International Journal of Pharmaceutics 416 (2011) 305–313

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Drug intercalation in layered double hydroxide clay: Application in the development of a nanocomposite film for guided tissue regeneration

Michelle Chakraborti[a], John K. Jackson[a], David Plackett[b], Donald M. Brunette[c], Helen M. Burt[a,*]

[a] Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC, Canada V6T 1Z3
[b] Risø National Laboratory for Sustainable Energy, Technical University of Denmark—DTU, Building 124, Frederiksborgvej 399, P.O. Box 49, DK-4000 Roskilde, Denmark
[c] Department of Oral, Biological and Medical Sciences, Faculty of Dentistry, University of British Columbia, 2199 Wesbrook Mall, Vancouver, BC, Canada V6T 1Z3

ARTICLE INFO

Article history:
Received 11 February 2011
Received in revised form 9 June 2011
Accepted 12 June 2011
Available online 17 June 2011

Keywords:
Layered double hydroxide clay
Guided tissue regeneration
Controlled release
Alendronate
Tetracycline
Poly(lactic-co-glycolic acid)

ABSTRACT

It has been proposed that localized and controlled delivery of alendronate and tetracycline to periodontal pocket fluids via guided tissue regeneration (GTR) membranes may be a valuable adjunctive treatment for advanced periodontitis. The objectives of this work were to develop a co-loaded, controlled release tetracycline and alendronate nanocomposite plasticized poly(lactic-co-glycolic acid) (PLGA) film that would form a suitable matrix supporting osteoblast proliferation and differentiation.

Alendronate release was successfully controlled, with complete suppression of the burst phase of release by intercalation of alendronate anions in magnesium/aluminum layered double hydroxide (LDH) clay nanoparticles and dispersed in the PLGA film matrix. Tetracycline, loaded as free drug into the film together with alendronate–LDH clay complex released more rapidly than alendronate, but showed evidence of intercalation in the LDH clay particles. The dual drug loaded nanocomposite films were biocompatible with osteoblasts and after 5 week incubations, significant increase in alkaline phosphatase activity and bone nodule formation were observed.

1. Introduction

Periodontal diseases such as gingivitis and periodontitis, are inflammatory disorders that affect the tissues supporting the teeth (Pihlstrom et al., 2005) and are caused by bacterial infections in the tissues adjacent to the teeth. As the inflammation progresses, periodontal pockets or crevices, form between the gingival tissues and the tooth root, causing degeneration of the periodontium and resorption of alveolar bone, which can lead to tooth loosening and eventually, tooth loss (Haffajee and Socransky, 1986). Significant bacterial loads and a broad range of microflora are commonly found in periodontal pockets (Pihlstrom et al., 2005).

The first-line, non-surgical approach to treating periodontal disease includes removal of dental plaque and calculus and the adjunctive localized delivery of antibiotics to the gingival crevicular fluid (GCF) within the periodontal pocket (Pihlstrom et al., 2005), via irrigation solutions or controlled release gels, fibres and implants (Agarwal et al., 1993; Webber et al., 1998). For more advanced disease, surgical strategies include guided tissue regeneration (GTR), a method in which a barrier film is surgically placed between the tooth and gingival connective tissue, allowing the detached root surface to be repopulated with regenerating cells such as osteoblasts and periodontal ligament cells (Gottlow et al., 1986; Nyman et al., 1982). The film acts as a mechanical barrier allowing undisturbed and guided bone tissue regeneration (Gottlow et al., 1986; Nyman et al., 1982; Wang et al., 2002). Bioabsorbable membranes composed of collagen, calcium sulphate or synthetic polyesters may promote periodontal regeneration, by providing a protected space for inward migration of regenerating cells, and the field has recently been reviewed by Villar and Cochran (2010). Retention of the physical integrity of the membrane was suggested to be about 6 weeks for the healing process, after which biodegradation and resorption would be optimal. However, it was noted that these biodegradable GTR membranes possess only a limited clinical efficacy since they have no biological effects on cellular proliferation or differentiation (Villar and Cochran, 2010). Furthermore, significant variability in the surgical outcomes of GTR procedures has been observed (Gottlow et al., 1986) frequently due to bacterial colonization of the membrane following its placement into the periodontal pocket, which may limit the proliferation of the regenerating cells like osteoblasts (Tempro and Nalbandian, 1993).

Our group has developed biodegradable, plasticized poly(lactic-co-glycolic acid) (PLGA) films loaded with either tetracycline or alendronate for potential application as GTR membranes (Long et al., 2009; Owen et al., 2010). Tetracycline and other similar broad spectrum antibiotics, including minocycline and doxycycline, have been used extensively in the treatment of periodontal disease. Tetracycline not only eliminates or reduces microbial load in the

* Corresponding author. Tel.: +1 604 822 2440; fax: +1 604 822 3035.
E-mail address: burt@interchange.ubc.ca (H.M. Burt).

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doi:10.1016/j.ijpharm.2011.06.016

Table 1
Target properties and formulation strategies of a co-loaded antibacterial/bisphosphonate guided tissue regeneration (GTR) membrane for application in periodontal therapy.

Feature of GTR membrane	Target properties and characteristics
Placement	Between tooth and gingival connective tissue
Tissue–membrane interface	Biocompatible for tissue integration
Lifetime of GTR membrane	Biodegradable PLGA-based film to avoid surgical removal Biodegradation lifetime >4–6 weeks for periodontal attachment
Mechanical properties	Initial handling: flexible, with some elasticity to conform and stretch around tooth. MePEG350 used as a plasticizer After placement: stiffening to protect defect space. MePEG diffuses out of film in aqueous fluids
Drug loading and approx release lifetime	
Antibacterial	Tetracycline: protect from bacterial invasion (release over ~2 weeks)
Bisphosphonate	Alendronate: enhance bone growth (release over ~4–6 weeks)
Reduction in alendronate burst phase of release	Alendronate bound to nano-clay particles and dispersed in polymer matrix
Other	Sterile Chemical stability maintained

GCF, but it also inhibits metalloproteinases (collagenases) that breakdown collagen and exacerbate inflammation (Golub et al., 1998, 1991; Rifkin et al., 1993; Weinberg and Bral, 1998). Alendronate, an amino-bisphosphonate, is an endogenous regulator of bone turnover and a potent inhibitor of osteoclastic resorption and may trigger the proliferation of osteoblasts (Fleisch, 1998; Im et al., 2004; Reinholz et al., 2000; von Knoch et al., 2005). Commonly, the drug is used for the treatment of bone disorders such as osteoporosis, Paget's disease and osteolytic bone metastases (Fleisch, 2007) and is proposed for use in the treatment of periodontal disease (Shinoda and Takeyama, 2006; Tenenbaum et al., 2002). Studies have shown that the local delivery of alendronate may improve the bone growth around dental implants (Yaffe et al., 1997, 2003).

