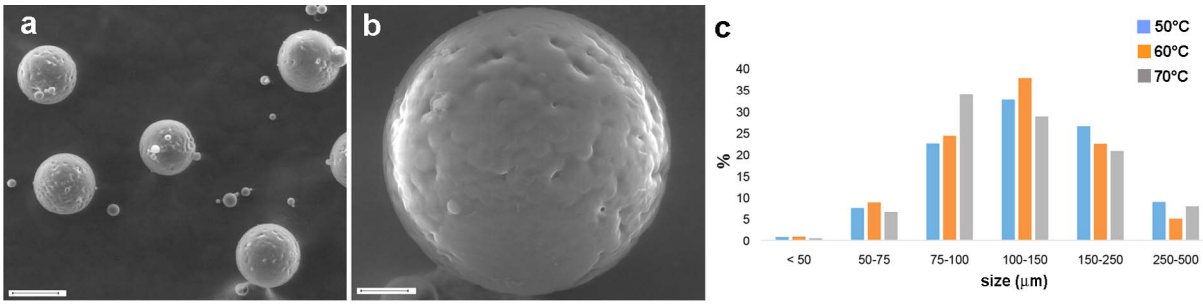


Fig. 1. Characterization of MPs-Cut. (a, b) SEM images of microspheres at two different enlargements. (b) is mixed signals from secondary electrons and back-scattered electrons. Scale bars: a = 100 µm; b = 25 µm. c: Particle size distribution of MPs-Cut. Different colors indicate different nozzle temperature.

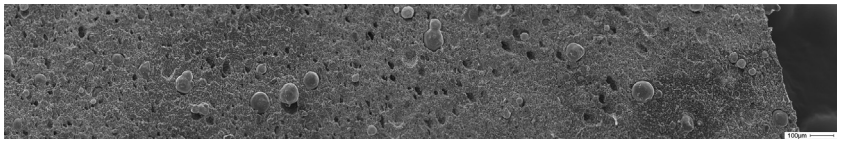


Fig. 2. SEM images of CPC-Cut; the bar represents 100 µm.

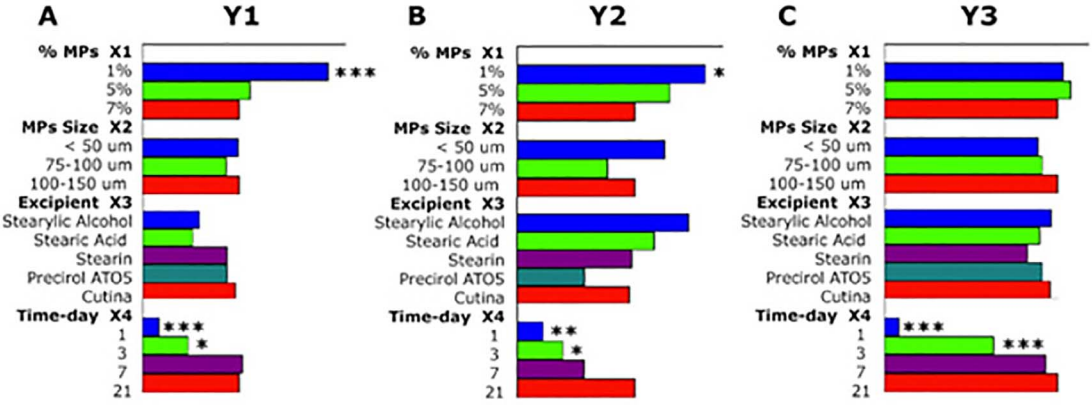


Fig. 3. Total effects of the three factors, each considered at different level, on the three chosen experimental responses ($^{***}p = .001$, $^{**}p = .01$, $^{*}p = .05$). Factor 4 was included to assess the "time effect" (after 1, 3, 7 and 21 days).

