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Formulation and preliminary in vivo dog studies of a novel drug delivery system for the treatment of periodontitis

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Abstract

A novel drug delivery system for the treatment of periodontitis was developed using two components. The first was tetracycline base loaded into the microtubular excipient halloysite, which was coated with chitosan to further retard drug release. Encapsulation efficiencies of 32.5% were achieved with the loading procedure, with tetracycline base showing in vitro release for up to 50 days in simulated gingival crevicular fluid. The second component developed was a vehicle for the drug loaded coated halloysite, which was primarily based on the thermoresponsive polymer, *poloxamer 407*. A concentration of 20% was chosen with the thermoresponsivity of the system modified using PEG 20,000 so that the mobile product at room temperature would gel by temperature rise following syringing into a periodontal pocket. Retention of the overall system in the pocket was further improved by the addition of octyl cyanoacrylate (OCA). The thermoresponsivity of the *poloxamer 407* system proved to be sensitive to the presence of added excipients with the levels of PEG 20,000 and OCA requiring modification in the presence of the halloysite component. A final formulation was developed which consisted of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan, suspended in 1 ml of *poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1.0% (w/w), water to 100%, adjusted to pH 4. The syringeability of this formulation at various temperatures was evaluated to ensure ease of delivery to the periodontal pocket. A stability study was performed to examine the change in thermoresponsivity over time, with the final formulation found to be stable for at least 9 months when stored at room temperature (∼20 °C). This formulation offered ease of delivery to the periodontal pocket and sustained release of the antibiotic for up to 6 weeks. The formulation had preliminary in vivo testing performed in dogs to determine levels of drug release, antimicrobial activity and retentive ability of the product. A wound pocket creation model was developed for the purposes of the trial. The product was easy to deliver to the pockets with application times of less than 1 min. Results showed the product was retained in the pocket for up to 6 weeks with effective tetracycline levels released locally over this time period, which achieved good antibacterial activity.

Keywords: Halloysite; Tetracycline base; Chitosan coating; Thermoresponsive retentive vehicle; Periodontitis treatment

1. Introduction

Periodontitis is a localized inflammation of the periodontal pocket caused by bacterial infection, which can result in tooth loss. The current microbiological treatment of periodontitis is through either the use of systemic antibiotics, or a localized delivery system incorporating an antibiotic. The use of systemic antibiotics raises a number of issues. Large doses must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pockets; this brings with it the associated side effects

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of antibiotics and problems regarding antibiotic resistance (Van Winkelhoff et al., 2000). There are also concerns regarding compliance, with problems increasing with frequent dosing or prolonged treatment. As a result of these issues, treatment of periodontitis with localized drug delivery systems is becoming more prevalent, with a number of products now commercially available. The concept of local drug delivery to the periodontal pocket began in the late 1970s with Goodson who developed a hollow fibre loaded with tetracycline HCl (Goodson et al., 1983). This ethyl vinylacetate fibre was eventually launched commercially under the trade name *Actisite*. The fibres are not resorbable and must be removed after 10 days. The application of this product is time consuming, taking up to 15 min to apply the fibre to one tooth (Tonetti et al., 1994). A second product that has been developed and launched on the market is *Periochip* and contains 2.5 mg of chlorhexidine gluconate in a biodegradable matrix (Killoy, 1998). Another commercially available product is metronidazole dental gel, which contains 25% (w/w) metronidazole in a glyceryl monooleate and sesame oil gel (*Elyzmol*) which is formulated as a suspension, but on contact with gingival fluid becomes a controlled release semi-solid. The longest release obtained with this system was 48 h (Norling et al., 1992). These commercially available delivery systems have a number of shortcomings, including limited duration of drug delivery, difficulty in application and poor retention. It was felt in this study that a more optimum formulation could be developed, offering more prolonged release of antibiotic and improved delivery to the periodontal pocket.

