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Research paper

Bioactivity of bone resorptive factor loaded on osteoconductive matrices: Stability post-dehydration

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ABSTRACT

Since calcium phosphate cements were proposed two decades ago, extensive research has been realized to develop and improve their properties. They have proved their efficiency as bone graft substitutes and their ability to incorporate and release drugs. However, to date, all 'resorbable' osteoconductive synthetic biomaterials are in fact simply soluble. In order to investigate a synthetic material capable of inducing osteoclast remodelling post-implantation, a formulation of calcium phosphate cement loaded with a pro-resorptive cytokine (RANKL) was studied. Many prior release studies on calcium phosphates did not confirm that the matrix had no detrimental effect on the molecule to be released during storage prior to use or that bioactivity was maintained during storage. In this report, the stability of our protein was tested after loading onto the cement, and various regimens to improve stability were compared. The presence of trehalose was shown to stabilize the bioactivity of RANKL adsorbed to brushite cement. The reduction of both moisture and oxygen in the storage vessel improved osteoclastogenic potential of the matrix compared with that stored in ambient atmosphere and temperature. No loss in activity was observed over the study period for the loaded matrix stored in dry nitrogen.

1. Introduction

Calcium phosphate-based synthetic bone grafts are widely used in orthopaedic and maxillofacial surgeries due to their osteoconductive properties. They are readily available, reproducible, and find many applications where they can substitute for autograft and allograft [1]. As people's life expectancy and levels of activity increase, the prevalence of musculoskeletal disorders (e.g. osteoporosis and osteoarthritis) also increases. Since these calcium phosphate biomaterials are porous and biodegradable *in vivo*, their combination with drugs and bioactive molecules may provide new drug delivery systems for the next generation therapies [2].

Calcium phosphate cements (CPCs) are characterized by their injectability, low-temperature self-setting, high and interconnected microporosity and osteoconductivity. These properties allow bioactive compounds to be incorporated into CPC matrices to create diffusion-controlled release devices. In this regard, association of CPCs with antibiotics is commonly used to prevent and to treat musculoskeletal infection [3,4]. Moreover, different growth factors and pharmacological compounds have been successfully combined with CPCs and were shown to initiate biological response [5].

Most CPCs undergo an initial cell-mediated process of degradation after implantation, but osteoconductive materials that are considered 'resorbable', such as monetite, brushite and calcium sulphate, are in fact simply soluble, whereas bone autograft is actively remodelled by osteoclasts.

Many prior release studies on calcium phosphates did not confirm that the matrix had no detrimental effect on the molecule to be released during storage prior to use or that bioactivity was maintained during storage. However, many biological and physico-chemical parameters, such as temperature, pH, and denaturant agents, may influence the stability of proteins – matrix formulations, and the effect of storage conditions on bioactivity of the formulation is rarely assessed.

Generally, bioactive proteins need to be stored at low-temperature (at or below 4 °C) to prevent denaturation. There are a variety of strategies to preserve protein activity during dehydration which typically involve the use of sugars that do not crystallise. Trehalose is such a sugar that is accumulated at high concentrations by many organisms capable of surviving complete dehydration [6] and may be used to preserve the native structure of proteins [7]. Other parameters that may affect the stability of the proteins are time, temperature, light exposure and composition of ambient atmosphere.

Having demonstrated the production of an osteoclastogenic bioceramic matrix [8], this study focuses on strategies to allow

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long-term storage of a dehydrated RANKL-loaded brushite cement formulation. The stability of the RANKL–CPC formulation was assessed following trehalose addition, exposure to light, and the effects of temperature, oxygen and humidity were investigated.

2. Material and methods

2.1. Preparation of calcium phosphate cement matrix

2.1.1. Brushite cement

Brushite cement powder was prepared from equimolar amounts of calcium phosphate monohydrate (Mallinckrodt Baker, Germany) and β-TCP as described previously [9]. The resulting powder was mixed with 0.8 M citric acid solution with a powder/liquid ratio 3.5 g/ml. The cement setting reaction is shown in Eq. (1):

$$Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 \cdot H_2O + 7H_2O \rightarrow 4CaHPO_4 \cdot 2H_2O. \quad (1)$$

For cell culture experiments, brushite cement was set at room temperature in cylindrical molds to form cylinders of 3 mm diameter and 3 mm height. The phase purity, density and specific surface area of set brushite cement were determined by X-ray diffraction by using a Siemens D5005 diffractometer (Siemens, Karlsruhe, Germany) with monochromated Cu Kα radiation, by using a helium pycnometer (AccPyc1330®, Micromeritics) and the Brunauer–Emmett–Teller (BET) method with helium adsorption–desorption (Tristar3000®, Micromeritics), respectively.