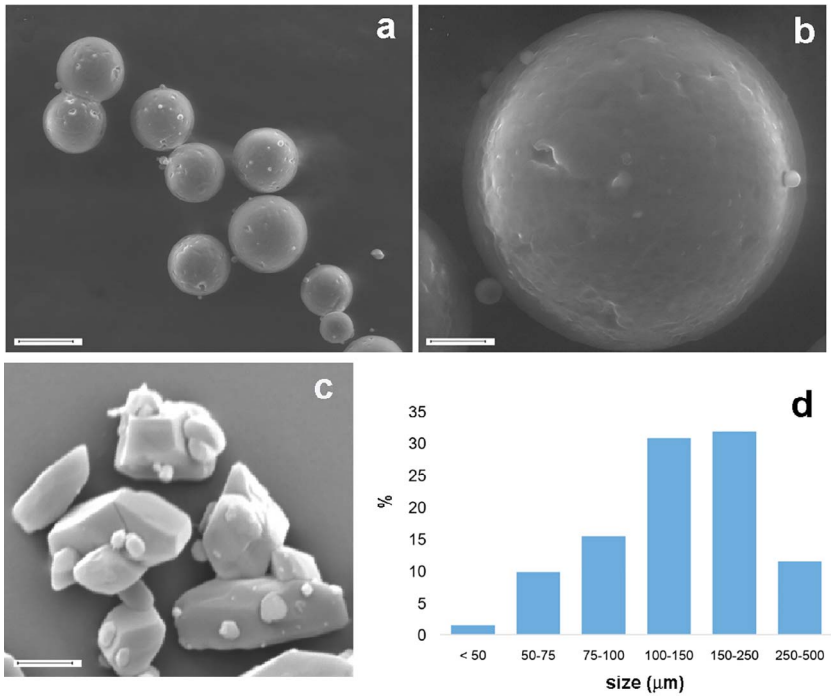


Fig. 4. Characterization of MPs-Cut-AL. SEM images of: (a, b) microspheres at two different enlargements; (c) commercial AL crystals. (b) is mixed signals from secondary electrons and back-scattered electrons. Scale bars: a = 100 µm; b = 25 µm; c = 10 µm. (d) Particle size distribution of MPs-Cut-AL.

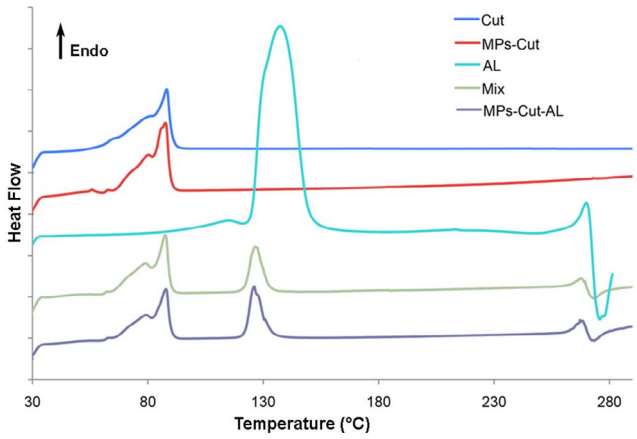


Fig. 5. DSC curves recorded on Cut-based samples.

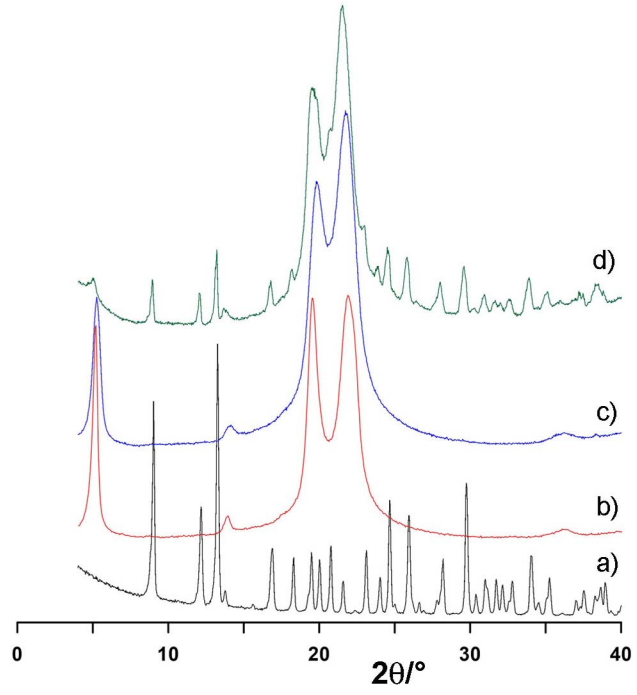


Fig. 6. Powder X-rays diffraction patterns of: (a) AL; (b) Cut; (c) MPs-Cut; (d) MPs-Cut-AL.

Table 3
Initial and final setting times measured for MPs- and MPs-AL cements.