Tetracycline was the drug chosen for the formulation as it is already widely used in the treatment of periodontitis, both systemically and locally, and has been shown to be effective against many of the common periodontopathic bacteria, in particular against *Prevotella intermedia* and *Porphyromonas gingivalis* (Miyake et al., 1995; Baker et al., 1985; Gordon et al., 1990). Systemically it has been used for the treatment of periodontitis (American Academy of Periodontology, 1996) at doses of 500 or 1000 mg daily.

Halloysite is an aluminosilicate material, chemically similar to kaolin. However, morphologically, it has a hollow tubular structure (Fig. 1), unlike kaolin, which has a stacked-plate structure. This hollow cylindrical structure can be drug loaded to produce gradual release. The in vitro release characteristics of a number of drugs from halloysite, including tetracycline HCl, have been previously examined by Price et al. (2001) and shown to have sustained release properties. Coating the halloysite using cationic polymers that bind onto the negative charges present on the tubule surface, i.e. charge neutralization, can further enhance the retardation of drug release. The

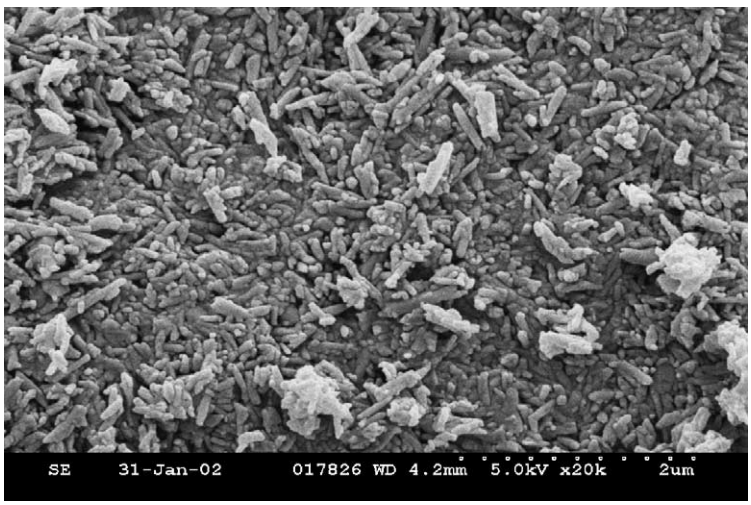

Fig. 1. SEM of halloysite (20,000×).

cationic polymer chosen for use in this study was chitosan as it had been shown previously to enhance the retardation of drug release from halloysite (Levis, 2000).

2. Materials and methods

2.1. Materials

Acetonitrile HPLC grade (Lab Scan), agar No. 1, brain heart infusion broth (Oxoid), calcium chloride dihydrate, sodium acid phosphate, sodium phosphate (Merck), chitosan—medium mol. wt. (Aldrich), doxycycline hydrochloride, ethylenediaminetetraacetic acid (EDTA), glacial acetic acid, tetracycline base, tetracycline hydrochloride (Sigma), ethanol, methanol HPLC grade (Riedel de Haen), halloysite G (New Zealand China Clays Ltd.), octyl cyanoacrylate (Henkel), *poloxamer 407* (BASF), polyethylene glycol 6000 and 20,000 (Fluka), potassium nitrate, sodium acetate (BDH) and water (glass distilled) were used. All reagents were GPR unless otherwise indicated.

2.2. Drug loading and coating of halloysite

The sieved halloysite (125 μm mesh) and drug solution were mixed in a 1:1 ratio. The halloysite was loaded with tetracycline base or tetracycline HCl (20 mg/ml) in ethanol and a vacuum was applied. Air bubbles were observed appearing on the surface of the mixture, being air drawn out of the hollow tubules. The vacuum was maintained until all the air in the tubules had been apparently displaced. The preparation was mixed again and the vacuum reapplied to remove any further trapped air. Drug-loaded samples were dried and sieved. The drug loading procedure was repeated, using the same 1:1 ratio. A suitable quantity of recovered double drug-loaded halloysite was mixed with the appropriate volume of chitosan solution (0.2% w/v solution, acetate buffer pH 4.2). This was blended for adequate time (90 s) to allow charge neutralization to occur between the halloysite and the chitosan. The preferred binding ratio of halloysite: chitosan was 1:0.114 (Levis, 2000). The suspension was centrifuged and the sediment dried, ground and sieved.