2.2. Incorporation of RANKL and RANKL–trehalose solutions onto cement matrix and storage conditions

A combination of a freshly reconstituted RANKL solution (50 μg/ml) and a D-(+)-trehalose (Sigma–Aldrich, USA) was prepared by adding dehydrated trehalose powder to 800 ng of RANKL solution (1.8 mg in 16 μl of RANKL protein solution) to a final concentration of trehalose of 300 mM. Then RANKL and RANKL–trehalose solutions were adsorbed onto the surface of dried set calcium phosphate cement and were stored at room temperature either for a short time period of 30 min or for 1 day, 3 weeks or 5 weeks.

2.2.1. Storage conditions for the long adsorption period

To test the influence of different storage parameters on the stability of RANKL–trehalose solution loaded onto brushite cement cylinders, formulations were stored at various times points at two different temperatures (at 4 °C and at −20 °C), and the effect of light exposure, humidity and oxygen were assessed. Samples were protected from light with aluminium foil and both atmosphere humidity and oxygen contents were depleted by the presence of silicate gel beads and addition of nitrogen gas (Nitrogen prepurified 99.99% with O_2 < 5 ppm and H_20 < 3 ppm, MEGS Specialty Gases, Inc., Montreal, Canada) in the storage container, respectively. These specific storage conditions were compared to the ambient storage conditions defined by the following parameters: a room temperature (RT) of 22 ± 3 °C, 10 ± 2 h of daylight exposure per day and atmospheric conditions (air is approximately 78% nitrogen, 21% oxygen, 1% of other gasses in volume and humidity <7%).

2.3. RAW 264.7 monocyte cell culture and osteoclastogenic assay

To test the biological activity in different formulations, we used an osteoclastogenic assay based on the formation of multinucleated TRAP positive cells from RAW 264.7 monocyte cell cultured for 7 days in the presence of RANKL [8].

2.3.1. RAW 264.7 cell culture and osteoclastogenic differentiation

The murine monocyte cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Rockville, USA). RAW 264.7 cells were cultured in 25 cm^2 tissue culture flasks in DMEM culture medium with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% sodium bicarbonate at 37 °C in a humidified atmosphere containing 5% CO_2 in air. After 24–48 h of culture, RAW 264.7 cells were detached using a cell scraper, and were seeded into 24-well plates at a density of 2.5×10^5 cells/cm^2.

The different RANKL–brushite formulations were added to the monocyte cell culture at day 1 and the medium was changed at days 1, 3, and 5. In control cultures, osteoclastogenesis was induced by the addition of freshly reconstituted soluble RANKL (50 ng/ml) to fresh medium at days 1, 3 and 5. RAW 264.7 cells cultured without RANKL or biomaterial addition were used as the negative control.

On day 7, cells were fixed using 4% paraformaldehyde for 15 min, washed with phosphate buffered saline (PBS) and stained for osteoclast marker Tartrate-resistant acid phosphatase (TRAP). TRAP staining solution (4% solution of 2.5 M acetate buffer (pH 5.2), 12.5 mg/ml naphthol AS-BI phosphoric acid, 0.67 M tartrate buffer (pH 5.2), and 15 mg of fast Garnet salt) was freshly prepared and filtered before use. The cultured cells were stained for 10–20 min at 37 °C and the numbers of multinucleated TRAP positive cells were assessed using a light microscope (Eclipse TS100, Nikon, USA).

