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Multi-drug delivery system based on lipid membrane mimetic coated nano-hydroxyapatite formulations

Damián Placente[a], Luciano A. Benedini[a], Mónica Baldini[b], Juan A. Laiuppa[b,c], Graciela E. Santillán[b,c], Paula V. Messina[a,*]

[a] INQUISUR – CONICET, Department of Chemistry, Universidad Nacional del Sur, B8000CPB Bahía Blanca, Argentina
[b] Department of Biology, Biochemistry and Pharmacy, Universidad Nacional del Sur, B8000ICN Bahía Blanca, Argentina
[c] INBIOSUR – CONICET, Argentina

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ABSTRACT

Local delivery systems from an osteoconductive biomaterial are suggested as a promising strategy to avoid simultaneously peri-implant traumas and to induce tissue regeneration. In this work, it is detailed the design and construction of a multi-drug delivery formulation based on lipid membrane mimetic coated nano-hydroxyapatite, LMm/nano-HA, as a bone-specific drug delivery approach. The optimal LMm/nano-HA formulation was selected after analysing the lipid/nano-HA interaction by dynamic light scattering (DLS), ζ-potential, transmission electron microscopy (TEM), polarized optical microscopy (POM), differential scanning calorimetry (DSC) and UV–vis spectroscopy. After the initial screening, Ciprofloxacin and Ibuprofen simultaneous -load and -release efficiency from selected LMm/nano-HA was assessed. pH-responsive kinetic profiles of local drug distribution were characterized and compared with currently applied systemic doses. Finally, the systems' biocompatibility and drug released activity were positively validated. The obtained results demonstrated that LMm/nano-HA formulations can represent a valuable multi-modal platform in bone tissue therapies.

1. Introduction

Tissue damages produced during a foreign body implantation involves an inherent risk of bacterial contaminations following by pain and loosening of bone/prosthesis interface (Xu et al., 2007). Situating the facts in numbers, at least half of the nosocomial infections involve graft insertions (Guggenbichler et al., 2011) and usually persist after the implant removal (Schierholz and Beuth, 2001). The routinely practice of systemic antibiotic and anti-inflammatory drugs release is not ideal because of the lack of targeting and bone-specificity (Xu et al., 2007). In addition, the distribution of bone throughout the body and the requirement of sustaining a pharmacological activity at the peripheral site involves the administration of high doses of drug producing a narrow toxic-therapeutic window (Hirabayashi and Fujisaki, 2003; Wang et al., 2015). To overcome this dilemma, many drug-target systems were proposed to the promise of improving therapeutic index (Hirabayashi and Fujisaki, 2003). Notwithstanding the steady advances, (Farokhi et al., 2016; Kim and Tabata, 2015) a true bone-specific delivery system is still under development on account of some obstacles that remains unsolved (Newman and Benoit, 2016): (i) limited accessibility of exogenous large substances to the bone interface, (ii) low expression of biomolecules with specific receptors for definite targets of mineralized tissues and finally, (iii) almost null affinity of target drugs to hydroxyapatite (HA), the essential constituent of bone (Hirabayashi and Fujisaki, 2003; Nguyen et al., 2010). The paradigm is the construction of a locally delivery system, applied direct to the implant, based on a bone-mimetic material. Thus, providing a favourable response from the host cells to attain tissue integration simultaneously with a directly release of active principles of the defective bone region avoiding postoperative complications. In preceding investigations (Andrés et al., 2017; D'Elia et al., 2017; D'Elia et al., 2015) we have demonstrated the *in vitro* bone regeneration ability of biodegradable, non-stoichiometric hydroxyapatite nano-rods (nano-HA, $Ca_{9.42}(PO_4)_{5.42}(HPO_4)_{0.58}(OH)_{1.42}$) synthesized in our laboratories. Here, we have verified their aptitude to act as local bone multi-drug delivery system. To solve the poor drug encapsulation efficiency of HA (Xu et al., 2007), nanoparticles were coated out of a vesicular lipid membrane whose role as drug carrier is well recognized (Nguyen et al., 2010). First we have investigated the adsorption of a lipid coating into nano-HA surfaces and established the formulation parameters. Following, based on a selected formulation of the initial screening, the simultaneous drug-load and -release efficiency were evaluated.

* Corresponding author.
E-mail address: pmessina@uns.edu.ar (P.V. Messina).