2.3. Preparation of poloxamer 407 containing samples

Polyethylene glycol (PEG) was added to distilled water and transferred to a cold room (4 °C). *Poloxamer 407* was added and left overnight to hydrate. The system was made up to final weight with distilled water. For systems containing OCA, the pH of the system was adjusted to 4.0 ± 0.05 to suppress polymerisation of the monomer. The coated drug loaded halloysite was incorporated into the *poloxamer 407* containing vehicle at a concentration of 200 mg/ml.

2.4. Syringeability of poloxamer 407 containing samples

The ease with which *poloxamer 407* containing samples could be expressed after filling into syringes and through their fitted needles, was measured using a texture analyzer XT.RA (Stable Micro Systems, UK) in compression mode. A filled 1 ml syringe was held in place with a clamp and the upper probe of the texture analyzer moved downwards until it came in contact with the syringe barrel base. A constant force of 0.5 N was applied to the base and the work required to expel the contents for a barrel length of 30 mm was measured. The area under the resulting curve was used to determine the work of expulsion.

2.5. Rheology

Rheological properties of various systems were evaluated using a Carri-Med rheometer CSL^2500 (TA Instruments, UK). All experiments used cone and plate test geometry (angle 4°). The linear viscoelastic region of the systems was first determined by torque sweep. Temperature ramps were performed over the range 15–45 °C, at a frequency of 1 Hz, with an oscillatory torque of 500 μN m.

2.6. Dissolution testing of halloysite drug loaded with tetracycline base or tetracycline HCl, with and without chitosan coating

Samples (250 mg) were placed in fine paper mesh bags and tied at the top to ensure retention of the sample. These were placed in 100 ml Duran flasks containing a defined amount of dissolution medium as a sim-

ulation of gingival crevicular fluid (phosphate buffer pH 6.8). The volume of dissolution medium used was dependent on the stage of the dissolution test. The Duran flasks were placed in a water bath at 37 °C, with samples being taken and the dissolution medium replaced at periodic intervals. After the last sample was taken, the residue of each product was suspended in buffer and left to stir for 7 days to determine 100% release.

2.7. HPLC assay for tetracycline base and HCl

The tetracycline base concentration in samples obtained from dissolution testing was determined by reverse phase HPLC using an assay modified from Knox and Jurand (1979). Samples were diluted using the buffer in which the dissolution test had been performed. 20 μl samples were injected onto a *Thermoquest* 25 cm × 4.6 mm column packed with *Hypersil* 5 μm octadecyl silica. The mobile phase consisted of water:acetonitrile:glacial acetic acid (71:18.5:10.5) v/v/v containing 0.011 M KNO_3 and 2.8×10^{-3} M EDTA, with pH adjusted to 3.0 using 0.5 M sodium acetate and a flow rate of 1.5 ml/min. The UV detector was set at 362 nm.

2.8. Formulation used for in vivo trial

The test system (final formulation) used in the in vivo study consisted of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan per 1 ml of vehicle composed of *poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w) and water to 100%, pH 4. The control system consisted of 200 mg of unloaded halloysite coated with chitosan per 1 ml of the same vehicle. After the test systems had been injected into the pockets on the dogs' teeth, samples remaining from each syringe were analysed to confirm the content of tetracycline base in the test samples.

2.9. Surgical procedure on dogs

In vivo properties of the product developed were examined using a wound pocket creation model to mimic periodontitis. Two healthy mature (2-year-old) male beagle dogs were selected for the study. Each dog had three pairs of contralateral maxillary teeth and mandibular teeth (canines and premolars) tested (Table 1). On canines, a mucoperiosteal flap was raised and pockets were surgically created of 4 mm corono-apically and 6 mm mesio-distally (surgical wound pocket creation model). Furcation defects were surgically created on the UP2, UP4, LP2 and LP4. Three pairs of pockets were used as vehicle controls. In the remaining contralateral pockets, the test samples were injected into the bottom of the pockets.