2.3.2. Time stability of RANKL and RANKL–trehalose-coated brushite cement

Cylinders of brushite cement coated either with 800 ng of RANKL or with a mixture of 800 ng RANKL and 300 mM trehalose were stored for either 30 min or 1 day, and were used during four consecutive 7-day cell cultures for a total of 28 days. RAW 264.7 cells were plated at a density of 2.5×10^5 cells/cm^2 with 1 ml of medium and the matrices were added to the cell culture at day 1. Cells were cultured for 7 days at 37 °C and the medium was changed at days 1, 3, and 5. At the end of this culture period, the number of TRAP positive cells was assessed, and the brushite cement cylinders were transferred to freshly plated monocyte cell cultures. This process was repeated four times.

2.4. Statistical analysis

All data were expressed as mean ± standard deviation or standard error of the mean. Statistical difference was evaluated by analysis of variance (ANOVA) with Fisher's probability least significant difference (PLSD) post-hoc test and was considered to be significant at $p < 0.05$.

3. Results

3.1. Brushite cement characterization

SEM micrographs of brushite cement cylinder (approximately 40 mg, 3 mm diameter and 3 mm height) showed a dense surface with plates and blocks of approximately 1–5 μm and an agglomeration of small particles (<1–1 μm) (Fig. 1a – i). At high magnification, a surface composed of micropores <1 μm (Fig. 1a – ii) was apparent. Adsorption of either RANKL solution (50 μg/ml, Fig. 1a – iii and iv) or trehalose solution (300 mM, Fig. 1a – v and vi) alone or in combination (Fig. 1a – vii and viii) did not induce any noticeable modification onto the surface of the set brushite cement. The diffraction pattern of the set brushite cement is shown in Fig. 1b and it corresponded well with the Inter-