In our previous work, we demonstrated that, although controlled release of alendronate could be achieved from 0.1% and 0.25% alendronate loaded PLGA films over 30 days, there was only a marginal increase in osteoblast cell numbers and a higher alendronate loading (0.5%) in the film produced evidence of osteoblast toxicity, thought to be due to the very large burst phase of alendronate release (Long et al., 2009). We hypothesized that a combination drug loaded GTR film formulation of tetracycline and alendronate, that would also eliminate the burst phase of alendronate release, would be effective in promoting osteoblast viability and proliferation. Our goal was to develop co-drug loaded films with target properties as summarized in Table 1. The use of anionic clay nanoparticles to form an intercalation compound with alendronate was explored to provide an additional controlled release strategy for alendronate.

Layered double hydroxides have the general formula: $M^{II}_{1-x}M^{III}_{x}(OH)_2Y^{n-}_{x/n} \cdot mH_2O$ where M^{II} and M^{III} represent the divalent and trivalent metal cations, respectively and Y^{n-} represents the hydrated exchangeable anions present in the interlayer space (Parello et al., 2010). They possess a basic structure of brucite [$Mg(OH)_2$]. The brucite-like sheets are positively charged due to the partial substitution of divalent metal cations by trivalent ions. The positive charge is balanced by interlayer anions like carbonates along with water molecules, therefore rendering the structure electrically neutral. The layered structure is stabilized by hydrogen bonding among the water molecules, anions and hydroxide layers (Gasser, 2009) and a schematic representation is shown in Fig. 1. Layered double hydroxides possess a high specific surface area and high layer charge density (2–5 mequiv./g), which results in strong electrostatic forces between the brucite sheets and anions. They also possess anion exchange properties (Choy et al., 2007; Del Hoyo, 2007). Recent studies have shown that LDH clay can bind with, and retain different drugs and modulate and/or delay their release (Aguzzi et al., 2007; Ambrogi et al., 2002; Evans and Duan, 2006; Li et al., 2004; Mohanambe and Vasudevan, 2005; Pihlstrom et al., 2005; Trikeriotis and Ghanotakis, 2007; Zhang et al., 2006; Zhang, 2004).

The objectives of this work were to develop a co-loaded, controlled release tetracycline and alendronate nanocomposite plasticized PLGA film, with the target characteristics appropriate for a GTR membrane (Table 1), eliminating the burst phase of alendronate release and forming a suitable matrix supporting osteoblast proliferation and differentiation.

2. Material and methods

2.1. Materials

Poly(D,L lactic-co-glycolic acid) (PLGA), with a weight percentage ratio of 85/15 (intrinsic viscosity of 0.61 dL/g) was obtained from Birmingham Polymers (Birmingham, AL). Methoxy poly(ethylene glycol) (MePEG) (molecular weight 350 g/mol) was obtained from Union Carbide (Danbury, CT). Alendronate sodium trihydrate and tetracycline hydrochloride were obtained from Sigma–Aldrich (St. Louis, MO). Aluminum/magnesium carbonate layered double hydroxide clay (LDH clay) was kindly provided by Dr. David Plackett (Risø DTU National Laboratory, Copenhagen). All solvents used were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ).

2.2. Size analysis and elemental analysis of layered double hydroxide clay

LDH clay samples contained particle aggregates and were therefore de-aggregated by ultrasonicating a suspension of LDH clay in ethanol before size analysis was performed using the Malvern Zetasizer Nano ZS (Malvern Zetasizer Ltd., UK) and elemental analysis using the Hitachi S-3000N scanning electron microscope-energy-dispersive X-ray spectroscopy analysis (SEM-EDX) (Tokyo, Japan). Samples were coated with a 60:40 alloy of gold:palladium using a Denton Vacuum Desk II sputtercoater (Moorestown, NJ) at 50 Torr.

2.3. Drug binding to layered double hydroxide clay

Increasing concentrations of alendronate in deionized water (15.625 μg/mL–4 mg/mL) were added to LDH clay (2 mg). The dispersion was vortexed for 2 min and incubated for 1 h in a rotary shaker (37 °C), followed by centrifugation for 5 min (18,000 × *g*). The supernatant (representing the unbound concentration of alendronate) was analyzed using HPLC with fluorescence detection (Long et al., 2009) as described below. The amount of alendronate bound was calculated by subtracting the unbound amount from the total amount of drug added. A binding study was carried out using tetracycline, as described above. Supernatants were analyzed using HPLC with UV detection (Owen et al., 2010) (see below) and the amount of tetracycline bound by clay determined.

2.4. Characterization of the alendronate–LDH clay complex

2.4.1. X-ray powder diffraction

X-ray powder diffraction patterns of alendronate loaded clay and control clay samples were obtained using a Bruker D8 advanced

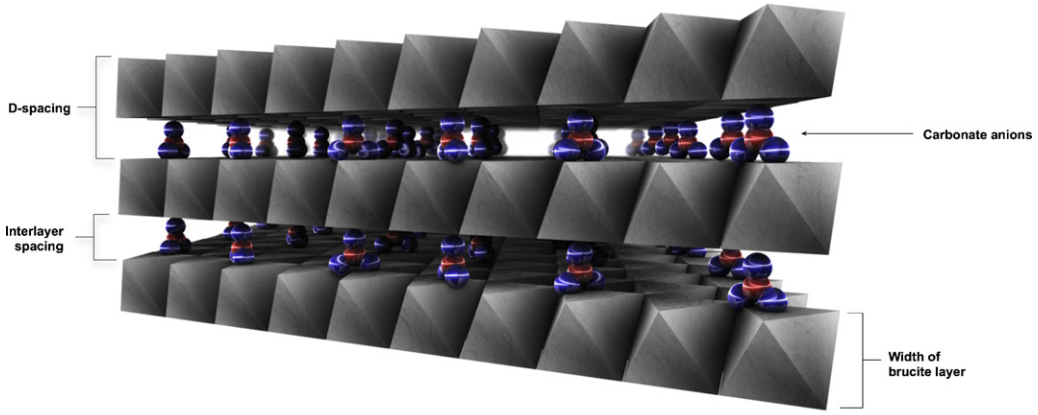

Fig. 1. Graphical representation of the structure of layered double hydroxide clay. The figure depicts the presence of carbonate anions in the interlayer space.

diffractometer with a Cu source at 25 °C. Approximately 100 mg of sample was packed into the sample holder and scanned from 2° to 60° 2θ using a step size of 0.020° and step time of 1 s/step.