2.10. Sampling of pockets

All pockets were sampled at days 14, 28, 42 and 56 for microbiological testing and tetracycline concentration determination using sterile orthodontic points, which were inserted into the bottom of each periodontal pocket until resistance was met and kept in place for 30 s. For drug residue testing, vials were weighed

Table 1
Canine teeth tested

Sample no.	Tooth	Dog 1	Dog 2
1	URP2 (upper right premolar furcation)	Test	Control
2	LRP2 (lower right premolar furcation)	Test	Control
3	LRC (lower right canine flat surface)	Test	Control
4	LRP4 (lower right premolar furcation)	Test	Control
5	URC (upper right canine flat surface)	Test	Control
6	URP4 (upper right premolar furcation)	Test	Control
7	ULP2 (upper left premolar furcation)	Control	Test
8	LLP2 (lower left premolar furcation)	Control	Test
9	LLC (lower left canine flat surface)	Control	Test
10	LLP4 (lower left premolar furcation)	Control	Test
11	ULC (upper left canine flat surface)	Control	Test
12	ULP4 (upper left premolar furcation)	Control	Test

on a microbalance and sterile orthodontic points were placed in each of the vials, which were then reweighed. After the pockets were sampled, the vials were once again weighed to determine the weight of residue obtained on the orthodontic points. For microbiological testing the sterile orthodontic points were shaken for 15 s and a 1 in 10 dilution was performed using half-strength Ringers solution. 200 μl quantities were plated on agar plates. The growth medium contained brain heart infusion broth (0.35%) gelled with 1% agar. Plates were stored under aerobic and anaerobic conditions with total colony forming units (CFUs) being counted after 24 h incubation at 37 °C for aerobic plates and 48 h at 37 °C for anaerobic plates.

2.11. HPLC analysis of residue samples

The samples were analysed using a HPLC system comprising a Shimadzu LC-5A HPLC unit equipped with a Shimadzu RF-10Axl fluorescence detector. The fluorescence detector was set with an excitation wavelength of 375 nm and an emission wavelength of 512 nm. The column used was a *Thermoquest* column packed with *Hypersil* 5 μm octadecyl silica. The mobile phase consisted of methanol and 0.1 M sodium acetate buffer containing 25 mM EDTA and 35 mM calcium chloride (pH 6.5) (40:60, v/v). Doxycycline HCl was used as the internal standard and was included in all standards and clinical samples. Triplicate injections were analysed on the HPLC system.

3. Results

3.1. Drug loading and release profiles from halloysite

There was a theoretical loading of 40 mg of tetracycline base per gram of halloysite, based on double drug loading using a solution of 20 mg tetracycline base/ml of ethanol. There was up to a 16-fold increase in the level of drug released from double loaded compared to single loaded halloysite. This dramatic increase can be explained by the fact that a large amount of the first loading may be irreversibly bound onto sites on the halloysite, making it unavailable for release. However, when the second drug loading is performed these sites are saturated so the majority of this drug loaded should enter the lumen of the halloysite tubules and would be available subsequently for release. The double loading of halloysite gave an encapsulation efficiency of 39.05% for tetracycline base and 49.3% for tetracycline HCl. When subsequent coating with chitosan was performed, the encapsulation efficiencies were reduced to 32.5% (13 mg/g of coated halloysite) for the base and 41% for the HCl (16.4 mg/g of coated halloysite).

Fig. 2 shows the release of tetracycline HCl and tetracycline base from halloysite in simulated gingival crevicular fluid (buffer pH 6.8). The solubility of both tetracycline base and tetracycline HCl was determined by dynamic solubility testing and was 0.843 and 3.45 mg/ml, respectively. Both systems