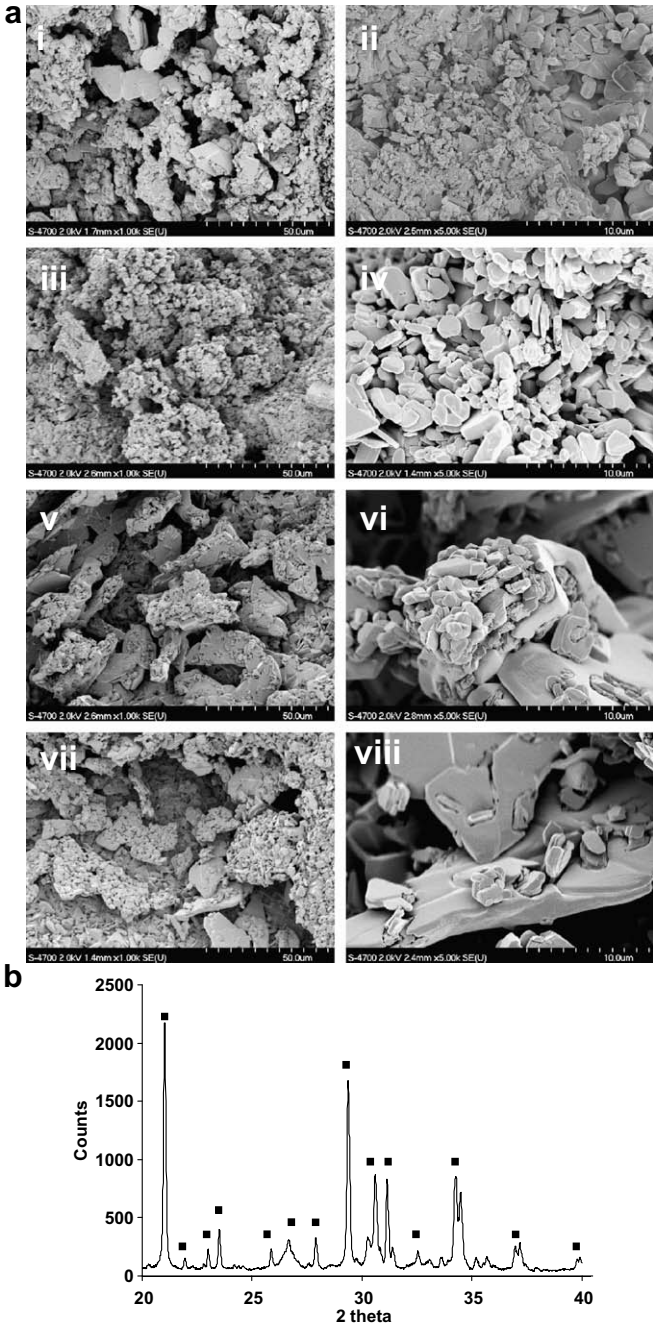

Fig. 1. Characteristics of the brushite calcium phosphate cement. SEM photographs of the set brushite cement cylinders (3 mm diameter, 3 mm in high) showing the micro surface of unloaded-cement (i and ii), RANKL-loaded cement (iii and iv), trehalose-loaded cement (v and vi) and RANKL–trehalose-loaded cement (vii and viii). Micrographs of brushite cement surface were captured at both low (1000×, i, iii, v, and vii) and high (5000×, ii, iv, vi, and viii) magnifications. (b) XRD patterns of the calcium phosphate brushite cement. Black squares indicate brushite peaks.

national Centre for Diffraction Data (ICDD) pattern. The set brushite cement showed an apparent density of 1.37 ± 0.02 g/cm^3 with a specific surface area of 5.57 ± 0.01 m^2/g and a total porosity of 41%.

3.2. Effects of brushite cement and trehalose on osteoclastogenesis

The effects of brushite cement and trehalose on the morphology and number of osteoclast formed after 7 days of RAW 264.7 cell culture are displayed in Fig. 2. Similar numbers of multinucleated TRAP positive osteoclastic cells were formed in monocyte cultures treated with soluble RANKL (50 ng/ml) or maintained in the presence of RANKL-coated brushite cement (Fig. 2b and d). Addition of trehalose (300 mM) to the culture medium (Figs. 2c and e) or to the coated RANKL solution (Fig. 2f) did not affect