2.4.2. Differential scanning calorimetry (DSC)

Samples (about 5 mg) of LDH clay or alendronate–LDH clay complex were placed in a crimped aluminum DSC pan and heated between 30 and 300 °C at a heating rate of 10 °C/min under nitrogen flow.

2.4.3. Alendronate release from LDH-clay complex

In vitro release studies were carried out in phosphate buffered saline (PBS) (0.1 mM; pH 7.4) at 37 °C. Alendronate (2 mg) was bound to LDH clay (2 mg) using the procedure described above. This was followed by the incubation of the complex with PBS (1 mL) for pre-determined time intervals, after which it was centrifuged (18,000 × *g*) for 10 min. The supernatant was withdrawn for analysis and replaced by PBS (1 mL).

2.5. Preparation of the dual-drug loaded nanocomposite film formulations

Film casting solutions were prepared by dissolving PLGA in dichloromethane at a concentration of 20% (w/v) of polymer solution, followed by the addition of MePEG (15%, w/w) to the polymeric solution. Tetracycline hydrochloride (5%, w/w) was pre-dissolved in a minimal volume of dimethylsulphoxide and added to the above prepared film casting solution. Alendronate (5%, w/w) was bound to LDH clay as described above. The alendronate–LDH clay complex did not disperse readily in the polymer solution, so that 100 μL polysorbate 20 (2%, v/v) was added to aid dispersion of the complex in the polymeric solution. The dispersion formed was added to the film casting solution and mixed vigorously by ultrasonication and vortexing until the complex was homogeneously suspended, following which, a 100 μL aliquot of the dispersion was cast on 1 cm × 1 cm Teflon templates applied to glass slides.

2.6. Characterization of the nanocomposite film formulations

2.6.1. Drug release studies

The films were weighed individually and placed in 20 mL glass vials. To the vials, were added 5 mL PBS (pH 7.4, 0.1 mM) and the samples were incubated in a rotary shaker at 37 °C. At predetermined intervals, all the PBS was withdrawn and replaced with fresh PBS. The samples were analyzed using HPLC analysis methods for alendronate and tetracycline.

2.6.1.1. Analysis of alendronate. Samples were assayed for alendronate using HPLC with fluorescence detection using previously described methods (Long et al., 2009). Briefly, samples were prepared by adding 100 μL of the drug release sample to 900 μL of 0.13 M EDTA buffer (pH 10) and 500 μL of fluorescamine dissolved in acetonitrile (2 mg/mL). The samples were then gently agitated until a yellow colored solution was formed (approximately 10 s), 1 mL dichloromethane was added and the samples were shaken vigorously. The solution separated into two layers. The yellow upper layer was withdrawn for HPLC analysis with fluorescence detection (λ_{ex} = 395 nm, λ_{em} = 480 nm). Analysis of alendronate was performed on a Waters HPLC system (Milford, MA, USA) consisting of a model 717 plus autosampler, 1525 binary HPLC pump and 2475 multi λ fluorescence detector. The mobile phase was composed of EDTA (1 mM)/methanol (97:3 v/v) mixture (pH 6.5) with a flow rate of 1 mL/min (Alltech nucleosil 100 C_{18} column).

2.6.1.2. Analysis of tetracycline hydrochloride. The samples were assayed for tetracycline hydrochloride using HPLC with UV absorbance detection at 358 nm (Owen et al., 2010). Analysis was performed on a Waters HPLC system (Milford, MA, USA) consisting of a model 717 plus autosampler, 1525 binary HPLC pump and 2487 dua λ absorbance detector. The mobile phase was composed of 12% (v/v) acetonitrile in 1.3 g/L oxalate solution (pH 2.1), having a flow rate of 1 mL/min (reverse phase C_{18} Novapak column).

2.6.2. Stress–strain determination

Stress–strain experiments were carried out at room temperature using a thermal mechanical analyzer (TMA) (TA Instruments, New Castle, DE) as previously described (Jackson et al., 2004). The rectangular films were measured using digital calipers (Mitutoyo, Japan). The film was subjected to increased force per unit length and the film extension was measured. Film recovery was observed before adding more weight. Measurements were discontinued when the membrane ceased to recover. Film extension was measured after incubation in PBS (pH 7.4) for predetermined time intervals. Film stiffness was calculated in terms of Young's modulus (stress/strain).

2.7. Osteoblast culture

Calvarial osteoblasts (from newborn Sprague Dawley rats) were kindly provided by Dr. Donald Brunette (Department of Oral, Biological and Medical Sciences, Faculty of Dentistry, UBC, Vancouver, Canada). The cells were subcultured by trypsinization as described previously (Long et al., 2009) and maintained in minimal essential medium (α-MEM) (Stem Cell Technologies, Vancouver,

Canada) that was composed of fetal calf serum (15%; Cansera International, Rexdale, ON, Canada), amphotericin B (Fungizone®) (3 μg/mL; Gibco, Grand Island, NY, USA), penicillin G (100 μg/mL) and gentamycin (50 μg/mL) (Sigma–Aldrich, St. Louis, MO, USA) in a humidified atmosphere with CO_2 (5%) at 37 °C. The experiments were performed in 24 or 48 well Falcon™ tissue culture treated plates (BD Bioscience). The polymeric films were sterilized by glow discharge using the plasma sterilizer (PDC 32-G connected to a high performance vacuum pump SPX, Owatonna, MN, USA). The osteoblasts used were between a 5 and 10 cell passage number.

2.8. Osteoblast viability

Cell viability was determined by the MTS assay kit (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay; Promega, WI, USA).

The films used in the study were prepared as described above and were composed of the following groups: free alendronate and alendronate bound to LDH clay in the concentrations, 0.03%, 0.1%, 0.75% and 4.2% (w/w). The films were placed in the individual wells of the plate ($n = 6$), followed by the addition of media (500 μL) and osteoblast cells (1×10^4) into each well. The plates were incubated for 2 days after which the media was removed from the wells and washed with PBS. Finally, the MTS reagent (200 μL) was added to each well and the plate was incubated for 3 h. The absorbance was measured at 492 nm (subtracting the blank reading at 600 nm) using the Labsystems Multiskan Ascent photometric plate reader (Labsystems, Helsinki, Finland).