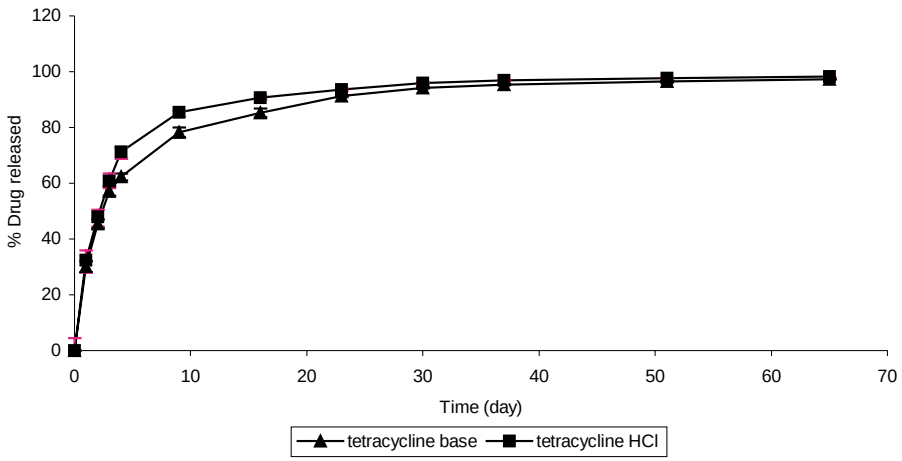

Fig. 2. Release of tetracycline base and tetracycline HCl from halloysite coated with chitosan into buffer pH 6.8 at 37 °C.

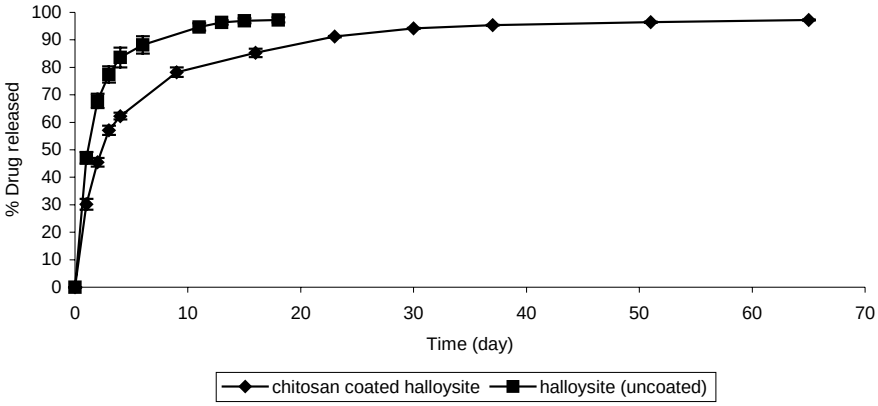

Fig. 3. Release of tetracycline base from halloysite (uncoated) and halloysite coated with chitosan into buffer pH 6.8 at 37 °C.

have been coated with chitosan to help retard drug release. The tetracycline HCl is released more quickly as would be expected taking into account its greater solubility (3.45 mg/ml) with 90% release at day 16. When the difference in release between tetracycline base and tetracycline HCl is examined statistically at days 4, 9 and 16, the results were found to be significant. A paired *t*-test with a confidence interval of 95% ($P < 0.05$) was performed on the results from these three time points and gave *P*-values of 0.0009, 0.04 and 0.034 for days 4, 9 and 16, respectively. After day 16 no significant difference was observed. The lower solubility of tetracycline base (0.843 mg/ml) can therefore be considered to have a significant impact on the sustained release properties of the system, reducing the release over the first 2 weeks. However, low levels of both tetracycline base and tetracycline HCl continued to be released until day 50 with the chitosan-coated halloysite.

The release profile of tetracycline base loaded halloysite vs. halloysite loaded with tetracycline base and coated with chitosan is shown in Fig. 3. When the halloysite is coated with chitosan this greatly retards the release of the drug. Both samples show a significant burst effect, with 30% drug being released from the coated halloysite and 45% being released from the uncoated halloysite in the first 24 h of the dissolution test. However, after the initial burst release, the chitosan-coated halloysite shows a dramatic reduction in release in comparison to the uncoated halloysite. At day 9 the uncoated halloysite has released 88% of its final drug load while the coated halloysite has released only 78% of its total drug load.