Excipient	CPC-MPs		CPC-MPs-AL	
	t_i (min)	t_f (min)	t_i (min)	t_f (min)
Stearylic alcohol	4 ± 1	10 ± 1	7 ± 1	14 ± 1
Stearic acid	3 ± 1	6 ± 1	6 ± 1	14 ± 1
Tristearin	5 ± 1	8 ± 1	6 ± 1	12 ± 1
Precirol® ATO5	4 ± 1	6 ± 1	6 ± 1	10 ± 1
Cutina® HR	3 ± 1	5 ± 1	4 ± 1	6 ± 1
none	2 ± 1	5 ± 1	nd	nd

3.2. Development of cements by use of experimental design

The screening of the effects of the formulation variables related to the presence of MPs inside the CPCs was planned using a Design of Experiments (DoE) and their influence on the CPCs properties was evaluated. This approach allows to obtain the highest amount of information reducing the number of the experiments and to avoid misleading conclusions that can be drown with a traditional experimental approach (simple trial and error varying one factor at a time) [39]. Three independent variables (X_i) were considered at different levels (Table 1): the percentage of MPs loaded in the CPCs (X_1 at 3 levels), the MPs size (X_2 at 3 levels) and the type of excipients, selected among the lipid used as carriers in the spray congealing technique (X_3 at 5 levels). Three critical CPCs properties were considered as dependent variables (Y_i, experimental responses): the compressive strength (Y_1), the elastic modulus (Y_2) and the conversion of a-TCP into CDHA (Y_3). Each dependent variable was measured at different soaking times (1, 3, 7 and 21 days). Furthermore, the initial and final setting times were recorded and morphological observations of the cements fractured surfaces were performed.

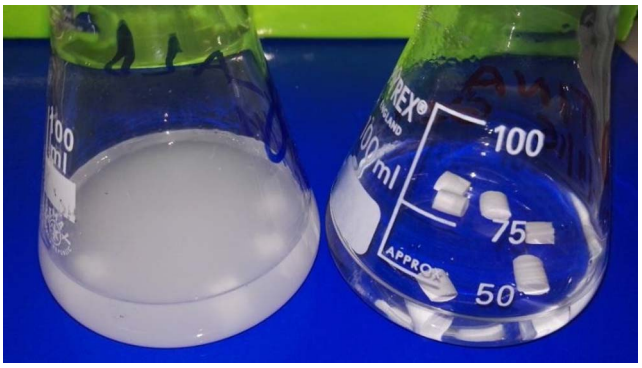


Fig. 7. Effect on cement pastes cohesion of 0.5% of AL directly dispersed into the starting powders (left) and loaded with -MPs-Cut-AL (right).

In order to obtain a cement paste containing well-dispersed microspheres, the excipients were added to the cement powders and mixed in an electric mortar for 60 s before addition of the liquid phase. Fig. 2 reports an image obtained from CPC-Cut: it can be inferred that the microspheres were homogeneously distributed along the whole surface.

Fifteen different cement compositions, corresponding to the random order reported in Table 2, were prepared and analyzed according to the DoE approach. To quantify the "weight" of every level of the independent variables here considered, the experimental data were processed by means of a multilinear regression model defined in terms of an arbitrary reference state. For a better interpretation of the influence of the different variable levels on the CPCs-MPs properties under study (Y_i), a graph-mode representation was used.

In Fig. 3, where the "total weights" graphs are shown, the highest level of each factor is taken as the level unit measure for each single factor. Therefore, all the highest factor levels (red bars) are represented with the same length. This graphical procedure enables the visualization and the simultaneous comparison of level effects on the dependent variables (Y_i). Thus, the factor levels that remarkably influence the final result can be easily identified [40]. In particular, in this study it is important to maximize all the considered experimental responses. For the data analysis, in order to assess the effect of soaking time on the three dependent variables, four different times (1, 3, 7 and 21 days) were considered as levels of a further "experimental" factor.

The results of the influence of X_i on the variable Y_1 (the CPCs-MPs compressive strength) (Fig. 3A) showed that the percentage of MPs (X_1) influenced Y_1: in particular, the 7% of MPs significantly decreased Y_1 respect to 1%. The MPs size (X_2) and the type of excipient (X_3) had not a significant effect on Y_1. As expected, the soaking time had a great influence on the CPCs-MPs compressive strength: the value of Y_1 after 3 days is not significantly different from 1 day, while the difference between 3 and 7 days would seem to be more relevant (even if not statistically significant in this case). This effect is probably due to the complete conversion of a-TCP into CDHA, which take place for all the tested compositions after 7 days. Quite similar trends were observed (Fig. 3B) for the experimental response Y_2 (CPCs-MPs elastic modulus):