2.9. Alkaline phosphatase activity

Bone specific alkaline phosphatase (ALP) is a membrane bound exoenzyme produced by osteoblasts. The presence of the enzyme indicates the onset of osteoblastic cell differentiation, thus it is used as a marker for bone formation (Long et al., 2009). Osteoblasts were grown for a period of 5 weeks in wells of tissue culture plates. Control wells had no films. Sterilized films, placed in individual wells, were composed of tetracycline, tetracycline and free alendronate, and tetracycline and alendronate–LDH clay complex. As described previously, the films in each well were seeded with 1×10^4 cells per well and reseeded after 2 days with another 1×10^4 cells in 500 μL media, supplemented with 10 mM sodium beta glycerophosphate and ascorbic acid (50 μg/μL). After 5 weeks of culture, the media was removed and each well was washed three times with PBS. The osteoblast cell extract was prepared by lysing the cells with a probe sonicator for 20 s in 1 mL Tris buffer (0.1 M, pH 7.2) containing 0.1% Triton-X 100 at 4 °C. The sonicated cell lysates were then centrifuged at 1500 × *g* (for 10 min), at 4 °C and the supernatant obtained was used in the assay for analysis of alkaline phosphatase activity. Alkaline phosphatase activity of the osteoblasts was determined spectrophotometrically at 405 nm by quantifying the conversion of p-nitro phenyl phosphate to p-nitro phenol, on the addition of 50 μL cell extract to 150 μL alkaline phosphatase reagent (Promega, WI, USA). ALP activity was normalized to the total protein content of the cells. Protein content was determined spectrophotometrically using a Micro BCA protein assay kit (Thermo Scientific, IL, USA).

2.10. Bone nodule formation

Bone nodules deposited by osteoblasts, were observed by alizarin red dye staining (Sigma–Aldrich, St. Louis, MO) of cells after 5 weeks of cell growth in wells of tissue culture plates. Control wells had no films. Sterilized films, placed in individual wells, were composed of tetracycline, tetracycline and free alendronate, and tetracycline and alendronate–LDH clay complex as described previously for the ALP assay (Long et al., 2009). Briefly, the osteoblast cells present were fixed with 1 mL para-formaldehyde solution (4%, w/v, Sigma) for 24 h, following which the fixative was removed and each well was washed three times with PBS. To each well, 500 μL alizarin dye in PBS (20 mg/mL) were added for 5 min, after which the dye was removed and each well was washed three times with PBS in order to remove excess stain.

The dye from the stained nodules was extracted using cetyl pyridinium chloride (10%, w/v, in PBS; Sigma–Aldrich) as previously described (Stanford et al., 1995). Briefly, cetyl pyridinium chloride (1 mL) was added to each well. After the dye was extracted, the solution was transferred into the 96-well plates, and its absorbance was measured at 540 nm using the Labsystems Multiskan Ascent photometric plate reader (Labsystems, Helsinki, Finland).

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism® version 5 (GraphPad Software, San Diego, CA). Significant differences among the groups was determined using a one way ANOVA followed by Turkey–Kramer's post hoc test with a significance level $P < 0.05$.

3. Results

3.1. Size and composition of LDH clay

The LDH clay particles possessed an average diameter of 557 ± 95 nm. Surface X-ray analysis showed the following elements and their amounts by weight %: carbon (18.24), oxygen (59.59), sodium (0.35), magnesium (15.68) and aluminum (6.13), indicating the clay sample was a magnesium aluminum hydroxycarbonate.

3.2. Adsorption isotherm and characterization of alendronate–LDH clay complex

Alendronate bound to LDH clay particles in a concentration dependent manner. Saturation binding was observed at an alendronate concentration of approximately 2 mg/mL, where about 600 μg alendronate was bound per mg LDH clay (~32%, w/w, drug bound) (Fig. 2).

The X-ray diffraction patterns for LDH clay and alendronate–LDH clay complex samples are shown in Fig. 3. LDH clay samples showed a strong X-ray diffraction peak at 11° (2θ) which corresponded to a d-spacing of 0.77 nm. Alendronate–LDH clay complex sample showed a smaller peak at 11° and an

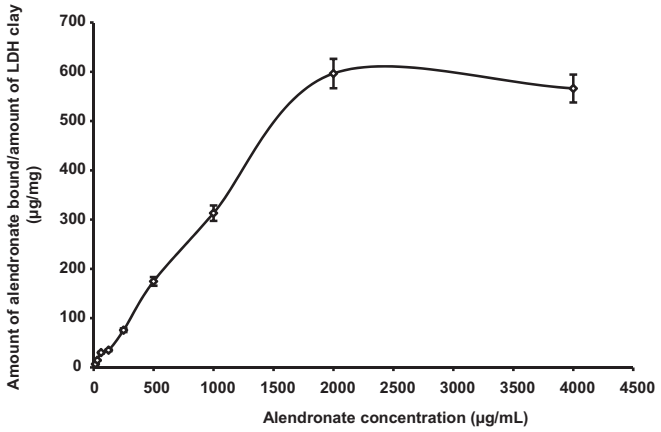

Fig. 2. Binding isotherm: alendronate bound to layered double hydroxide clay at room temperature.

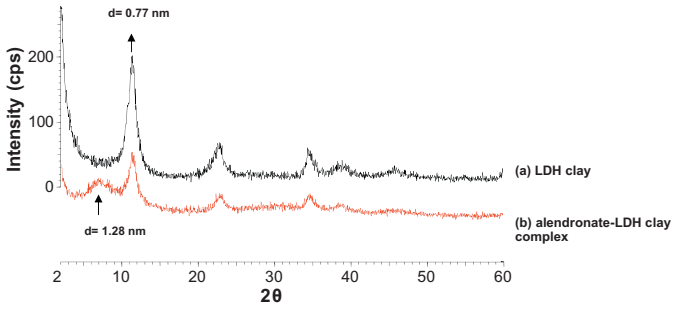

Fig. 3. X-ray diffraction patterns of: (a) layered double hydroxide clay and (b) alendronate-layered double hydroxide clay complex. The arrows indicate the d-spacing at 11° and 7° 2θ, respectively.

additional peak at 7° (2θ) corresponding to a d-spacing of 1.28 nm (Fig. 3).

DSC scans for both LDH clay and alendronate–LDH clay complex showed very similar broad endotherms beginning at about 70 °C, with the peaks occurring at 127 °C (data not shown) indicative of loss of adsorbed and intercalated water from the clay particles. These LDH type clays, with and without intercalated drug, are well known to exhibit this distinctive decrease in water content between 70 and 200 °C (Gasser, 2009).

Fig. 4 shows the alendronate release profile from alendronate–LDH clay complex. There was a very large initial burst release of alendronate (~45%) from the LDH clay particles over the first 6 h, followed by a period of slower sustained release, with approximately 80% drug released in 6 days.

