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Scavenging effect of Trolox released from brushite cements

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ABSTRACT

In this study a brushite cement was doped with the chain-breaking antioxidant Trolox. The effect of the antioxidant on the physical properties of the cement was evaluated and the release of Trolox was monitored by UV spectroscopy. The ability of the Trolox set free to scavenge reactive oxygen species (ROS) released by macrophages was determined in vitro using a luminol-amplified chemiluminescence assay. Trolox did not modify the crystalline phases of the set cement, which mainly formed crystalline brushite after 7 days in humid conditions. The setting time, compressive strength and morphology of the cement also remained unaltered after the addition of the antioxidant. Trolox was slowly released from the cement following a non-Fickian transport mechanism and nearly 64% of the total amount was released after 3 days. Moreover, the capacity of Trolox to scavenge the ROS released by macrophages increased in a dose-dependent manner. Trolox-loaded cements are expected to reduce some of the first harmful effects of acute inflammation and can thus potentially protect the surrounding tissue during implantation of these as well as other materials used in conjunction.

1. Introduction

Implantation of a biomaterial in a living tissue causes post-surgical inflammation [1,2]. The origin of this inflammation is partially due to the surgical injury, which interferes with the homeostatic mechanisms that lead to cellular cascades of wound healing [1]. The typical healing response occurs in a very efficient manner and is characterized by four distinct, but overlapping phases: hemostasis, inflammation, proliferation and remodeling [3].

A biomaterial itself can also affect inflammation. Certain chemical or physical properties of biomaterials may trigger a potent and uncontrolled inflammatory reaction. This may cause damage to the surrounding cells and tissue due to prolonged exposure to pro-inflammatory cytokines, reactive oxygen species (ROS) and destructive enzymes. For instance, ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($^{\cdot}OH$) released by immune cells are known to damage proteins and lipids within the cell membrane and to cause strand breaks and nucleobase modifications in DNA due to their powerful oxidant potency [4–8]. A very potent inflammatory phase could cause a chronic inflammation that might lead to implant failure [9].

By physically or chemically modifying a material (surface and/or bulk) it is possible to enhance specific cell responses, beneficial for biomaterial integration, and thus improve implant performance [10]. This knowledge can be combined with the concept of drug delivery systems [11]. In order to cope with inflammation and protect the surrounding tissue during implantation, antioxidants can be loaded into the biomaterial to delay or inhibit the oxidation of lipids, proteins or DNA [12]. Trolox (Fig. 1) is a synthetic compound and a derivative of the vitamin E family of chain-breaking tocopherols and tocotrienols. The hydrophobic side-chain attached to the 2-position of the chromanol entity in the naturally occurring compounds has been replaced with a carboxylic acid. Trolox can therefore be viewed as a more water-soluble form of vitamin E [13]. Trolox has been proven to prevent DNA fragmentation of cells grown in the presence of H_2O_2 [14] and Satoh et al. showed that the antioxidant property of Trolox can surpass that of α-tocopherol [15]. However, to the authors' knowledge, no clinical studies using this compound have yet been performed.

Calcium phosphate cements (CPCs) are commonly used as bone void fillers [16], but also to enhance screw fixation in poor-quality bone [17]. The two most commonly encountered end products are precipitated hydroxyapatite at pH > 4.2 and brushite/monetite at pH < 4.2 [16–18]. Brushite cements are resorbed quicker than apatite cements [19] due to the higher solubility of brushite compared to that of calcium-deficient hydroxyapatite [20], which may be an advantage for bone regrowth. CPCs are used as bone fillers due to their chemical similarity to the mineral phase of bone and excellent biocompatibility. However, for brushite cements, integration

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with the surrounding tissue appears to depend on the β-tricalcium phosphate (TCP)/monocalcium phosphate (MCPM) ratio, and hence the resulting pH surrounding the cement, with a lower ratio giving higher acidity and possibly fibrous tissue surrounding the implant rather than osteointegration [21]. Limiting the inflammatory reaction may especially be desirable when using brushite cements of a certain composition to optimize properties such as mechanical strength [22]. This may be of even higher interest when used in conjunction with other implants, e.g. metallic screws, and/or in the case of open surgery, since these procedures increase the chances of an infection and subsequently inflammation. It should also be noted that biomaterials loaded with vitamin E may reduce bacterial adhesion [23].

CPCs are able to set at body temperature and result in an intrinsically porous material that allows drug diffusion [24]. It is important to study thoroughly each drug–carrier couple since, on the one hand, the drug may cause physicochemical modifications to the cement paste (e.g. setting time, porosity and mechanical properties) [25]; on the other hand, the cement may modify the active principle of the drug due to, for example, local changes of pH or ionic concentration [24]. However, several biologically active molecules or drugs are able to retain their activity within the cement paste [24], making these materials potentially good drug carriers [11].

The aim of this work was to produce and characterize a brushite cement loaded with Trolox. The release of Trolox with time was monitored and the capacity of the released drug to scavenge reactive oxygen species produced by activated inflammatory cells was determined through an in vitro study.