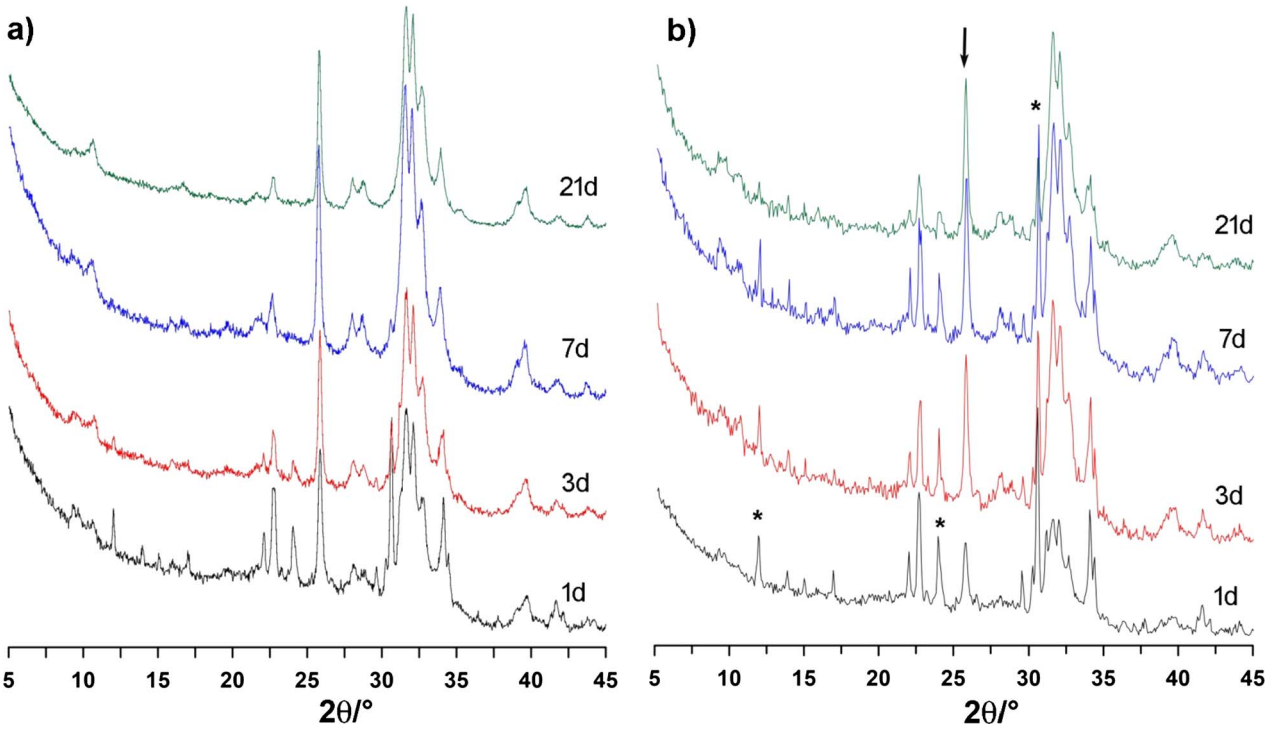


Fig. 8. Powders X-Rays diffraction patterns of: (a) CPC-Cut and (b) CPC-Cut-AL after different times of soaking. The arrow indicates 002 reflection of CDHA, while the asterisks indicate some characteristic reflections of α-TCP.

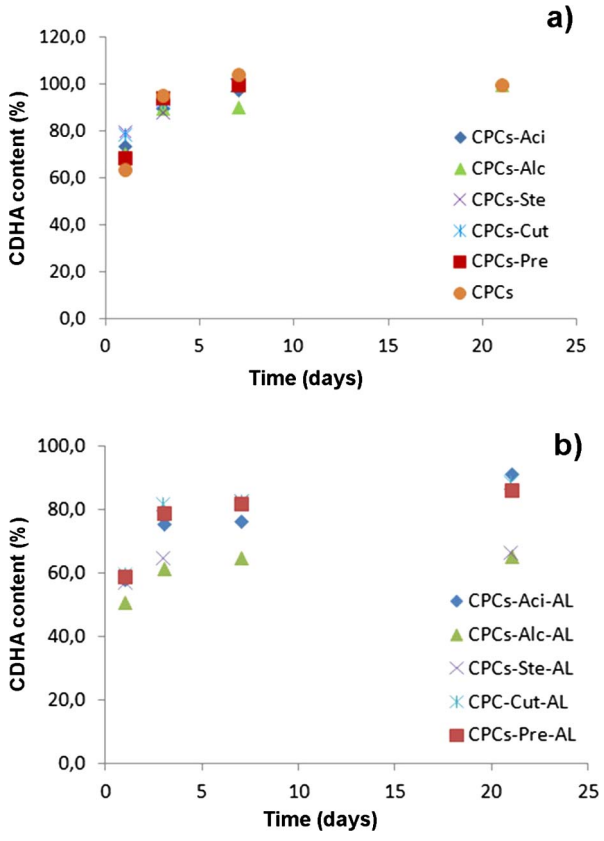


Fig. 9. Relative amount of CDHA content as a function of soaking times, for cements containing MPS (a) and MPsAL (b).

by increasing the amount of MPs from 1% to 7%, Y_2 decreased significantly, while the MPs size was an irrelevant variable. As regards excipient type, Stearylic alcohol would seem to increase the CPCs-MPs elastic modulus in comparison to Precirol ATO5. As regards the experimental response Y_3 (conversion of a-TCP into CDHA), the graphs in Fig. 3C show that the variables percentage of MPs, MPs size and excipient type were not relevant. As expected, the soaking time significantly influenced this dependent variable till 7 days.