3.3. Characterization of the nanocomposite film formulation

The film matrix used in these studies was a miscible blend of 15% MePEG in PLGA. Previous studies in our lab have shown that the addition of MePEG to PLGA causes a concentration dependent decrease in the T_g. The MePEG was added as a plasticizing agent and produced a film with a glass transition temperature (T_g) of 11 °C (Jackson et al., 2004). The films were prepared by the solvent evaporation method, with co-loading of the drugs accomplished by addition of free tetracycline and alendronate as the alendronate–LDH clay complex.

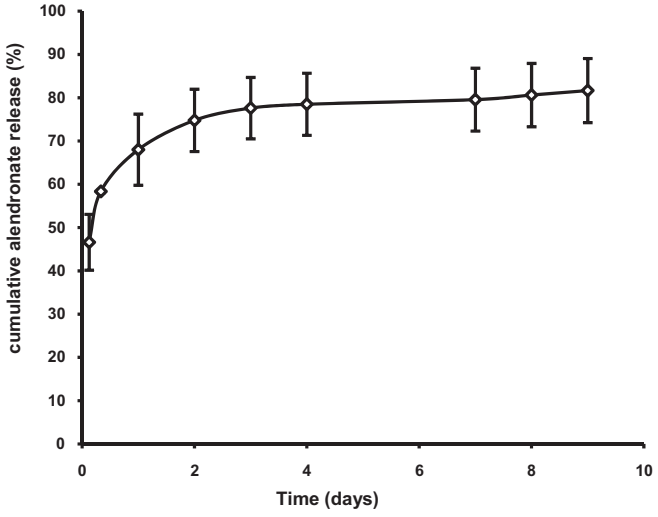

Fig. 4. Cumulative alendronate release from alendronate-layered double hydroxide clay complex in PBS (pH 7.4) at 37 °C under gentle agitation. Error bars indicate standard deviation among $n = 3$ samples.

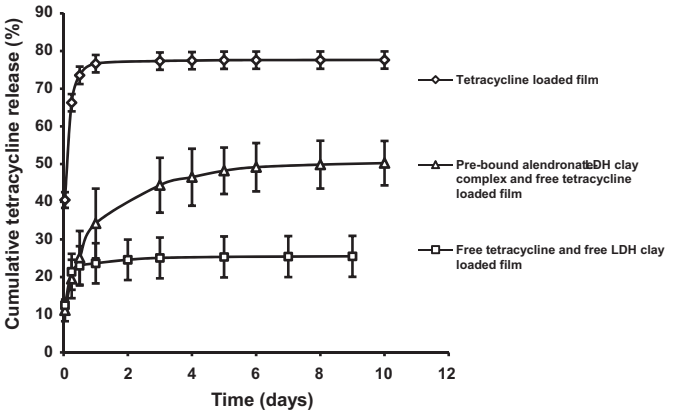

Fig. 5. Cumulative release of tetracycline from films composed of a polymeric blend of PLGA (85:15) and MePEG (15%, w/w) loaded with (%, w/w): (◊) 5% tetracycline, (△) 5% tetracycline and 4.2% alendronate–LDH clay complex, (□) 5% tetracycline and LDH clay [at 37 °C; in PBS (0.1 mM)] ($n = 5$).

3.3.1. Tetracycline and alendronate release from film formulations

No absorbance or fluorescence interference was observed between drugs during the absorbance (tetracycline) or fluorescence (alendronate) HPLC assays of these drugs. The release profiles for tetracycline from films loaded either with free tetracycline, free tetracycline and free LDH clay particles or nanocomposite films co-loaded with free tetracycline and alendronate–LDH clay complex are shown in Fig. 5. Films loaded with free tetracycline showed a burst phase of release of about 65% in the first 6 h and almost 80% drug released over the next 3 days. However, the films loaded with free tetracycline and free LDH clay showed a significantly reduced tetracycline burst phase, down to about 10% drug release at 6 h and 25% released over 7 days. Similarly, the tetracycline/alendronate co-loaded nanocomposite film produced a significantly reduced tetracycline burst phase down to about 10% with 50% of tetracycline released over 7 days (Fig. 5).

On completion of the release studies, the films were dissolved in order to determine the recovery of intact tetracycline by HPLC. About 80% (w/w) tetracycline was released from tetracycline loaded films and upon dissolution of the films, no additional tetracycline was detected. From the dual drug loaded nanocomposite films 50% (w/w) tetracycline was released and on dissolving these films, about 28% (w/w) of intact tetracycline was recovered, indicating that about 80% (w/w) of the total drug present could be accounted for.

Given the greatly reduced tetracycline release from nanocomposite films compared to tetracycline when loaded as the free form in the films, it was possible that tetracycline was adsorbed or bound by the LDH clay particles loaded in the nanocomposite films. Tetracycline was shown to bind to LDH clay particles with a binding curve very similar to that of alendronate (Fig. 2), with maximal uptake of about 600 μg tetracycline bound per mg of clay at a concentration of 4 mg/mL tetracycline (data not shown).

Alendronate release profiles from free alendronate loaded films, showed 35% burst phase with a slower release phase of up to 80% after 10 days (Fig. 6) whereas nanocomposite films co-loaded with free tetracycline and alendronate–LDH clay complex produced no burst phase and a slow controlled release of alendronate of about 15% over 10 days. When LDH clay was included in films loaded with free alendronate, the release rate of alendronate showed a significantly lower burst phase release of 6%, followed by a slow increase in release up to 25% over a period of 9 days.

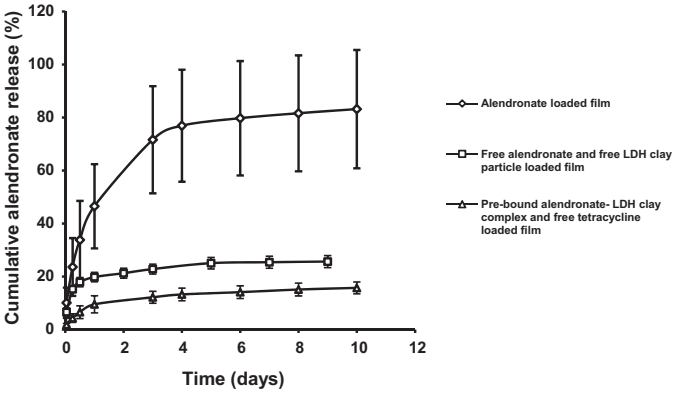

Fig. 6. Cumulative release of alendronate from films composed of a polymeric blend of PLGA (85:15) and MePEG (15%, w/w) loaded with (%, w/w): (◊) 4.2% alendronate, (△) 5% tetracycline and 4.2% alendronate–LDH clay complex, (□) 4.2% alendronate and LDH clay [at 37 °C; in PBS (0.1 mM)] ($n = 5$).

The $t_{1/2}$ values for alendronate and tetracycline release from the lead formulations (i.e. free tetracycline loaded into films containing alendronate pre-bound to LDH clay) were 91 days and 9 days, respectively.