3.2. Delivery vehicle development

Poloxamer 407 (*Lutrol F127*) is an example of a polyoxyethylene–polyoxypropylene copolymer. The main characteristic of this class of polymers, relative to this study, is thermoreversible behaviour, with aqueous *poloxamer 407* solutions being liquid below 20 °C and gelling at higher temperatures, if at concentrations of 20% (w/w) and above. These gels retain their characteristics and properties throughout repeated heating and cooling processes, with their gel strength and viscosity dependent on polymer concentration. The *poloxamer 407* gel systems were characterized rheologically using their storage modulus (G') and apparent viscosity (η_{app}). The G' (Pa) is a measure of the elasticity of the system which reflects the solid-like component of the gel. Therefore, the larger the storage modulus the more solid like behaviour the system possesses, aiding better retention in the periodontal pocket. It is desirable to have a gelling temperature for the vehicle of between 25 and 30 °C, so as to avoid the need for refrigerated storage, to facilitate ease of administration, but also to have a high storage modulus on achieving body temperature (37 °C).

Two concentrations of *poloxamer 407* 25% (w/w) and 20% (w/w) were initially studied. The 25% (w/w) system showed a gelling temperature of approximately 15 °C, which was considered too low. It was therefore decided to develop a formulation based on the 20%

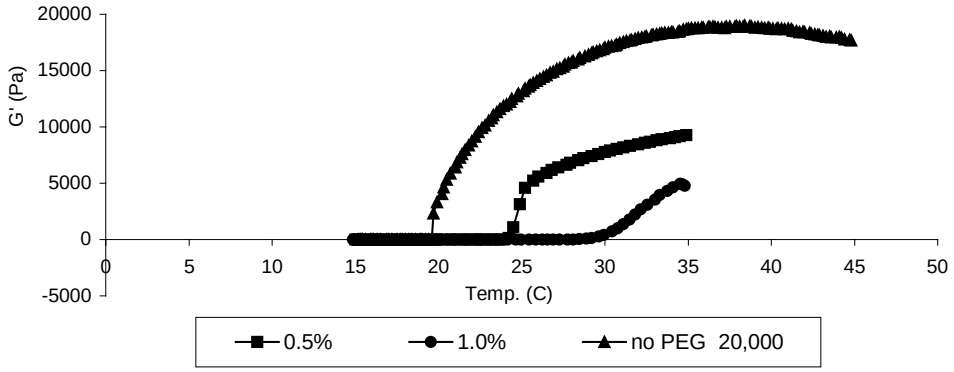

Fig. 4. Storage moduli of *poloxamer 407* 20% (w/w) containing 0.5% (w/w) PEG 20,000, 1% (w/w) PEG 20,000 and no PEG.

(w/w) concentration, which showed a sol–gel transition temperature of just below 20 °C (Fig. 4). The 20% (w/w) *poloxamer 407* showed a 30% reduction in the storage modulus at 37 °C compared to the 25% (w/w), however the storage modulus was still high at 18,810 Pa (Fig. 4).

In order to increase the gelling temperature of *poloxamer 407* 20% (w/w) to a more desirable range, addition of polyethylene glycols (PEG 6000 or 20,000) was examined. It was found that high concentrations of PEG 6000 were required to give a small increase in gelation temperature and that the transition temperature was broadened by these high concentrations. PEG 20,000 required low concentrations to give significant changes in gelation temperatures, with the transition temperature remaining narrow. PEG 20,000 1% (w/w) addition brought the gelation temperature to about 30 °C with a broader transition and a much lower storage modulus (Fig. 4). A concentration of PEG 20,000 0.5% (w/w) gave a gelation temperature of 25 °C when combined with a concentration of *poloxamer 407* 20% (w/w) (Fig. 4). This was considered suitable if the product was to be stored and used at room temperature. At 37 °C the storage modulus of the system containing 20% (w/w) *poloxamer 407* and 0.5% (w/w) PEG 20,000 was only 10,000 Pa, which is a 45% reduction in the storage modulus compared to *poloxamer 407* 20% (w/w) alone.