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Adsorption and kinetic profiles of Ciprofloxacin and Ibuprofen were analyzed; maximum load capacity and the delivery rate on physiological and open wound conditions (Bennison et al., 2017) were determined. Ibuprofen activity was determined by Albumin denaturation test and systems biocompatibility were confirmed by interaction with primary rat calvarial osteoblasts. Finally, the antibiotic potential was validated against drug-resistance strains responsible to habitual bone infections. The obtained results demonstrated that LMm/nano-HA formulations have essential qualities to fulfill the requests for bone defects reconstruction: prevention of bacterial infections, inflammation suppression and implant integration.

2. Material and methods

2.1. Reagents

Hexadecyl-trimethyl ammonium bromide (CTAB, 99%, Sigma -Aldrich); poly (propylene glycol) (PPG, Sigma-Aldrich, average MW = 425 g/mol, δ = 1.004 g/mL at 25 °C); sodium phosphate (Na_3PO_4, 96%, Sigma – Aldrich); calcium chloride ($CaCl_2$, 99%, Sigma – Aldrich); sodium nitrite ($NaNO_2$, 97%, Sigma – Aldrich); acetic acid ($C_2H_4O_2$, 99%, Sigma – Aldrich); sodium acetate trihydrate ($C_2H_3NaO_2{\cdot}3H_2O$, 99%, Sigma – Aldrich); sodium hydroxide (NaOH, 97%, Sigma – Aldrich); phosphotungstic acid hydrate (PTA, Sigma – Aldrich); phosphate buffer saline (PBS tablets, Na_2HPO_4/NaH_2PO_4, Sigma – Aldrich); methanol (CH_3OH, anhydrous 99.8%, Sigma – Aldrich); ethanol (EtOH, 96%, Sigma – Aldrich); hydrochloric acid (HCl, 37%, Sigma – Aldrich); chloroform (CH_3Cl, anhydrous 99%, Sigma – Aldrich); bovine serum albumin (BSA, Fraction V, 95%, Carl Roth); sodium azide (NaN_3, 99.5%, Sigma-Aldrich); urea (CON_2H_4, 98%, Sigma-Aldrich); Lipoid S75 (LS75, Lipoid AG Sennweidstrasse 44 CH-6312 Steinhausen/ZG Switzerland) and neutral red ($C_{15}H_{17}ClN_4$, BioReagent, Sigma – Aldrich) were used without further purification. Ciprofloxacin hydrochloride and Ibuprofen were from Parafarm, Droguería Saporiti S.A.C.I.F.I.A., Av. del Campo 1449, C1427APB, C.A.B.A, Argentina. For solutions preparation, only Milli-Q water was used.

2.2. Lipid membrane mimetic coated nano-hydroxyapatite (LMm/nano-HA) formulations

2.2.1. Preparation and selection of the optimal LMm/nano-HA formulation

Bone-like HA nanoparticles were prepared and characterized following previously described procedures (D'Elía et al., 2013; D'Elia et al., 2017; D'Elia et al., 2015), details were shown in **section SM1** of the supplementary material (SM). For the preparation of lipid membrane mimetic coatings, LS75 was used. It contains 69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine, 2.1% lysophosphatidylcholine and 18.8% fatty acid (17–20% palmitic, 2–5% stearic, 8–12% oleic, 58–65% linoleic and 4–6% of linolenic acid) (Gibis et al., 2013) and, was currently used to construct membrane mimetic models because of its composition (Benedini et al., 2016). Multilamellar vesicles (MUVs) were made according to the thin-film hydration method as follows (Lasic, 1993): the appropriate amount of LS75 was dissolved in a $CHCl_3$/MetOH (4/1) mixture to obtain a final concentration of 0.25 mg mL^{-1} and dried in a rotary evaporator under N_2 stream. The resultant lipid film was kept under N_2 for about 1 h, then, hydrated for 2 h with PBS (pH = 7.4), homogenized with vigorous vortexing – sonication cycles at a temperature 10 °C above the gel to liquid-crystalline phase transition point (T_c = 50–55 °C) and finally kept in the refrigerator (4 °C) overnight. As a final point, the dispersion was filtered through polycarbonate filters of 100 nm pore size with an extrusion syringe (15 times) to form small unilamellar vesicles SUVs. 63.4 ± 1.7 nm diameter SUVs were obtained and was freshly prepared each time before to use, Fig. 1c and Fig. SM3. To obtain the LMm/nano-HA formulations, nano-HA powder was suspended in PBS to obtain a 10 mg mL^{-1} stock concentration and sonicated for 30 min. The optimal LMm/nano-HA formulation was selected after identifying the maximum adsorption capacity of LS75 SUV to nano-HA. Adsorption experiments were conducting by adding the nano-HA suspension to liposome dispersion at RT. The properly amounts of nano-HA and LS75 SUVs dispersions were mixed and diluted with PBS as required to obtain a final 4 mg mL^{-1} nano-HA and 0–0.28 mg mL^{-1} lipid concentrations in centrifugal test tubes. LS75 SUV/nano-HA mixtures were kept in contact during 20 h in refrigerator (4 °C) and then centrifuged at 4000 rpm for 5 min at RT. Centrifuged bottom layer was separated from the supernatant, hydrated with PBS and centrifuged again. The process was repeated three times to eliminate not adhered lipid. After centrifugation, the supernatants were transferred to glass vials and conserved to posterior lipid quantification by reverse phase high pressure liquid chromatography, RP-HPLC. The amount of lipid adsorbed to nano-HA was computed as the difference between the initial and the supernatant concentration. The adsorption capacity, q (mg_{LS75}/mg_{HA}), was calculated with the equation:

$$q = \frac{([LS75]_0 - [LS75]) \times V}{m} \quad (1)$$

where $[LS75]_0$ and [LS75] are the initial and supernatant concentrations of LS75 expressed in mg mL^{-1}, V (mL) is the total dispersion volume and m the mass of HA (mg).

2.2.2. Lipid quantification by RP-HPLC

For the determination of q (mg_{LS75}/mg_{HA}), a 20 µL aliquot of each samples were manually injected into a Waters 600E MDS HPLC (Waters, Mississauga, ON, Canada). The RP-HPLC was equipped with a C_{18} reverse phase column (Nova-Pak C18 Column, 60 Å, 4 µm, 3.9 mm x 150 mm, 1/pkg, WAT086344) and a UV–vis detector (Water 486) set at 205 nm. The mobile phase was methanol – 1 mM PBS, pH 7.4, 9.5:0.5 (v/v) pumped at a flow rate of 1 mL min^{-1} (Smith and Jungalwala, 1981). The lipid concentration in all samples was determined by comparing the UV absorbance of the phosphatidylcholine with a standard curve (R^2 greater than 0.998) generated under the same conditions. Samples and solvents were filter prior to use.

2.2.3. Particle size distribution and ξ-potential measurement

Size distributions and ξ-potentials of LS75 SUVs and LMm/nano-HA formulations were determined at 25.0 ± 0.1 °C using a Malvern Zeta Sizer Nano (ZSP) with a He-Ne laser (633 nm) as a source of incident light. The mean particle size was obtained by quasi-elastic light scattering (DLS) analysis from the measured intensities at scattering angle of 90°. All samples were 1/10 diluted with filtered hydration medium to an appropriate counting rate prior to analysis. Reported values were the result of ten independently determinations.

2.2.4. Thermal characterization and storage stability of LMm/nano-HA formulations

The thermal characterization of LMm/nano-HA formulations were determined using a Q20 Differential Scanning Calorimeter (TA Instruments). The temperature and enthalpy scales were calibrated using standard samples of indium (CAS No.7440-74-6/1, T_m = 156.6 °C, ΔH_m = 3.295 kJ mol^{-1}) and zinc (CAS No.7440-66-6/3, T_m = 419.53 °C, ΔH_m = 7.103 kJ mol^{-1}). The LMm/nano-HA samples, which had been weighted with a ± 0.00001 g precision balance, were sealed in a hermetic aluminum pan to prevent any loss of moisture during differential scanning calorimetric (DSC) measurement. The samples had been previously cooled to − 20 °C during 1 min. Afterwards they were heated up to 600 °C at a rate 0.08 °C seg^{-1}. Ultrapure nitrogen was used as purge gas at a rate of 20 mL min^{-1} during the measurement. The baseline subtraction was made to correct any heat capacity difference between the sample and the reference furnace. All the experiments were done in triplicate. The specific heat C_p in J g^{-1} K^{-1} of every sample was calculated from the DSC