3.3.2. Stress–strain determination by thermal mechanical analysis (TMA)

PLGA films plasticized with 15% MePEG have excellent mechanical properties (Table 1) being flexible, with some elasticity. Previous work in our laboratory has shown that following incubation of MePEG/PLGA films in PBS, the water soluble MePEG is rapidly released from the film (Owen et al., 2010), producing a film that, when handled, appears stiffer with less flexibility. Young's modulus (Y) defined as the ratio of stress (force per unit area) to strain (change in length per unit length) is a measure of the material stiffness. Films incubated for different time periods in PBS (Fig. 7) showed an increase in Young's modulus over 2 h, indicating enhanced stiffness of the films.

3.4. Osteoblast viability

Osteoblast viability was evaluated in free alendronate loaded films and alendronate–LDH clay complex loaded films. There

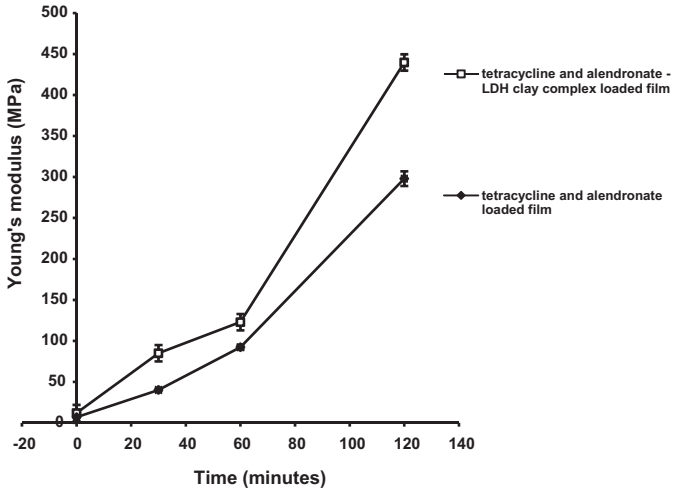

Fig. 7. Variation of Young's modulus with an increase in incubation time in aqueous media (0.1 mM PBS; 37 °C) on films composed of a polymeric blend of PLGA (85:15) and MePEG (15%, w/w) loaded with (%, w/w): (♦) 5% tetracycline and 4.2% alendronate (□) 5% tetracycline and 4.2% alendronate–LDH clay complex ($n = 3$).

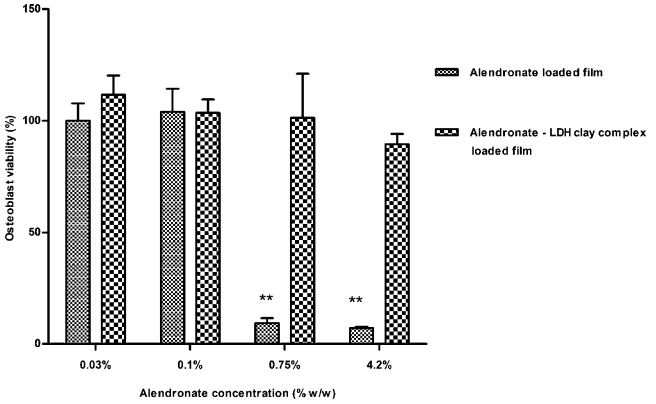

Fig. 8. Osteoblast viability after a two day incubation period on films composed of a blend of PLGA (85:15) and MePEG (15%, w/w) loaded with 0.03, 0.1, 0.75 and 4.2 (%, w/w): (a) alendronate and (b) alendronate–LDH clay complex. Percent osteoblast viability expressed as mean ± S.D ($n = 6$). $^{**}P < 0.05$.

was no change in osteoblast viability at low alendronate concentrations (up to 0.1%, w/w) for the free or bound alendronate films (Fig. 8). However, when the alendronate concentration was increased to 0.75% and 4.2%, a significant decrease in cell viability was observed in films containing free alendronate but cell viability was unchanged in the films containing the same concentration of alendronate bound to LDH clay (1:1 ratio).

3.5. Alkaline phosphatase activity (ALP) and bone nodule formation

The effect of alendronate released from film formulations on the ALP activity (Fig. 9) and bone nodule formation of osteoblasts (Fig. 10) was evaluated. There was no significant difference in the ALP activity and bone nodule formation in the plates containing no film controls, free tetracycline loaded films and free tetracycline plus free alendronate loaded films. However, a significant

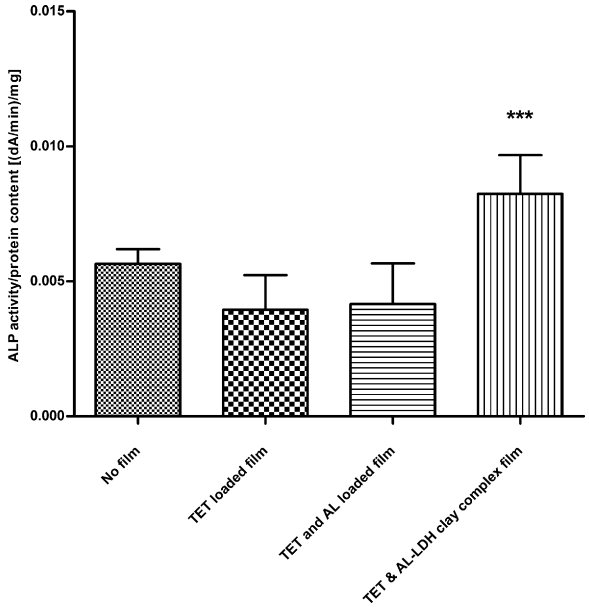

Fig. 9. Alkaline phosphatase activity of osteoblasts grown for 5 weeks on (A) no films; (B) films composed of a blend of PLGA (85:15) and MePEG (15%, w/w) loaded with (%, w/w) (a) 5% tetracycline, (b) 5% tetracycline and 4.2% alendronate and (c) 5% tetracycline and 4.2% alendronate–LDH clay complex. Data expressed as mean ± S.D ($n = 12$). $^{***}P < 0.05$. TET and AL: abbreviations used for tetracycline and alendronate, respectively.