The retention of the product in the periodontal pocket was of major concern as it was necessary to ensure that the product would remain there for the intended period of drug release from the contained drug loaded coated halloysite, i.e. a period of up to 6 weeks. It was felt that due to the significant drop in the storage modulus, as a result of the addition of PEG 20,000, the *poloxamer 407* containing vehicle might not be rigid enough to ensure retention in the periodontal pocket for the required time period. To try and improve the retention time it was decided to include 1% OCA in the formulation, which is a powerful tissue adhesive (Refojo et al., 1971). Polymerization of such alkylcyanoacrylates can be suppressed by maintaining an acidic environment (pH 4 or below). The natural buffering capacity of the periodontal pocket should over the course of time cause the gel to reach a more neutral pH, allowing polymerization of the OCA to occur and increasing tissue adhesion as a result, leading to better retention to the product. To lower the pH of the *poloxamer*/PEG system to 4, adequate 10% glacial acetic acid was added prior to addition of the OCA. Again both 20 and 25% (w/w) *poloxamer* systems were examined, but the 20% (w/w) still showed a more favourable gelation temperature with the OCA incorporated. The presence of the OCA however did affect gelation temperature, causing a slight increase (Fig. 5). Overall it was still the concentration of PEG 20,000 0.5% (w/w) that gave the best results in the presence of OCA 1% (w/w).

The final step was to incorporate the drug loaded coated halloysite. This incorporation was carried out on a number of different *poloxamer* systems, as the thermoresponsivity of the *poloxamer 407* seemed to be highly sensitive to added excipients. On halloysite addition, a thick yet syringeable liquid was obtained at room temperature for all systems. On performing a temperature ramp, significant differences were ob-

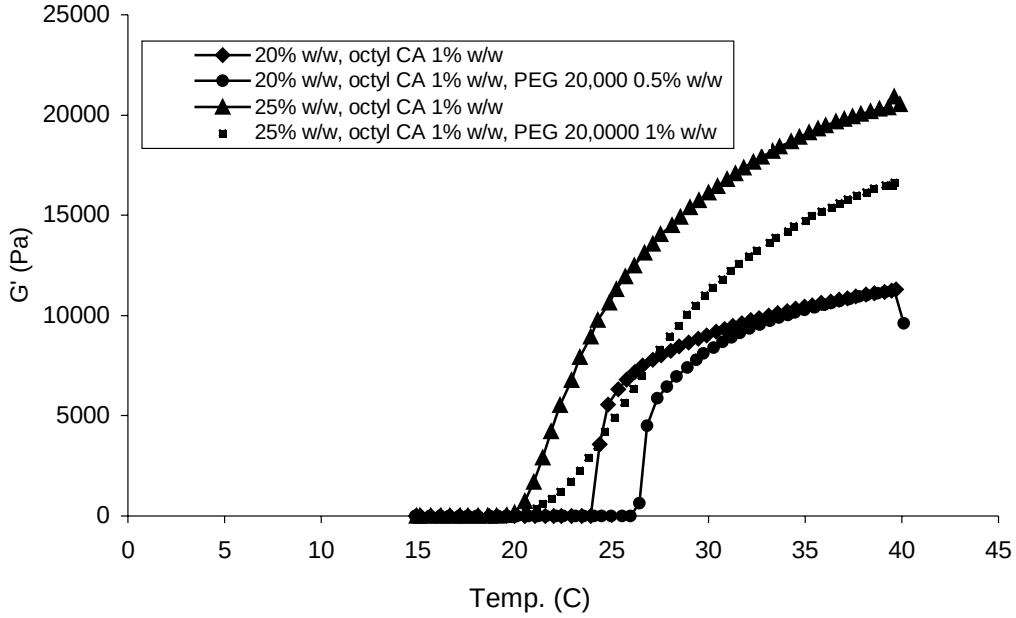

Fig. 5. The effect of OCA on gelation temperature of *poloxamer 407* 20 and 25% (w/w) systems.