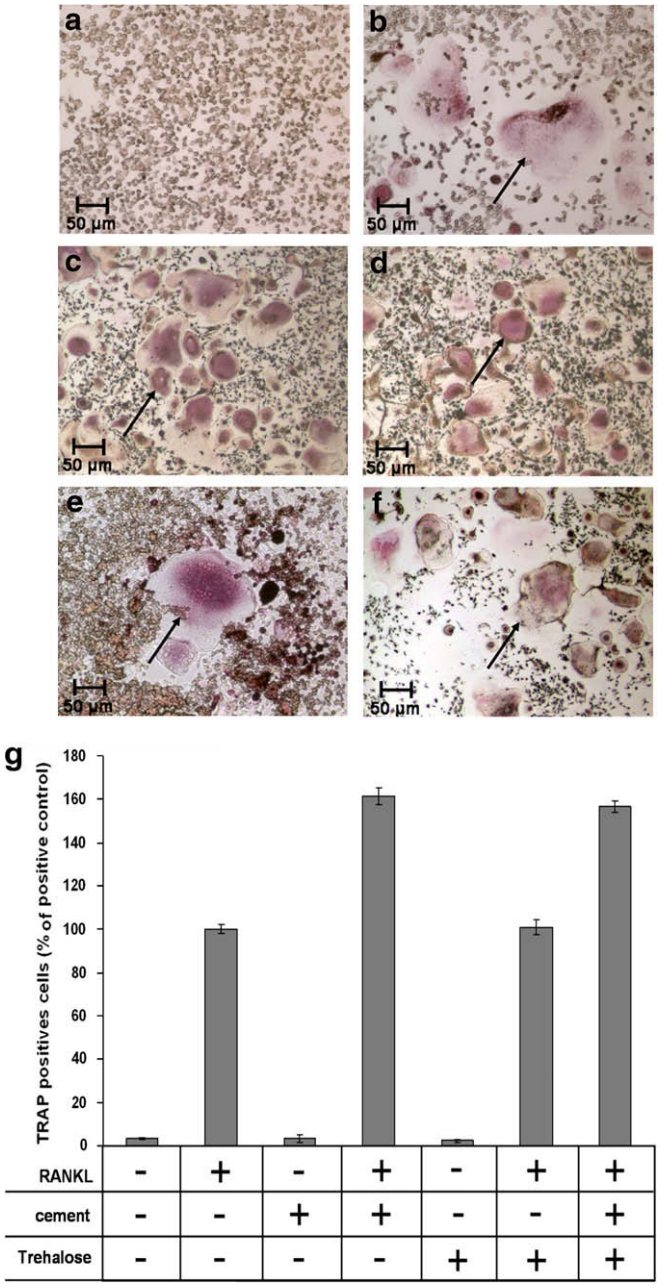

Fig. 2. Characteristics of osteoclastogenesis after 7 days of RAW 264.7 cell culture in the presence of calcium phosphate cement, RANKL and trehalose. After 7 days of culture, cells were fixed with a 4% paraformaldehyde solution and pictures were obtained by using light microscopy with bright field after TRAP staining. RAW 264.7 cells were observed in (a) untreated cell culture (medium alone) and osteoclast formation was induced either in (b) RANKL (50 ng/ml)-treated cell culture, (c) RANKL and trehalose (300 mM)-treated cell culture solution in the medium, (d) the presence of RANKL-coated brushite cement cylinder, (e) the presence of RANKL-loaded cement and trehalose or (f) in the presence of RANKL/trehalose-loaded material. (g) Percentage of TRAP positives cells was assessed for each cell culture condition and normalized to the positive control (RANKL +/cement −/trehalose −). (RANKL +/cement +/trehalose ±) conditions indicate that RANKL solution with or without addition of trehalose was coated onto brushite cylinder. Data are mean ± SEM, $n = 3$ independent experiments.

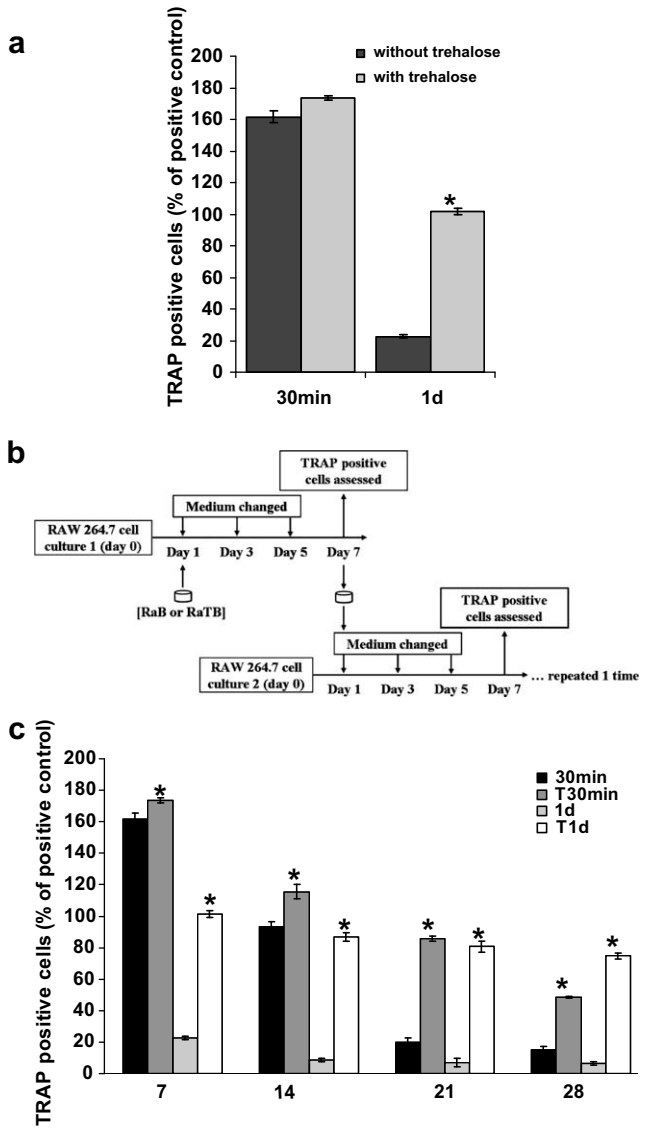

Fig. 3. Evaluation of the stability of RANKL solution coated onto brushite cement with or without addition of trehalose. (a) Percentage of TRAP positive cells formed after 7 days of RAW 264.7 cell culture in the presence of matrices loaded with either RANKL or RANKL–trehalose solution, and normalized to the positive control (culture medium + freshly reconstituted RANKL). These two formulations were stored at ambient conditions for either 30 min or 1 day (1d). Data are mean ± SD, $n = 3$ replicates. Comparison of the osteoclastogenic activities of RANKL–brushite or RANKL–trehalose–brushite (T) cement during 28 days of culture. Matrices were stored at ambient conditions for either 30 min or 1 day. (b) Representation of the evaluation process to determine the stability of the different formulations during four successive 7 days monocyte cell culture. (c) Percentage of TRAP positive cells normalized to the positive control (culture medium + freshly reconstituted RANKL) and formed after four successive 7 days culture periods of RAW 264.7 cell in presence of the different matrices. Data are mean ± SD, $n = 3$ replicates. The differences were evaluated by analysis of variance (ANOVA) with Fisher's probability least significant difference post-hoc test and were considered to be significant at $p < 0.05$.