The results of the explorative analysis indicated that it is possible to load lipid MPs inside the CPCs maintaining suitable cement mechanical properties. Increasing the amount of empty MPs loaded into the CPCs from 1% to 7% significantly decreased both the CPCs compressive strength and elastic modulus, while the MPs size and the type of excipient did not affect the CPCs mechanical properties.

Therefore, in the subsequent part of the work, a 5% of MPs and of MPs-AL, prepared using all the 5 carriers, were embedded into CPCs formulation and a complete characterization of the cement properties was carried out.

3.3. *Characterization of MPs and MPs-AL*

The incorporation of drug (10% w/w) did not alter the size distribution (see Fig. S2) and the spherical shape of the microparticles, which still appeared round-shaped with a smooth surfaces. As an example of AL loaded microparticles, Fig. 4 reports the SEM images recorded on commercial AL and on MPs-Cut-AL, together with the particle size distribution. SEM analysis of the drug (Fig. 4c) showed that the commercial AL crystals had an irregular shape with length between 5 and 30 μm. The incorporation of drug (10% w/w) did not alter the spherical shape of the microparticles, which still appeared round-shaped with a smooth surfaces (Fig. 4a and b), and the size distribution (Figs. 4d and S2).

In order to detect possible drug or carrier modification due to the spray congealing process or to interaction between AL and the excipients, the microspheres were analyzed by means of DSC and X–ray diffraction. Fig. 5 reports the DSC curves of the raw excipient Cutina HR (Cut), of the corresponding microparticles with and without AL (MPs-Cut-AL and MPs-Cut, respectively) and of Cut and AL physical mixture (Mix). The DSC curve of pure AL presents two endothermic peaks: an

Table 4
Compressive strength (σ_b), of the different samples as a function of soaking time in PB.

Excipient	CPC-MPs σ_b (MPa) (Mean ± SD)				CPC-MPs-AL σ_b (MPa) (Mean ± SD)			
Day	1	3	7	21	1	3	7	21
Stearylic alcohol	7 ± 2	7 ± 1	7 ± 1	9 ± 1	4 ± 1	7 ± 1	7 ± 1	6 ± 1
Stearic acid	7 ± 1	10 ± 2	11 ± 2	12 ± 3	8 ± 1	8 ± 1	9 ± 1	9 ± 1
Tristearin	6 ± 1	8 ± 1	9 ± 2	12 ± 3	7 ± 1	8 ± 1	8 ± 1	6 ± 3
Precirol® ATO5	9 ± 2	9 ± 1	13 ± 3	11 ± 2	6 ± 1	10 ± 1	10 ± 1	10 ± 3
Cutina® HR	8 ± 1	8 ± 2	8 ± 1	12 ± 2	8 ± 1	10 ± 1	10 ± 2	10 ± 2
None	12 ± 2	12 ± 1	9 ± 1	11 ± 3	nd	nd	nd	nd

irregular peak with shoulders centered at 133.38 °C, and a peak at 266 °C, which correspond, respectively, to loss of structural water, and to the melting process of the drug [41]. These peaks are clearly appreciable in the thermogram of MPs-Cut-AL, together with the peaks due to excipient melting.

Comparing the thermograms obtained from MPs-Cut-AL and from the corresponding physical mixture, no differences were evidenced, suggesting that the spray congealing process did not modify the solid state of the drug and of the carrier.

Similar results were obtained for the MPs and MPs-AL prepared with the other excipients, except for tristearin microparticles, as shown in Fig. S3. It is well known that triglycerides can crystallize in at least three polymorphic forms: the least thermodynamically stable α form, the metastable β^I form and the stable β form. DCS curves of tristearin revealed a single endothermic peak at 75.5 °C attributable to the melting of stable β form. The thermogram of AL-Tristearin physical mixture presented the original characteristic peaks of the carrier and of the drug. On the contrary, the DSC curves of both empty and AL-loaded MPs evidenced the endothermic peak of the α form at 60.3 °C, followed by the exothermic peak of the recrystallization of the α form into the β form and by the melting of β form. In addition, DSC of AL-loaded MPs presented the two characteristic endothermic peaks of AL, suggesting the permanence of the drug in its original crystalline form. These results suggested that the rapid cooling from the melt which occurred during the spray congealing process resulted in a transformation from the original tristearin stable form into a different polymorph, according to data reported in literature [42].

