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Abstract

Calcium hydroxide ($Ca(OH)_2$) loaded poly(DL-lactide-co-glycolide) acid (PLGA) microspheres (MS) might be employed for apexification requiring a sustained release of Ca++. The aim of this study was to formulate and characterize $Ca(OH)_2$-PLGA-MS. The $Ca(OH)_2$-loaded MS were prepared by either oil-in-water (O/W) or water-in-oil/in-water (W/O/W) emulsion solvent evaporation technique. MS produced by the O/W technique

exhibited a larger diameter (18.63 ± 7.23 μm) than the MS produced by the W/O/W technique (15.25 ± 7.37 μm) (Mann Whitney U test $P < 0.001$). The $Ca(OH)_2$ encapsulation efficiency and Ca++ release were calculated from data obtained by absorption techniques. Ca++ release profile was evaluated for 30 days. The percentage of encapsulation efficiency of the O/W-produced MS was higher (24%) than the corresponding percentage of the W/O/W-produced MS (11%). O/W- and W/O/W-produced MS released slower and lower Ca++ than a control $Ca(OH)_2$ paste with polyethylene glycol 400 (ANOVA 1 way, Tukey HSD $P < 0.01$). O/W-produced MS released higher Ca++ than W/O/W-produced MS (statistically significant differences with t-Student test). We concluded that $Ca(OH)_2$-PLGA-MS were successfully formulated; the technique of formulation influenced on the size, encapsulation efficiency and release profile. The MS were better sustained release system than the $Ca(OH)_2$ paste.

Key words: apexification, calcium hydroxide, microspheres, poly(DL-lactide-co-glycolide) acid, sustained drug delivery system.

Introduction

Apexification is the induction of apical closure to produce favorable conditions for conventional root canal filling [1]. Calcium hydroxide ($Ca(OH)_2$ is widely used for apexification treatment because of its ability to induce mineral tissue formation and apical closure [1, 2]. The $Ca(OH)_2$ releases calcium ions (Ca++) and hydroxyl ions; the Ca++ and the local increase of pH (~12.5) in the tissues induce cellular activity promoting the mineral tissue formation [3-5]. Because the apexification is a long-term treatment, it requires sustained release of Ca++ from the $Ca(OH)_2$ and usually to achieve that condition, the $Ca(OH)_2$ is replaced on multiple appointments [2]. Thus a biodegradable sustained drug delivery system (SDDS) loaded with $Ca(OH)_2$ might be useful for apexification because the SDDS would release Ca++ during long time with a single application.

Among SDDS technologies, microspheres (MS) formulated with polymers have showed efficacy to promote sustained release of Ca++ [6-8]. Hunter et al. manufactured calcium citrate loaded poly(ethylenglycol)-MS within a range size of 180-2000 μm releasing Ca++ for 3-4 days but for pulp capping [6, 7]. For apexification, Strom et al. produced alginate-based MS loaded with $Ca(OH)_2$ to promote long term release of Ca++; the MS showed a longer sustained Ca++ release profile than that of a $Ca(OH)_2$ paste prepared with distilled water [8]. Despite the few approaches to research polymer-based MS for Ca++ sustained release, the studies have ignored the use of a biomaterial with advantageous physicochemical properties for such purpose: the poly (DL-lactide-co-glycolide) acid (PLGA). The PLGA is a biodegradable and biocompatible polymer approved for human use by the FDA; its degradation in tissues initiates by hydrolysis of the ester linkages of the polymer chain giving the innocuous lactic acid and glycolic acid [9]. Acting as matrix of a

MS, the PLGA might entrap a biomolecule that will be released while the polymer degradation occurs, this action results in a sustained release that depends on PLGA properties but also on the characteristics of the MS [10, 11]. Accordingly we suggest to explore the formulation of a sustained release system of $Ca(OH)_2$ loaded PLGA-MS for apexification.

The aim of this study was to formulate and characterize $Ca(OH)_2$ loaded PLGA-MS (CMS). To achieve this purpose, we performed two techniques − oil-in-water (O/W) single emulsion or water-in-oil-in-water (W/O/W) double emulsion– based on the solvent evaporation method to compare physical properties of the CMS and Ca++ release profile.

Materials and Methods

Poly (DL-lactide-co-glycolide) (ratio lactide:glycolide 75/25; mol wt 66,000-107,000 kDa), dichloromethane anhydride (DCM) ≥99.8% solvent, Polyethylene glycol (PEG; Mn 400) and polyvinyl alcohol (PVA) (87.90 % hydrolyzed) were purchased from Sigma-Aldrich (St Louis, MO,USA). Calcium hydroxide was purchased from Viarden (Mexico City, DF, MX). All reagents were of analytical grade.

Preparation of the microspheres

Oil-in-water single-emulsion solvent evaporation technique (W/O)

The O/W technique was based and adapted from methodologies previously reported [12, 13]. Briefly, 20 mg $Ca(OH)_2$ and 200 mg PLGA were added into a 10 mL glass tube containing 2 mL DCM, this oil phase (drug/matrix dispersion) was vortexed with a Maxi Mix II vortex (Thermo Scientific, Pittsburgh, PA, USA) at maximum speed for 3 min. Immediately after that, the oil phase was added drop-wise into a 250 mL glass baker

containing a 100 mL 1% PVA (continues phase) under stirring at 800 rpm (25°C) (Corning PC 4200 stirring hot plate, Corning, NY, USA). After that 100 mL of distilled water was added. Then, the stirring continued for 3 h to promote evaporation of DCM. After finishing the stirring, the formed MS were recovered by filtration through a filter paper (2 μm Filter Paper Ahlstrom, Monterrey, NL MEX) and were profusely washed with distilled water. Finally the MS were freeze-dried (Freeze Dry System Freezone 6, Labconco, Kansas City, MI, USA) for 4 h and stored at 4°C until its characterization and evaluation of Ca++ release. The $Ca(OH)_2$ loaded MS were identified as oCMS. Blank control MS were produced following the same method without $Ca(OH)_2$.

Water-in-oil-in-water method double-emulsion solvent evaporation technique (W/O/W)

The WOW procedure was based and adapted from techniques already reported [12, 13]. Briefly, a 20 mg/mL $Ca(OH)_2$ dispersion was prepared with double distilled water. This dispersion and 200 mg PLGA were added into a 10 mL glass tube containing 2 mL DCM, this oil phase (drug/matrix dispersion) was vortexed with a Maxi Mix II vortex (Thermo Scientific, Pittsburgh, PA, USA) at maximum speed for 3 min. After that, the procedure was performed as described in the paragraph above (see O/W process). The $Ca(OH)_2$ loaded MS were identified as wCMS. Blank control MS were produced with the same method without $Ca(OH)_2$.

Characterization of the MS

Morphology of the MS

Morphology was observed using 1-2 mg MS. They were put on an adhesive tape, and then it was coated with gold (20 mA for 4 minutes). The gold-coated MS were observed by a scanning electronic microscope (Philips XL-30, Philips, Hillsboro, OR, USA).

Particle size analysis of the microspheres

Microspheres were imaged at 100x using a light microscope with a digital camera (Olympus, Center Valley, PA, USA). Images were analyzed with ImageJ Software (Version 1.45, National Institutes of Health, Bethesda, MD, USA); one hundred of MS were measured and its average diameter was calculated. A Mann Whitney U test was applied to identify a possible statistical significant difference between CMS and blank MS as well as between oCMS and wCMS. Statistical significance was set at $p < 0.05$.

Encapsulation efficiency (Ee)

To calculate the Ee, $Ca(OH)_2$-loaded MS were dissolved in 1 M NaOH. In this dissolution, a calcium colorimetric marker *(*Calcio Arsenazo III, Bio Simex, Guadalajara, JAL, MEX) was added for 5 minutes to react with Ca++. The reagent caused a blue color in the samples thus absorbance of the samples was measured by a UV-VIS system at 650 nm (Cary 50 UV-Visible Spectroscopy System, Agilent Technologies, Mexico City, MEX). A calibration curve was previously performed to obtain the calcium concentration in relation to absorbance values. Ee was calculated as the ratio of the experimental loading to the theoretical loading, of the drug in the microspheres.

Ca++ release

Twenty mg of either oCMS or wCMS was suspended into 1 mL deionized water in an Eppendorf tube (1.5 mL) and incubated at 37°C for 30 days and shook at 150 rpm (Incu-Shaker Mini, Benchmark Scientific; Edison, NJ, USA). The deionized water of the Eppendorf tubes was collected at different times; then 1 mL fresh deionized water was added in the Eppendorf tube containing the CMS. For Ca++ measuring, the Ca++ in the collected supernatant was marked with a calcium colorimetric marker (Calcio Arsenazo III,

Bio Simex, Guadalajara, JAL, MEX) and measured by a UV-VIS system at 650 nm (Cary 50 UV-Visible Spectroscopy System, Agilent Technologies, Mexico City, MEX). Ca++ concentration was calculated from the absorbance in the basis of a calibration curve performed previously. Ca++ release of experimental groups was compared with a Ca++ release of a control paste prepared with $Ca(OH)_2$ and PEG 400 (1.5 mg/10 μL). We incubated the $Ca(OH)_2$ paste in a 1.5 cm length dialysis tubing cellulose membrane (Avg. flat width 10 mm; typical molecular weight cut-off = 14,000; Sigma-Aldrich, St. Louis, MO, USA) with a seal in each extremity. The membrane was used only for the control paste to avoid its immediate dispersion in the deionized water. The membrane with the paste was suspended into 1 mL deionized water in an Eppendorf tube (1.5 mL) and incubated at 37°C for 30 days and shook at 150 rpm. The measurement of Ca++ was identically as described for the CMS. All experiments were done in triplicate. ANOVA one way test and Tukey HSD test were applied to identify possible statistical significant differences between Ca++ release profiles of control and CMS. Statistical significance was set at $P < 0.01$. T-student was applied to identify possible statistical significant differences between Ca++ release profiles of oCMS and wCMS.

Results

CMS with a similar spherical morphology were obtained with two emulsion solvent evaporation techniques. The CMS exhibited different topographical characteristics depending on the preparation method (Figure 1). The oCMS showed an average diameter of 18.63 ± 7.23 μm, while the wCMS showed a diameter of 15.25 ± 7.36 μm. No statistical difference in diameter was found between CMS and blank MS. Significantly statistical difference was noticed ($P < 0.001$) between average diameter of oCMS and wCMS.

Calculation of Ee was 24% for oCMS, while it was 11% for the wCMS. Compared with the control paste, the CMS showed a lower and longer Ca++ releasing activity; the figure 2 shows the Ca++ releasing activity of the CMS and the control.

Discussion

We explored the formulation of CMS by the solvent extraction/evaporation method. In this method a drug/matrix oily dispersion is partitioned into microdroplets when it is added in an aqueous phase under shear forces. Then, extraction/evaporation of the solvent induces PLGA crystallization transforming the microdroplets into solid MS. We employed two variants of the method, O/W and W/O/W techniques. They differed in the physical presentation of the $Ca(OH)_2$ for the dispersed phase, which was employed as a powder for the O/W and diluted in distilled water for the W/O/W techniques. The wCMS showed a rough like-porous surface while the oCMS showed a smooth surface; the average diameter of the MS was another property varying between the wCMS and the oCMS, these latter were the largest ones. The size of the MS depends on factors controlled by the formulation technique, for instance speed of stirring, temperature of the aqueous phase, or mass content in the dispersed phase [12]. In our study, the phase for the O/W presented larger solid content than the dispersed phase for the W/O/W. In that condition, a dispersed phase is more resistant against the shear forces causing its partitioning into droplets, and consequently larger MS are produced [14]. The oCMS entrapped over twice $Ca(OH)_2$ than the wCMS. Higher Ee in oCMS is explained because higher DMC volume in the O/W dispersed phase reduces the flux of the used biomolecule ($Ca(OH)_2$ in our case) to aqueous phase during solvent extraction/evaporation, and also accelerate PLGA crystallization with a consequent increase in the entrapment of the drug [15]. It should be considered that $Ca(OH)_2$ is an hydrophilic molecule that easily might go to the aqueous phase from the

dispersed phase causing a low Ee. We determined whether CMS released lower Ca++ compared to Ca++ released from a $Ca(OH)_2$ paste formulated with PEG 400, a fluid polymer used to promote slow and sustained release of Ca++ [2, 16, 17]. The paste showed a burst release of 42.6% Ca++ after 1 day of evaluation; then the paste showed a release of 86.3% Ca++ after 3 days and finally it showed a release of 100% Ca++ after 6 days. The PEG is a highly hydrophilic polymer, thus it was rapidly dissolved in the PBS under our experimental conditions resulting in a fast release of the Ca++. The CMS behaved as a more efficient slow and sustained release system compared to the $Ca(OH)_2$ paste indeed. In the CMS, the $Ca(OH)_2$ was entrapped into the PLGA matrix during the MS formulation and it was released in a slow and sustained manner because of the gradual degradation of the polymer matrix during the evaluation time. The CMS kept a Ca++ release for 30 days. During first 9 days of evaluation a similar Ca++ release profile was noticed for both oCMS and wCMS, but since day 12th, the Ca++ release profiles behaved different in both CMS. We correlated the difference between release profiles to the surface properties of the MS. A rough like-porous surface favors a rapid drug release because fluids penetrate easier into the MS matrix and facilitates degradation of PLGA, releasing the entrapped drug; in the contrary, a smooth surface delays drug release [18,19]. It can also be correlated the fact that wCMS loaded less $Ca(OH)_2$, as demonstrated by the percentage of Ee, and this was reflected on the release profile.