either positively or negatively the osteoclastogenic process induced by soluble RANKL or RANKL-coated brushite material (Fig. 2g).

3.3. Effects of trehalose addition on RANKL/RANKL–brushite formulation stability

Stability of RANKL solution coated onto brushite cement and effects of trehalose addition to the coated RANKL solution were evaluated.

Results for the stability of RANKL (800 ng) coated onto the cement cylinders and the effect of trehalose addition (300 mM) to the coated RANKL formulation are displayed in Fig. 3a. After 7 days of culture, 800 ng of RANKL-coated onto the cement cylinders and stored for 30 min in ambient conditions induced differentiation of 162 ± 4% of the number of osteoclasts in the positive control (standard osteoclast differentiation protocol [10]). In contrast, biological activity of RANKL-coated on cement cylinder and stored for 1 day in ambient conditions prior to use only induced 23 ± 1% of the number of osteoclasts in the positive control. The additional presence of trehalose significantly increased the bioactivity of the RANKL after 1 day's storage to 101 ± 2% compared with the positive control.

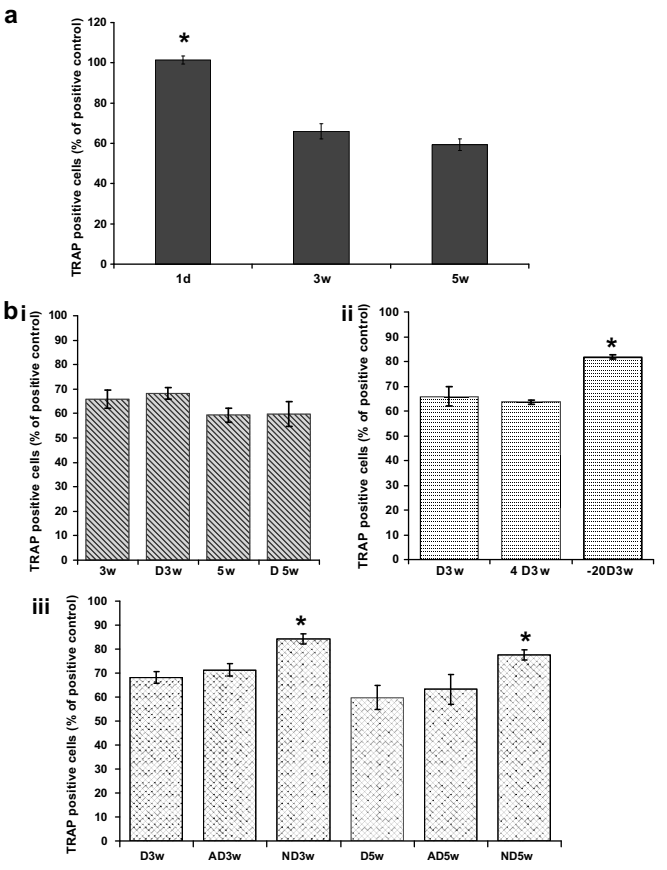

Fig. 4. Evaluation of the stability of RANKL–trehalose-coated material under different storage conditions. Cylinders of brushite cement loaded with RANKL–trehalose were stored for various time points (1 day, 3 weeks or 5 weeks), either in ambient or specific storage conditions: protected from light or not, at two different temperatures (at 4 °C or at −20 °C), without humidity or without oxygen. Influence of storage conditions on the bioactivity of the matrices was determined by assessing the percentage of osteoclast formation after 7 days of monocyte cell culture and normalized to the positive control (culture medium + freshly reconstituted RANKL). (a) Influence of storage time on loaded matrices' bioactivity. Loaded cement was stored in ambient conditions for 1 day (1 d), 3 weeks (3 w) or 5 weeks (5 w). Data are mean ± SD, $n = 3$ replicates. (b) Effect of ambient condition variations on the stability of RANKL protein–trehalose-loaded brushite cement. (i) Influence of light exposure. Materials were stored for 3 weeks or 5 weeks protected from light (D3 w, D5 w) or not (3 w, 5 w). Data are mean ± SD, $n = 3$ replicates. (ii) Influence of temperature. Loaded matrices were stored protected from light for 3 weeks either at 4 °C (4D3 w) or at −20 °C (−20D3 w). Data are mean ± SD, $n = 3$ replicates. (iii) Effect of atmosphere composition variation. Coated brushite cements were stored protected from light for 3 weeks or 5 weeks in dried air (AD3 w, AD5 w) or in nitrogen gas (ND3 w, ND5 w) conditions. Data are mean ± SD, $n = 3$ replicates. The differences were evaluated by analysis of variance (ANOVA) with Fisher's probability least significant difference post-hoc test and were considered to be significant at $p < 0.05$.