Additional information on the solid state structures of the drug and the carriers were obtained through X–ray diffraction analysis. Fig. 6 reports the XRD patterns of the raw excipient (Cutina HR), that showed the characteristic reflections at 5.2, 19.5 and 21.95° of 2θ, together with those of AL (PDF 00-050-2301), MPs-Cut and MPs-Cut-AL. The XRD pattern of Cut compared to that of MPs-Cut doesn't show any differences, revealing no structural changes after the spray congealing process, while the MPs-AL shows the characteristic diffraction peaks of alendronate together with those characteristic of the excipient. XRD spectra were recorded for all the excipients and reported in Fig. S4. No differences were found for all the excipient, except for Tristearin which, according to obtained DSC profiles, clearly shows a different diffraction pattern before and after spray congealing, due to a different polymorph formation.

In addition, the HSM analyses on the MPs-AL obtained from all the excipients were performed and reported in S5. In all the samples, crystals of AL were clearly evidenced after the fusion of the low-melting excipients, confirming the permanence of the drug in its crystalline structure after the spray congealing process.

3.4. CPCs-MPs-AL characterization

As the MPs sizes were not relevant for the CPCs properties, the MPs dimensions were selected in the range 100–150 μm, which was the prevalent fraction for the majority of the excipients (see Fig. S2). The amount of AL loaded into MPs-AL was 10%, which means that AL content in the cements corresponds to 2.5-fold the maximum amount previously inserted in CPCs [19].

The analysis of the drug content of the MPs evidenced that, independently from the excipient, the actual AL contents were close to the theoretical values (10% w/w) for all the formulations, allowing to obtain encapsulation efficiency (EE) values higher than 90%. These results are consistent with those reported in previous studies [35,36], confirming the usefulness of the spray congealing as technology to efficiently incorporate drugs into the matrix of MPs.

Two different controls were used: CPCs and CPCs-MPs (100–150 μm, 5% w/w). Table 3 reports the initial and final setting times for all the MPs- and MPs-AL cements, and for the reference samples.

We have previously demonstrated [18,19] that the presence of bisphosphonates increases the setting times of the cements and that AL contents greater than 0.2% (w/w) prevent setting. In the present study, the setting times of CPC-MPs are only slightly different with respect to CPC (Table 3), thus evidencing that the addition of Solid Lipid Microparticles doesn't interfere with the setting process. When cements are loaded with AL-containing microparticles, the setting times are still comprised into the interval of clinical interest. However, the values are comparable to those of the CP-MPs when Precirol And Cutine are used as excipients, whereas longer setting times are observed for Stearylic alcohol, Stearic Acid and Trystearine. This result suggests that the approach used here is successful and allows the incorporation of a relatively high amount of Sodium Alendronate. In fact, adding the same amount of AL as that loaded into MPs directly to the starting cement powders, prevents setting and cohesion of the paste, as showed in Fig. 7.

The X-ray diffraction patterns of the cements collected after different soaking times in PB are reported in Fig. 8 and showed the reflections due to α-TCP together to those characteristic of poor crystalline hydroxyapatite (CDHA): some of the most representative reflections of both phases are indicated.

The diffraction patterns recorded on CPC-Cut (Fig. 8a) and on CPC-Cut-AL (Fig. 8b), show that the conversion of the starting powders into CDHA was complete after seven days of soaking for the cement containing MPs, whereas the presence of Alendronate, loaded into the MPs, delayed the conversion and a small amount of a-TCP was still detectable after 21 days. A similar trend was observed for all the formulations. The extent of conversion of α-TCP into CDHA as a function of soaking time has been evaluated from the powder X-ray diffraction patterns using the calibration curve obtained through the analysis of the diffraction patterns of weighted mechanical mixtures of hydroxyapatite and α-TCP, as reported in Section 2. The results plotted in Fig. 9 put into evidence that the conversion of α-TCP is delayed in the MPs-AL containing cements and proceed to a maximum extent of about 90% after 21 days of soaking when Pre, Cut and Aci are employed as excipients.