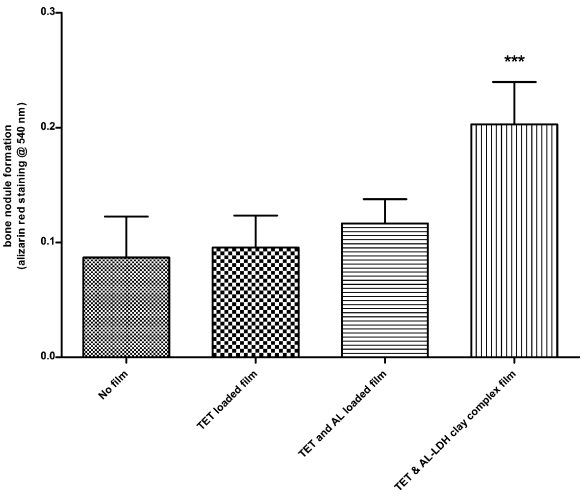

Fig. 10. Bone nodule formation. Osteoblasts grown for 5 weeks on (A) no films (B) films composed of a blend of PLGA (85:15) and MePEG (15%, w/w) loaded with (%, w/w) (a) 5% tetracycline, (b) 5% tetracycline and 4.2% alendronate and (c) 5% tetracycline and 4.2% alendronate–LDH clay complex. Data expressed as mean ± S.D ($n = 12$). $^{***}P < 0.05$. TET and AL: abbreviations used for tetracycline and alendronate, respectively.

increase in both the ALP activity and bone nodule formation was observed for the free tetracycline and alendronate–LDH clay complex nanocomposite film compared to the free drug loaded film formulations.

4. Discussion

Untreated periodontal disease is characterized by advanced bacterial infection that leads to the spread of inflammation into the deeper tissues and alveolar bone resorption. Therefore, the objective of our study was to co-deliver both an anti-bacterial agent, tetracycline, and a bone resorption inhibitory agent, alendronate, into the periodontal pocket using a biocompatible and biodegradable film with a proposed degradation/drug release lifetime of about 4–6 weeks, suitable mechanical properties and importantly, allowed suppression of the burst phase of release of the water soluble alendronate from the film to avoid osteoblast toxicity issues (Table 1).

PLGA, the biodegradable polymer used in the study, formed the major component by weight of the film matrix. Although degradation of this polymer is associated with acidity, we do not anticipate that low levels of acid production over an extended time course of degradation may cause any irritation in the gingival cavity in vivo. The crevicular fluid present in the periodontal pocket has a high turnover rate (Etienne, 2003) and it may buffer any acidity produced. Also the thin nature of the PLGA film may prevent the creation of internal pools of acid that might release spontaneously. A recent study by Ho et al. (2011), using PLGA nanoparticles loaded with lovastatin in the rat mandibular defect suggested that the buffering capacity of the tissue fluid in vivo may be one of the reasons for not observing a drop in the pH (caused due to PLGA acidity) in the surrounding fluid. Furthermore, there are currently no available reports or studies suggesting that the acidity of PLGA polymers in commercially available periodontal formulations lead to irritation/harmful responses that may affect periodontal tissue regeneration in the periodontal cavity (Villar and Cochran, 2010).

Extensive binding of alendronate to the LDH clay particles of around 32% (w/w) was observed, similar to other reports of binding of anionic drugs to LDH clays, including captopril (Zhang et al., 2006), non-steroidal anti-inflammatory compounds (Ambrogi et al., 2002; Mohanambe and Vasudevan, 2005) and antibiotics (Trikeriotis and Ghanotakis, 2007). The binding has been shown to be due to the anion exchange properties of the LDH clay and replacement of the interlayer anions with drug anions. Ambrogi et al. (2001) showed the binding of ibuprofen to an Mg/Al-based LDH clay composed of exchangeable chloride ions to be 0.5 g ibuprofen per 1 g of LDH-ibuprofen intercalated product. The drug anions were incorporated until all the clay chloride ions had been exchanged by ibuprofen.

These guest–host intercalation structures can be analyzed by X-ray diffraction, as shown in Fig. 3. LDH clay showed three relatively intense peaks at low 2θ, indicative of a crystallized form of LDH clay. The interlayer distance of the LDH host particles is represented by the peak at 2θ of 11° with a d-spacing of 0.77 nm. As a consequence of intercalation of alendronate ions, the interlayer distance increased to a d-spacing value of 1.28 nm. The thickness of the brucite layer, shown schematically in Fig. 1 has been reported by several groups as 0.48 nm (Ambrogi et al., 2001; Zhang et al., 2006). Overall, an expansion of about 0.5 nm was observed upon alendronate intercalation which is lower than for ibuprofen intercalation into a nitrate exchanging anionic clay (Ambrogi et al., 2001) but likely reflects easier accommodation of the alendronate anion into the interlayer space and less requirement for lattice expansion. The peak at 11° 2θ did not disappear completely for the alendronate–LDH clay complex, indicating that not all interlayer sites exchanged with alendronate. Ambrogi et al. (2002) also showed that the original anionic clay (with exchangeable chloride ions) showing a d-spacing of 0.78 nm was retained in the X-ray diffraction pattern following intercalation of diclofenac into the clay.

The release profile of the alendronate–LDH clay complex in PBS is characterized by an initial burst phase release followed by a slower sustained release of the remaining drug over 4–6 days (Fig. 4). Alendronate release from the LDH clay occurred as phosphate and/or chloride ions in the PBS buffer exchanged with alendronate and allowed alendronate anions to be released from the clay structure into the release medium. Transport of alendronate ions out of the matrix is thought to be controlled primarily via diffusion out of the clay matrix and is related to the rigidity of the layers and the diffusion path length (Ambrogi et al., 2001). The kinetic treatment of the data (plotted as a square root function of time) demonstrated linearity, further confirming that the release process occurs by diffusion (data not included). It has been suggested that the initial large burst phase of release is due to anion exchange near the external regions of the clay particles, with concurrent decrease in interlayer distance in these regions. Release then becomes slower and more controlled as a result of the diffusional path length and tortuosity becoming increased for diffusion of the larger drug anions out of more internal regions of the clay particles (Ambrogi et al., 2001). Interestingly, most reports of intercalated drug release from anionic LDH clays show release to be very rapid and complete within minutes (Ambrogi et al., 2001; Li et al., 2004; Trikeriotis and Ghanotakis, 2007; Zhang et al., 2006) to several hours (Ambrogi et al., 2002). Gasser (2009) also noted that, whereas most drugs are shown to release rapidly via ion exchange from the clays, the release of vitamin C from intercalated LDH clays was not completed within 120 min. Gasser (2009) suggested that the much slower release of vitamin C from the LDH clays was due to drug molecules being deeply embedded in the LDH host structure.