On the basis of this observation, we investigated how long trehalose could preserve the bioactivity of RANKL in brushite cement matrices (Fig. 3b). The percentage of TRAP positive cells induced by RANKL-coated onto brushite cement continuously decreased from 162 ± 4% after the first 7 days culture period to 15 ± 2% of positive control after 28 cumulative days of culture. The additional presence of trehalose significantly increased the osteoclastic formation to 75 ± 2% after 28 cumulative days of culture (Fig. 3c).

3.4. Effects of storage conditions on RANKL–trehalose–brushite stability

The effects of different storage parameters prior to culture on the osteoclastogenic activity of RANKL–trehalose-loaded brushite cement are presented in Fig. 4.

The percentage of osteoclast formation induced by RANKL–trehalose-loaded cement stored in ambient conditions for 3 and 5 weeks significantly decreased compared with the same formulations stored for 1 day with 66 ± 4% and 59 ± 3% of osteoclast formation compared to positive control, respectively. However, there was no further deterioration of the osteoclastogenesis after 3 weeks (Fig. 4a).

To determine the parameters responsible for the decrease of efficiency of RANKL protein–trehalose combinations, the effects of light, temperature, humidity and oxygen were evaluated. Exclusion of light had no effect (Fig. 4b – i). Reducing the storage temperature to −20 °C and the absence of oxygen were found to be critical in improving the long-term activity of RANKL (Fig. 4b – ii and iii), for example, 78 ± 2% of activity was retained after 5 weeks storage in dry nitrogen compared with that of the positive control, which was a significant improvement over storage in ambient conditions (cf 59 ± 3%, Fig. 4a).

4. Discussion

Calcium phosphate cements (CPCs) were proposed two decades ago by LeGeros et al. [11] and Brown and Chow [12] as synthetic bone substitutes. Since then, several formulations have been developed and have proven to be efficient as bone substitutes due to their physico-chemical properties. The specific properties of CPCs such as injectability, mouldability and low-temperature *in situ* setting make them very suitable to fill bone defects [13]. During the last decade, both commercial and experimental CPCs have been investigated as drug delivery systems; CPCs offer the advantages of setting at low-temperatures and the set cement matrix is micro-porous which enables controlled release of drugs dissolved in the liquid cement phase. Most studies are focused on the release of robust and easy to measure molecules such as antibiotics [14], and anti-osteoporotic agents such as bisphosphonates [15,16]. Other widely used bioactive molecules combined with CPCs are bone morphogenic proteins (BMPs) that impart bone regenerative properties since calcium phosphate cements generally lack the osteogenic potential to promote bone healing of critical-sized defects [17]. Nevertheless, lyophilized stocks of BMPs have to be stored below −20 °C to maintain their osteogenic activities, and after reconstitution, they cannot be stored for longer than 7 days at 4 °C [18,19]. Thus, formulations combining CPCs and delicate proteins such as BMPs should be stored at the temperature of conservation of the biomolecule (usually below −20 °C) in order to maintain their bioactivity for a prolonged time.