The values of compressive strength, σ_b, of the cements at different

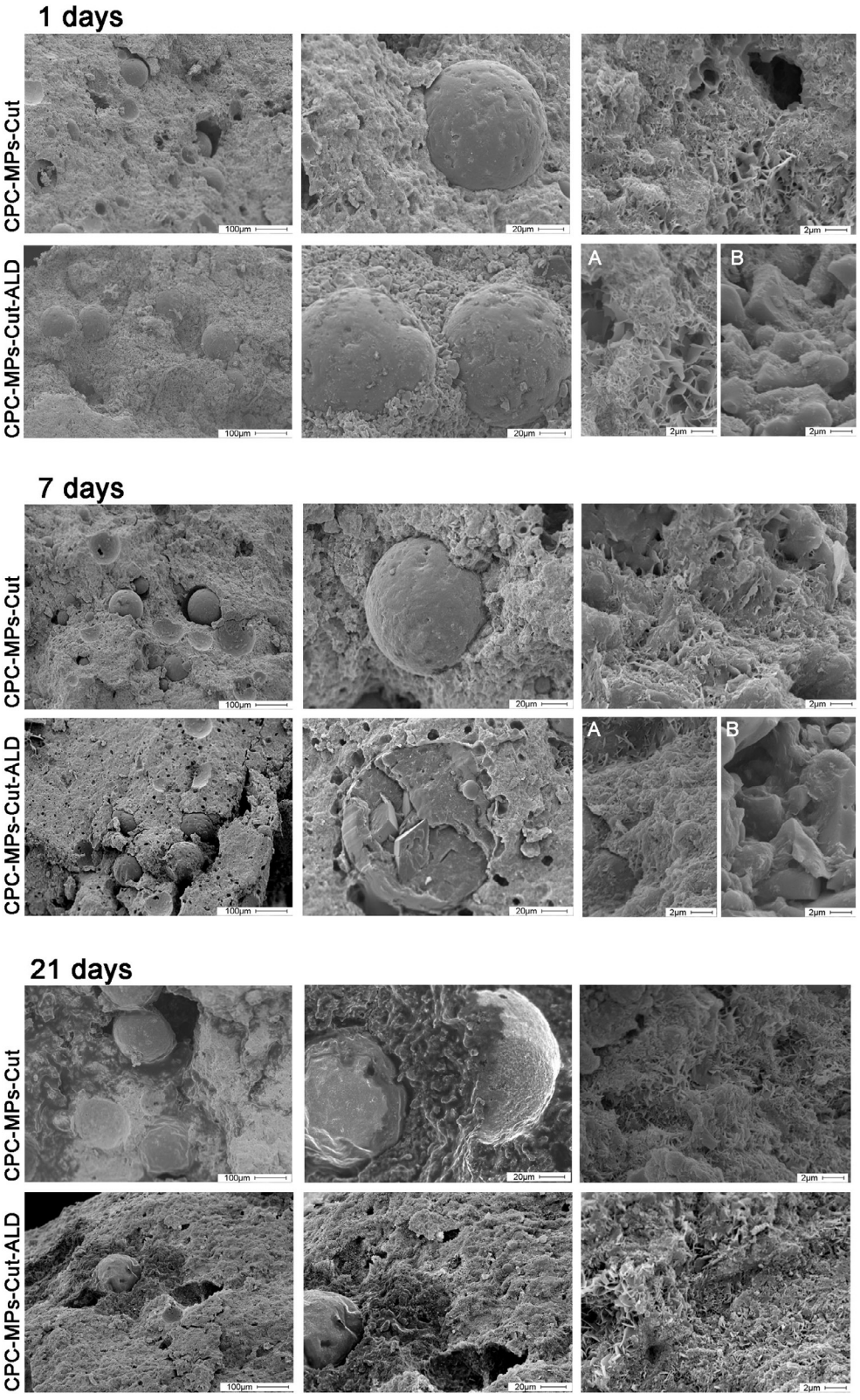


Fig. 10. Scanning electron microscopy performed on fractured surfaces of CPCs samples containing MPs-Cut and MPs-Cut-Al after different times of soaking.

soaking times up to 21 days are reported in Table 4. The values recorded on the CPCs-MPs are significantly different ($p < .05$) compared to CPC for soaking times ranging from 1 to 3 days, whereas did not differ significantly from the reference samples ($p > 0.05$) for longer times. The presence of AL-containing MPs did not alter the mechanical properties of CPC-Cut_AL and CPC-Pre-AL even after 21 days of soaking, while the σ_b values obtained from cements loaded with AL-containing MPs of Aci, Alc and Ste evidenced a worsening of the mechanical properties.

The fractured surfaces of CPCs-Cut and CPCs-Cut-AL after different times of soaking were reported in Fig. 10. The MPs are clearly embedded into the cement matrix and keep unchanged their rounded shape up to seven days of soaking. After this time, the MPs gradually lose their morphology.