Our study is the first one researching $Ca(OH)_2$ loaded PLGA-based MS for apexification. Others have explored MS for the same purpose. Strom et al. produced $Ca(OH)_2$ loaded alginate MS (CAMS) crosslinked by the Ca++ in the polymer matrix [8]. They compared the CAMS to a $Ca(OH)_2$ paste (with distilled water; CP) and Ultracal XS calcium

hydroxide paste (UC) [8]. After 4 days, the CAMS released similar Ca++ amount to that released from UC but lower to that released from the CP [8]. After 10 days the CAMS released significantly lower Ca++ than both CP and UC; at 1 month the CAMS released ∽18% of its Ca++ content [8]. Although the CAMS and our CMS are different in their characteristics, both systems showed a better sustained release profile when compared to a $Ca(OH)_2$ paste.

We choose PLGA to produce the MS because of its biocompatibility and biodegradability. The CMS were a micro-granular material ad hoc to be introduced into a root canal by a double-ended spatula for cement. When needed, the CMS might be easily removed from the root canal by flushing with distilled water followed by aspiration. If the CMS were located at extra-radicular area –as might occur for apexification– they will be biodegraded by action of the tissue fluid [12]. Thus the CMS might be placed into the root canal at apical level intending a single application of a SDDS. Using CMS might overcome disadvantages of $Ca(OH)_2$ paste for apexification such as replacement of paste requiring multiple visits but also risk of root fracture by $Ca(OH)_2$ dressing [20-22].

We are aware of the limitations of this study. Evaluation time for 30 days was suitable to explore the release profile but it was short for a clinical reality; also the evaluation was short to know the time in which both oCMS and wCMS releases the total content of Ca++. We obtained an Ee 3 times lower than the total amount of $Ca(OH)_2$ employed in the formulation of the CMS, the technique should improve to get a higher Ee.

We concluded that CMS were successfully formulated, the techniques employed to produce them influenced on their characteristics. The CMS showed a size suitable for its application

into a root canal. The CMS showed a Ca++ sustained releasing activity for 30 days and it was better than that of the $Ca(OH)_2$ paste.

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Figure captions

Fig 1 SEM images (1000x) showing the morphological properties of the two types of $Ca(OH)_2$ loaded microspheres. Oil-in-water produced microspheres (oCMS) shows a spherical morphology with a smooth surface (A). Water-in-oil/in-water produced microspheres (wCMS) shows a spherical morphology with a porous and like-rough surface (B). Blank microspheres (C)

Fig 2 Ca++ accumulative release profile (A) and release percentage (B). The control ($Ca(OH)_2$ with polyethylene glycol 400) released 401.1 ± 128.5 μg/mL, 700.3 ± 38.9 μg/mL and 811.2 ± 26.3 μg/mL after 1, 3 and 6 days of evaluation, respectively. At 6 days, the control released 100% of Ca++ (B). The control showed a Ca++ release profile shorter than that of the oCMS (oil-in-water produced microspheres) and wCMS (water-in-oil/in-water produced microspheres); statistically significant differences between the control and oCMS and wCMS were found at (*) (A). The oCMS and wCMS exhibited a similar release profile after 9 days of evaluation (A). But at (#) significantly statistical differences were found between the Ca++ released amounts from oCMS (430.5 ± 63.4 μg/mL) and wCMS (292.8 ± 50.7 μg/mL), the differences were observed up to the end of the experiment. The total released Ca++ was 590.1 ± 89.0 μg/mL for the oCMS and 297.9 ± 50.6 μg/mL for the wCMS; that represented 76.6% and 90.9% of Ca++ for the oCMS and wCMS, respectively