The induction of cellular remodelling of CPCs might be achieved by incorporating molecules playing a key role in the bone turnover process. This study aimed to modify a synthetic brushite cement bone graft with the pro-resorptive factor RANKL, as a potent stimulator of bone remodelling process, mixed with the conservative agent trehalose to increase the stability of RANKL-coated cement formulation and the release from a brushite matrix. A vital criterion for the clinical use of these protein-loaded bioceramics is the maintenance of the biological protein activity during preparation and handling of the materials. According to the manufacturers, reconstituted solution of RANKL is usually stored below −20 °C [20,21]. After loading RANKL solution onto the surface of calcium phosphate brushite cement, the sustained material showed a high osteoclastogenic activity after 30 min of storage but lost 90% of its stability after even 1 day of storage (Fig. 3a). This deterioration of the osteoclastogenic potential of the loaded matrices may be explained by the lost of bioactivity of the loaded protein during the storage process in ambient conditions.

In order to improve protein stability, trehalose was added to the RANKL solution prior to cement modification. Trehalose is a natural alpha-linked disaccharide found in many organisms, which is also known as a fragile glass-forming agent that acts to protect proteins [22,23]. Recent studies suggest that trehalose is effective because it reduces the molecular mobility of water molecules and creates clusters of more organized water [24]. Thus, there has been considerable interest in the use of trehalose as a protective agent during conventional freezing but also to improve desiccation tolerance [25,26]. Addition of trehalose to a drug delivery system associating biomaterials and bioactive molecules will be highly valuable to increase and improve the conservation and the stability of loaded factors. But, to our knowledge, only two studies were previously published describing the effects of trehalose on calcium phosphate-based device for controlled drug delivery [27,28]. No chemical interaction was found between trehalose and a calcium phosphate material in dried conditions [27], and trehalose was shown to significantly reduce degradation of the loaded drug [28]. The stabilizing effect of trehalose was confirmed in this study. After 1 day of storage in ambient conditions, addition of trehalose increased RANKL-loaded brushite stability by 80%, with an osteoclastogenic activity comparable to that of the positive control (Fig. 3a). Moreover, during successive cell cultures, presence of trehalose improved the bioactivity of RANKL-coated cement (up to 1100%), and was shown to maintain a high activity of the matrices after 28 days of culture (Fig. 3c).

Mechanistically, several parameters such as temperature, pH, denaturant agents, and the binding to ligands are influencing protein stability and degradation. Several storage factors were examined in this study such as storage time, light exposure, temperature, humidity and oxygen [29,30]. The storage time was the most critical parameter decreasing RANKL activity. After 3 weeks and 5 weeks of storage, RANKL–trehalose–brushite combinations lost 40% of their activity compared with 1 day storage (Fig. 4a). Stored either protected from light, at lower temperature of 4 °C or in dried air, RANKL–trehalose-loaded matrices did not significantly retain their activity (Fig. 4b – i–iii), whereas storage at −20 °C or in a dry nitrogen atmosphere increased the stability of RANKL–trehalose formulation by 15% and 20%, respectively (Fig. 4b – ii and iii).

Addition of trehalose to the loaded matrices reduced the deterioration of the bioactivity of the molecules during the storage at ambient conditions and improved the efficiency of this released material during successive uses. Moreover, by reducing the temperature to −20 °C and by removing the oxygen molecules during the storage, protein oxidation was prevented and the stability of RANKL–trehalose-loaded matrices was increased. All these improvements combined together allow storing the matrices with retaining the activity of the loaded molecule.

5. Conclusion

Regeneration of bone defects can be accelerated by implanting a resorbable synthetic bone substitute that could locally de-

liver an appropriate amount of bioactive molecules at a desirable rate and concentration. A vital criterion for this protein-based approach is the sufficient stability of the adsorbed protein during storage and handling the biological protein activity. RANKL stability was improved by the presence of a conservative agent, trehalose, added to the RANKL solution before loading. This formulation retained up to 60% of its activity even after 5 weeks of storage under ambient conditions. This study identified storage temperature and the presence of oxygen as main critical parameters influencing the stability of the pro-resorptive cytokine RANKL adsorbed on preset brushite cement specimens. By optimizing complex parameters controlling the release and the stability of bioactive molecules for synthetic bone grafts, new drug delivery devices will become of a high interest to regenerate damaged orthopedic tissue.

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