2. Materials and methods

2.1. Cement preparation

Cement powder was prepared daily by mixing 54 wt.% β-TCP (β-$Ca_3(PO_4)_2$, Sigma Aldrich, ref. no. 21218, St Louis, MO), 44 wt.% MCPM ($Ca(H_2PO_4)_2{\cdot}H_2O$, Scharlau, ref. no. CA0211005P, Port Adelaide) and 2 wt.% SPP (sodium pyrophosphate, $Na_4P_2O_7$, Fluka, ref. no. 71499, St Louis, MO) in a powder mixer (Turbula®, Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) for at least 20 min. SPP was included as a retardant of the reaction. The particle size of β-TCP was 13.6 ± 0.10 μm. MCPM was previously sieved (Retsch, ref. no. 60131000075, Haan, Germany) and only particles <75 μm were used. The cement paste was prepared by manually mixing the powder with distilled water with a spatula in a glass mortar, maintaining a liquid-to-powder ratio of 0.32 ml g^{-1}.

Brushite cement was loaded with Trolox (Sigma Aldrich, ref. no. 238813) by adding 4 mg of Trolox to 1 g of cement powder (0.4 wt.%). The powder was blended in a powder mixer (Turbula®) before adding the liquid phase (distilled water). A liquid-to-powder ratio of 0.32 ml g^{-1} was used.

2.2. Cement characterizations

Teflon rings (diameter = 12.5 mm, h = 5 mm) were filled with cement paste and the setting time was evaluated using Gillmore needles [26] at room temperature (21 ± 1 °C). The initial setting time was defined, according to the standard, as the time elapsing after the start of mixture of the powder and liquid phases until no indentation could be observed on the cement surface when the needle exerting less pressure was used; the final setting time was obtained following the same procedure but applying a heavier and thinner needle. Setting times were evaluated in triplicate.

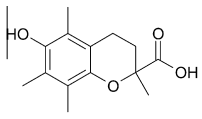

Fig. 1. Formula of Trolox.

When testing mechanical properties, cement paste was molded in cylindrical silicon molds (diameter = 6 mm, h = 12 mm) and soaked in PBS solution for 7 days. After this time, cement cylinders were manually polished by fitting them in a stainless steel mold and using a silicon carbide paper, P#1200 (Struers, ref. no. 40400023, Cleveland, OH). The mechanical testing was performed in a universal materials testing machine (AGS-X, Shimadzu, Kyoto, Japan) equipped with a load cell of 5 kN at a crosshead speed of 1 mm min^{-1}. Eight specimens of each formulation were tested.

Specimens molded in silicon molds (diameter = 12 mm, h = 2 mm) were set in 100% humidity conditions. After 7 days a few specimens were sputtered with a mixture of gold and palladium and their microstructure was evaluated by means of a scanning electron microscope (Tabletop Microscope TM-1000, Hitachi, Tokyo, Japan). Another group of samples was finely crushed with a mortar and analyzed by powder X-ray diffraction (XRD, D5000, Siemens) using Bragg–Brentano geometry and Cu K_α radiation. Scanning was performed with a time step of 1.0 s and a scan step of 0.02° min^{-1} between 20° and 50°. The diffraction patterns were compared with the Joint Committee on Powder Diffraction Standards for $CaHPO_4{\cdot}2H_2O$ (brushite, JCPDS No. 009-0077), $CaHPO_4$ (monetite, JCPDS No. 009-0080), β-TCP (JCPDS No. 009-0169) and MPCM (JCPDS No. 009-0347).

2.3. Release of Trolox

For the drug release study, a Trolox-loaded cement was set in silicon molds (diameter = 12 mm, h = 2 mm) for 1 h at room temperature. Afterwards each cement disc was gently placed into a glass flask containing 50 ml of PBS. As controls, cement discs without Trolox and Trolox alone (4 mg) were included. The flasks were kept at room temperature on a shaker (125 rpm) for 5 days. Sampling was performed at specific time points (every 15 min for the first hour, every hour for the first 7 h and afterwards twice per day), 1 ml aliquots were removed and replaced by 1 ml of fresh PBS to maintain the initial volume. The concentration of Trolox in each aliquot was quantified immediately in a UV spectrophotometer (UV-1800, Shimadzu) at a wavelength of 290 nm, the absorbance being interpolated from a standard curve ranging between 5 and 500 μM. The remaining aliquots were frozen for the in vitro study. Each sample group was included in triplicate and the whole experiment was carried out three times.

The measured concentrations of Trolox released in PBS were corrected in two ways. The dilution caused by removing aliquots and replacing them with PBS at each time point was compensated for (sampling). In addition, the degradation of Trolox in solution was taken into account by using degradation data from Trolox alone. Data is shown as concentration and percentage of Trolox released after correcting for sampling and degradation.

The release of Trolox was modeled through the Korsmeyer–Peppas equation (Eq. (1)), where C_t is the drug concentration at time t, C_∞ is the drug released as time approaches infinity, k is a constant incorporating the characteristics of the substrate, and n is the diffusional exponent which is indicative of the transport mechanism [27]:

$$\frac{C_t}{C_\infty} = kt^n \tag{1}$$

To determine the mechanism by which Trolox was released, its concentration was plotted following Eq. (2), derived from the Korsmeyer–Peppas model: