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Tetracycline nanoparticles loaded calcium sulfate composite beads for periodontal management

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ABSTRACT

Background: The objective of this study was to fabricate, characterize and evaluate in vitro, an injectable calcium sulfate bone cement beads loaded with an antibiotic nanoformulation, capable of delivering antibiotic locally for the treatment of periodontal disease.
Methods: Tetracycline nanoparticles (Tet NPs) were prepared using an ionic gelation method and characterized using DLS, SEM, and FTIR to determine size, morphology, stability and chemical interaction of the drug with the polymer. Further, calcium sulfate ($CaSO_4$) control and $CaSO_4$-Tet NP composite beads were prepared and characterized using SEM, FTIR and XRD. The drug release pattern, material properties and antibacterial activity were evaluated. In addition, protein adsorption, cytocompatibility and alkaline phosphatase activity of the $CaSO_4$-Tet NP composite beads in comparison to the $CaSO_4$ control were analyzed.
Results: Tet NPs showed a size range of 130 ± 20 nm and the entrapment efficiency calculated was 89%. The composite beads showed sustained drug release pattern. Further the drug release data was fitted into various kinetic models wherein the Higuchi model showed higher correlation value ($R^2 = 0.9279$) as compared to other kinetic models. The composite beads showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The presence of Tet NPs in the composite bead didn't alter its cytocompatibility. In addition, the composite beads enhanced the ALP activity of hPDL cells.
Conclusions: The antibacterial and cytocompatible $CaSO_4$-Tet NP composite beads could be beneficial in periodontal management to reduce the bacterial load at the infection site.
General significance: Tet NPs would deliver antibiotic locally at the infection site and the calcium sulfate cement, would itself facilitate tissue regeneration.

1. Introduction

Periodontal disease causes severe inflammation, alveolar bone loss, tooth mobility, tooth loss and other serious complications if left untreated. Replacement of bone loss due to inefficient bone healing, and controlling the microbial infections are some of the noteworthy clinical challenges [1]. The main goal of treating the periodontal infections includes the reduction of bacterial load at the infection site which if left unattended may delay healing and affect new bone formation and periodontal ligament re-attachment [1,2]. The conventional treatment procedures involve the systemic delivery of high dosage antibiotics; however these led to systemic toxicities of the liver. In order to avoid such toxicity issues, and to improve the prognosis of the patients with such infections, local antibiotic delivery systems were developed. The main reason for using such local antibiotic delivery vehicles is the ability to achieve very high local concentrations of antibiotics without associated systemic toxicity [3,4]. Initially, studies used non-biodegradable bone cements with materials like poly(methyl methacrylate) (PMMA) [5–7], however these bone cements had one major disadvantage of requiring a second surgical intervention solely for their removal [8]. In order to avoid such disadvantages, the use of biodegradable materials, both natural and synthetic, came into play [9]. Ceramics are widely used as bone cements for the repair and reconstruction of diseased and damaged

Abbreviations: ALP, alkaline phosphatase; BCA, bicinchoninic acid; *O*-CMC, *O*-carboxymethyl chitosan; $CaCl_2$, calcium chloride; $CaSO_4$, calcium sulfate; $CaSO_4 \cdot 1/2H_2O$, calcium sulfate hemihydrate; CFU, colony forming units; DAPI, 4′,6-diamidino-2-phenylindole; DD, degree of deacetylation; DLS, dynamic light scattering; *E. coli*, *Escherichia coli*; EthD-1, ethidium homodimer-1; EtO, ethylene oxide gas; FBS, fetal bovine serum; FTIR, Fourier transform infrared spectroscopy; HAp, hydroxyapatite; hPDL, human periodontal ligament cells; L.B. broth, Luria Bertani broth; MEM, minimal essential media; NPs, nanoparticles; PBS, phosphate buffered saline; Pen–Strep, penicillin–streptomycin; PNPP, *p*-nitrophenylphosphate; K_2SO_4, potassium sulfate; SEM, scanning electron microscope; *S. aureus*, *Staphylococcus aureus*; Tet, tetracycline hydrochloride; XRD, X-ray diffraction

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parts of the body, and are generally termed as bioceramics. The most widely used bioceramics are calcium phosphate [10], and calcium sulfate [11]. Though calcium phosphate has been widely used as a bone void filler, calcium sulfate is a better option because of its biodegradable nature (resorption rate of calcium phosphate is very slow) [10].

Calcium sulfate hemihydrate ($CaSO_4 \cdot 1/2H_2O$) commonly known as Plaster of Paris has been used as a bone void filler since 1928. The increasing attention to calcium sulfate being used as bone cement is largely due to its biodegradable, biocompatible, and its injectable nature. It is also characterized by osteointegrity and new bone formation ability [11]. The dissolution property of calcium sulfate makes it possible to be used for drug release, such as antibiotics [11–14]. In addition, the dissolution rate of calcium sulfate in comparison to calcium phosphate can exceed the ability for new bone formation. However, the mechanical characteristics for most calcium sulfate compounds, show low compressive strength and a brittle appearance, thus are generally applicable to small bone defects [15].

Antibiotics such as tetracyclines, a group of broad spectrum antibiotics, exhibit activity against both gram positive as well as gram negative bacteria, and are clinically very efficient in controlling periodontal bone infections [16]. They are generally bacteriostatic in nature. Tetracycline (Tet) shows its antibacterial activity by entering through the bacterial cell wall and reversibly binding to the 30S ribosomal subunit thereby inhibiting protein synthesis and leads to bacteriostatic effect. In addition, Tet also reduces the inflammation at the infection site by blocking a protein, collagenase that destroys bone and other connective tissues [16,17].

In contrast to the work so far published, drug-nanoparticle based composite cement beads were developed to obtain the necessary antibacterial effect to treat bacterial infections and promote tissue regeneration. These drug based nanoparticles would help us to achieve the desired antibacterial effect at the lowest possible dosage of the drug. Besides drug encapsulation, polymeric nanoparticle is a well suited vehicle for antimicrobial drug delivery that allows sustained release of the drug, minimizes the toxicity associated with the conventional administration of bare drug and increases the bioavailability of the drug [18]. In the present study we have developed Tet-*O*-CMC NPs using *O*-carboxymethyl chitosan (*O*-CMC) with calcium chloride ($CaCl_2$) as a cross-linking agent. *O*-CMC has been widely used as the sustained drug-release carrier in pharmaceutical field and acts as a good antibiotic carrier. *O*-CMC is very advantageous due to its non-toxicity, biodegradability, biocompatibility, antibacterial, antifungal and bioactivity and therefore is a promising polymer derivative for bio-medical applications [19–21]. These Tet-*O*-CMC NPs (at minimal concentration of 2 wt.%) in the composite beads would maintain a local concentration of the drug with an extended duration of release without exceeding systemic toxicity thereby ensuing the necessary antibacterial effect [18,22]. Consecutively, the calcium sulfate on degradation/dissolution would promote bone tissue regeneration.

Hence the objective of this study was to fabricate a delivery system with ceramic bone cement, wherein, calcium sulfate loaded with antibiotic tetracycline nanoformulation would deliver tetracycline locally to achieve the required antibacterial effect. In addition, the bone cement would itself facilitate tissue regeneration.