We have suggested that the targeted mechanical properties of the GTR film require a flexible, elastic film for initial placement, which was achieved by the blending of 15% MePEG into the PLGA (Table 1). The Young's modulus values for these plasticized films loaded with either free tetracycline/alendronate or tetracycline/alendronate–LDH clay complex were between 7 MPa and 12 MPa. Following exposure of the film to aqueous fluids, the Young's modulus of both films increased dramatically up to 2 h

(Fig. 7), corresponding to a time dependent loss of the water soluble MePEG from the film matrix (Jackson et al., 2004). This increase in Young's modulus of the film was considered to be an important feature of the GTR film, to impart stiffness to create a seal around the tooth, protect the defect space and prevent gingival cells from populating the void space during healing. Furthermore, increased membrane stiffness could prevent collapse of the GTR film in the void space, which is often observed as the films are not able to bear the pressure of the growing cells (Owen et al., 2010). Although the Young's modulus for the LDH-containing films was higher than the films with no clay following incubation, there were no differences in the films in the dry state (before incubation). It is possible that MePEG was released faster from the LDH clay-containing films because of either greater water uptake or increased porosity of films containing clay nanoparticles.

Alendronate release profiles from PLGA films (with 15% MePEG) loaded with either free alendronate, free alendronate and free LDH clay or alendronate–LDH clay complex particles (4.2%, w/w, loading) are compared in Fig. 6. Tetracycline was loaded in all groups of films at 5% (w/w). The data demonstrates that alendronate release is dramatically influenced by the combination effect of the anion exchanging clay and the polymer matrix, such that the burst phase of alendronate release is essentially eliminated when pre-bound to LDH clay and release rates significantly lowered. The $t_{1/2}$ values for alendronate release from the lead formulation (PLGA films containing free tetracycline and alendronate pre-bound to LDH clay) were 91 days indicating a preferred (extended) therapeutic profile for in vivo applications.

Interestingly, the rate of release of free alendronate from films containing free LDH clay particles fell between the rates measured for free alendronate (no LDH clay) and alendronate pre-bound to LDH clay. This suggests that as water enters the film, free alendronate may dissolve and then intercalate with the dispersed LDH clay present in the PLGA matrix. This in situ binding may then result in a decrease in the overall release profile of the drug as compared to free drug released from films not containing LDH clay. The tetracycline release profiles from the plasticized films described above, loaded with either free tetracycline, free tetracycline and free LDH clay or free tetracycline/alendronate–LDH clay complex are compared in Fig. 5. Although tetracycline was loaded in the free state into all films, tetracycline release rate was markedly decreased by the presence of the alendronate–LDH clay complex. Further binding studies showed that there was significant tetracycline binding by the LDH clay nanoparticles. The binding is suggested to be due to intercalation within the clay particles, given that it has been reported that in addition to anionic compounds, neutral and zwitterionic species may also undergo intercalation (Choy et al., 2007). Hence, the decreased tetracycline release rate from the dual drug loaded nanocomposite films was likely caused by the uptake of PBS buffer into the film, followed by diffusion of tetracycline onto available sites in the interlayer spacing of the LDH clay nanoparticles. The release profile of tetracycline from films loaded with free clay particles further supports the concept of in situ interaction of tetracycline with LDH clay. Dissolution of films on completion of release studies and recovery of tetracycline in the film, showed significant levels of intact drug (28% of the initial loading) remaining, suggesting that tetracycline was intercalated in the clay particles and retained in the film. The effect of film thickness on tetracycline release was also investigated and the rate of drug release was found to decrease as a function of film thickness. At day three, the amount of drug released (%) from films with thicknesses of 440 μm, 801 μm and 1100 μm was 67 ± 4, 42 ± 4 and 17 ± 2 (%), respectively. This effect results from an increased diffusional path length for both incoming water and outgoing dissolved drug. Generally, in these studies, a film thickness of 400 ± 25 μm was used throughout because these films are thought to have the appropriate handling properties for periodontal applications so further investigations of thickness effects on drug release were not pursued.

Nanocomposite film formulations loaded with alendronate were evaluated for biocompatibility and the ability to provide a surface for maintaining viability of osteoblast cells (Fig. 8). Tetracycline was not included in the films for the viability studies as tetracycline interfered with the experimental conditions. Initial studies with co-loaded films, showed that as significant amounts of tetracycline were released from the films within the first 2 days, yellow precipitates were observed over the cell layers and cells could not be visualized. The plasticized PLGA films could be loaded with alendronate–LDH clay complex nanoparticles up to 4.2% without any decrease in cell viability, whereas free alendronate in the films showed evidence of osteoblast toxicity at a loading level of 0.75% and higher.

The viability studies were followed by long term studies, where the effects of the nanocomposite formulations were observed on ALP and bone nodule formation over a period of 5 weeks. Bone specific alkaline phosphatase (ALP) is a membrane bound exoenzyme produced by osteoblasts. It is required during bone formation for osteoid formation and matrix mineralization (Mohamadnia et al., 2007). An increase in ALP levels signifies an increase in osteoblastic activity. Following cell differentiation, mineralization of the matrix takes place and calcium deposits are laid down in the extracellular matrix. The deposits of calcium in the matrix are referred to as bone nodules and they are an indication of early cell maturation (Long et al., 2009). Figs. 9 and 10 demonstrate that either free tetracycline or free alendronate loaded into the films had no effect on ALP activity and bone nodule formation in the cell cultures. Although alendronate has been shown to increase proliferation and enhance osteoblastic differentiation at submicromolar concentrations (Long et al., 2009), we believe that for the films containing free co-loaded drugs, the burst phase of alendronate release was toxic to osteoblasts, thus inhibiting osteoblast proliferation and differentiation. However, after the initial burst phase of release was complete, the reduced amounts of alendronate released may have been favorable for cell growth, allowing the cells to recover and proliferate.

Tetracycline was released rapidly from the nanocomposite films and showed a large burst phase and this is suggested to be optimal for maintaining high concentrations of the antibiotic as wound healing occurs (Owen et al., 2010).

5. Conclusion

A plasticized biodegradable PLGA film formulation based on dual drug loading of free tetracycline and alendronate intercalated within LDH clay nanoparticles was developed with the target properties suitable for potential application as a GTR membrane. The alendronate–LDH clay complex in the polymer film matrix successfully controlled the release of alendronate from the film and suppressed the burst phase of release. The long term increase in osteoblastic activity observed may be favorable in a periodontal setting as the amount of bone that regenerates in presently used GTR membranes (along with bone grafts) has not been sufficient to bring about total healing (Samuel et al., 1999).

Acknowledgements

The authors would like to acknowledge the following individuals for their contributions:

- Anita Lam from UBC chemistry for her assistance with the X-ray diffraction experiments.
- SeaBass Studios for providing illustrations.

The authors gratefully acknowledge the work of Nathalie Gerds, PhD student at the University of Copenhagen Faculty of Life Sciences, in synthesizing the LDH carbonate (LDH-CO3) used in this research.

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