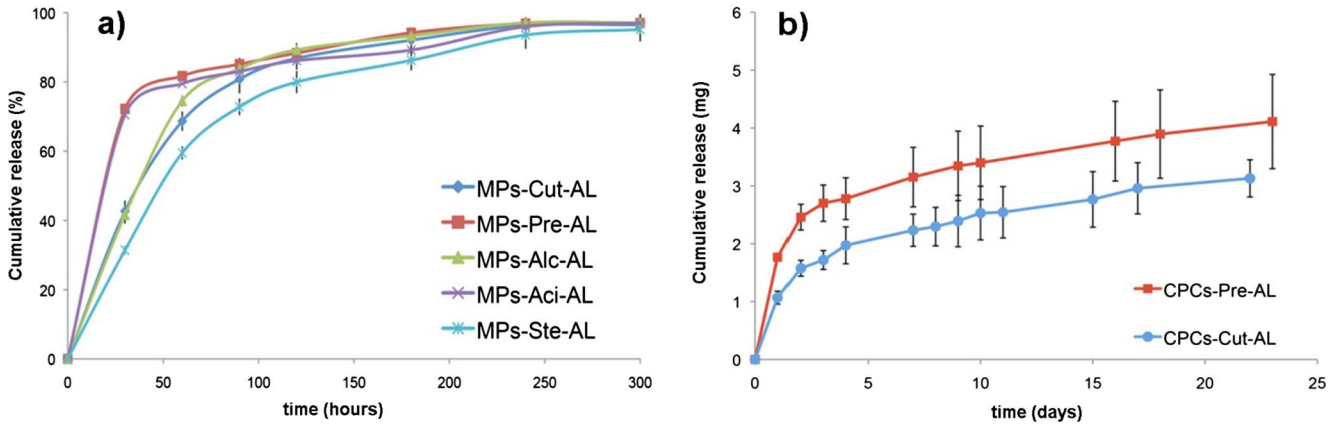


Fig. 11. Cumulative Alendronate release as a function of time from: (a) MPs; (b) CPCs-Cut-AL and CPCs-Pre-AL. Each value is the mean of three determination and is reported with its standard deviation.

The alendronate released strongly influenced the hardening of the cement near to the MPs: as an example, micrographs A and B show an enlargement of the microstructure of the inorganic phase just around the particles microparticles for CPCs-Cut-AL. The different morphologies indicate the simultaneous presence of in situ formed CDHA (A) and of residual α-TCP (B) up to seven days, whereas the presence of α-TCP is not appreciable in the images from CPS-Cut, in agreement with the results obtained from X-rays diffraction patterns.

3.5. Determination of alendronate release

The last step was the evaluation of AL release behaviour in PBS at pH 7.2 for the microparticles and the cements. The pure AL completely dissolved within few minutes, according to its great water solubility (dissolution profile not reported). The results (Fig. 11a) showed that the drug encapsulation within the microparticles allowed to control the release, due to the hydrophobicity of the excipients selected for the preparation of the systems. The type of carrier slightly affected the drug release profiles only in the initial part (90 min): AL released from tristearin (most lipophilic carrier) MPs was slower than from others MPs. All the AL-MPs released more than 90% of the drug within 4 h. Precirol ATO5 or Cutina HR microparticles, having slightly different release profiles, were then embedded into the cements to obtain a multi-composite system. As expected, the release profiles of AL from the two systems (Fig. 11b) were slower than that obtained from the MPs, evidencing that both systems allowed to obtain a controlled release of the drug at least for 21 days.

4. Conclusion

The results of this study demonstrate that Solid Lipid Microparticles (MPs) of different sizes produced by Spray Congealing technology can be added into cement formulations up to 7% wt without worsening of the setting properties of the final materials. Furthermore, the capability of MPs as carriers for controlled delivery of API was successfully proven by using a Bisphosphonate, namely Alendronate, as a model drug. Alendronate-containing CPCs could be of great interest for the delivery of this therapeutic agent directly into the bone environment, in order to minimize the numerous adverse side effects of bisphosphonates systemic administration. Until now, only low amount of AL has been loaded into CPCs formulations, because of its ability to sequester calcium from calcium phosphates, thus preventing the setting reaction of bone cements. [43]. The approach followed in this study, that implies the incorporation of the drug into MPs, hinders its direct contact with the cement and allows to achieve an alendronate content 2.5-fold greater than that previously reported for a cement of similar composition [18,19], without provoking significant delay of the cementitious reaction. The release of the highly water soluble drug from the MPs is further controlled by their incorporation into CPC and as, a consequence, a sustained release of the drug is provided. The obtained results suggest the possibility of using Solid Lipid Microparticles embedded in cement as drug delivery system for the local administration of drugs to bone tissues.

In particular, the best results, in term of setting times and mechanical properties, were obtained using Precirol and Cutine as eccipients. The results of this preliminary study could be improved by loading higher AL content and the composite system tailored for the local administration of a variety of drugs (i.e. antibiotics, anti-inflammatory agents and anticancer drugs) to bone tissues. Furthermore, biological properties of some selected composition are under evaluation.

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Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2017.10.002.

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