2. Materials and methods

2.1. Materials

O-CMC (degree of deacetylation — 61.8% and degree of substitution — 0.54) was purchased from Koyo Chemical Co. Ltd. Japan, calcium chloride ($CaCl_2$), minimum essential medium (MEM), Triton X-100, paraformaldehyde, *p*-nitrophenylphosphate (PNPP) and potassium sulfate (K_2SO_4) were purchased from SIGMA Aldrich Company, calcium sulfate ($CaSO_4$) from Fischer Scientific, tetracycline hydrochloride (Tet), *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) and *Escherichia coli* (*E. coli*) (ATCC 25922) strains were provided by Microbiology Lab of Amrita Institute of Medical Sciences, Kochi, India. Luria Bertani broth (L.B. broth), agar–agar and alkaline phosphatase (ALP) were purchased from Himedia, India. Human periodontal ligament cells (hPDL) were obtained from Science Cell, USA. Glutaraldehyde was purchased from Fluka. Alamar blue, trypsin-EDTA, DAPI (4′, 6-diamidino-2-phenylindole), penicillin–streptomycin (Pen–Strep), fetal bovine serum (FBS), acridine orange and ethidium bromide were obtained from Gibco, Invitrogen Corporation. All chemicals were used as such with no further purification.

2.2. Preparation and characterization of Tet-O-CMC NPs

0.1% *O*-CMC solution was prepared as reported in literature [23]. 0.001% tetracycline (Tet) solution was prepared by dissolving Tet in ethanol–water mixture. This 0.001% Tet solution was added to 0.1% *O*-CMC solution in a ratio of 5:10 under constant stirring for 4 h. The solution was further cross-linked using 0.05% $CaCl_2$ solution that was added dropwise under continuous stirring until the formation of an opalescent suspension was observed [19]. The reaction of the Ca^{2+} ions (of the cross-linking agent) with the carboxyl group (of *O*-CMC) yielded tetracycline-carboxymethyl chitosan nanoparticles (Tet-*O*-CMC NPs) [22]. The NP suspension thus obtained was purified by centrifugation for 15 min at 15,000 rpm, lyophilized and used for further studies [19].

The particle size distribution of the NPs (in triplicates) was analyzed using dynamic light scattering (Malvern Zeta Sizer, Nano Series) and morphological evaluation was performed using a scanning electron microscope (JEOL JSM-6490LA Analytical SEM). The potential interactions between the constituents of the nanoparticle system (in triplicates) were analyzed using Fourier transform infrared spectroscopy (PerkinElmer Co, SPECTRUM RX1, FTIR).

2.3. Entrapment efficiency

The entrapment efficiency and loading efficiency were determined as reported in the literature [19,24]. It was calculated using the formula:

$$\text{Entrapment efficiency}(\%) = \frac{\text{Total amount of Tet added} - \text{Free Tet}}{\text{Total amount of Tet added}} \times 100$$

$$\text{Loading efficiency}(\%) = \frac{\text{Total amount of Tet}}{\text{Total amount of nanoparticles}} \times 100.$$

2.4. Preparation and characterization of $CaSO_4$ control and $CaSO_4$-Tet NP composite beads

100 mg of calcium sulfate ($CaSO_4$) was thoroughly mixed with required quantity of 3% potassium sulfate (K_2SO_4) solution to obtain a paste [25]. The paste was further molded into spherical bead form which served as the $CaSO_4$ control beads. For the composite beads, 2 wt.% of lyophilized Tet-*O*-CMC or Tet NPs (~89% encapsulation efficiency and 48% loading efficiency, wherein, the Tet quantity was found to be ~1 wt.%) were mixed with 100 mg $CaSO_4$ and 3% K_2SO_4 solution to obtain a paste further molded into spherical beads of 1 mm diameter. Several such beads were prepared for analytical and *in vitro* studies. These beads would facilitate periodontal regeneration by reducing gingival inflammation (gums) followed by restoration of normal alveolar bone level and periodontal ligament fibers in the affected area.

The structural morphology of the $CaSO_4$ control and $CaSO_4$-Tet NPs composite beads (in triplicates) was analyzed by SEM. The presence of Tet NPs in the composite bead (in triplicates) was confirmed through X-ray diffraction (XRD) (PAN analytical X'Pert PRO X-ray diffractometer) and FTIR analysis.