served with the system consisting of 200 mg of halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% (w/w) PEG 20,000 1% (w/w), OCA 1% (w/w) and water to 100%, losing nearly all thermoresponsivity. The system without PEG 20,000 showed a sharp transition, but at a lower temperature (21.9 °C) (Fig. 6). The comparative system containing PEG 20,000 0.5% (w/w) showed a broader transition at a slightly higher temperature (23 °C) (Fig. 6). The tetracycline base containing component appeared to have the effect of further decreasing the gelation temperature, which is similar to the result reported (Esposito et al., 1996). The effect of the broader transition is significant as the stor-

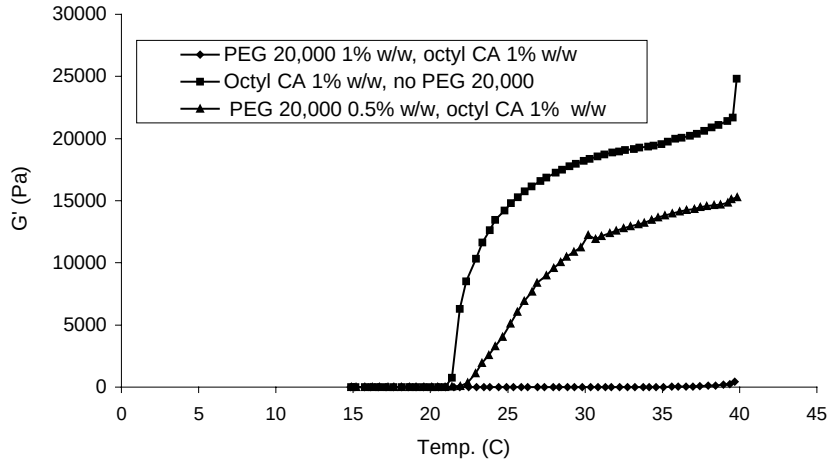

Fig. 6. Effect of addition of 200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% (w/w) systems on storage modulus and gelling temperature.

age modulus of the *poloxamer 407* 20% (w/w), OCA 1% (w/w), water to 100%, system reaches 6288 Pa at 21.9 °C, whereas the storage modulus of the *poloxamer* 20% (w/w), OCA 1% (w/w), PEG 20,000 0.5% (w/w), water to 100%, only reaches 5117 Pa at 25.2 °C. This should allow the syringeability to be sufficient up to a temperature of 25 °C. At 37 °C the storage modulus of this system is 14,480 Pa, which is significantly higher than for the system less the drug component. This is advantageous, as it should aid better retention in the periodontal pocket.

3.3. Syringeability

Syringeability of various *poloxamer 407* systems was examined to determine the effect that both temperature and the addition of excipients have on the force required to expel the product. This is of importance as the product must be able to be delivered from a syringe through a needle, in order to fulfil the requirement of ease of application. The systems examined for syringeability are listed in Table 2, along with the syringeability values obtained. The effect of temperature on syringeability of the system can be clearly seen. System 1 has a syringeability of 21.68 N at 4 °C and 185.28 N at 20 °C, which is an 8.5-fold increase in resistance to syringing over this temperature range. Concentration also has a significant impact with syringeability increasing from 21.68 to 41.77 N with increasing concentration from 20 to 25% (w/w) for *poloxamer* addition at 4 °C. The syringeability of the 25% (w/w) *poloxamer* containing system at 20 °C could not be determined due to the solid nature of the polymer dispersion at this concentration and temperature. On addition of PEG 20,000 at a concentration of 0.5% (w/w) to the *poloxamer 407* 20% (w/w), the syringeability of the system at 20 °C is almost halved. This would correlate well with the increase in gelling temperature seen with the addition of PEG 20,000 0.5% (w/w). The system incorporating the OCA shows an interesting result with syringeability being almost the same at both 4 and 20 °C. This is clearly due to the effect that both PEG 20,000 and OCA have on the gelling temperature and as a result, the apparent viscosity of the system. The combination of these two components significantly increases the gelling transition temperature of the system, indicating that the apparent viscosity of the system does not begin to rise until higher temperatures have been reached. The addition of tetracycline base loaded halloysite does not change this syringeability at 20 °C. The final syringeability shown for system 5 at 20 °C is only a quarter of the syringeability of system 1 at 20 °C and only double the syringeability of system 1 at 4 °C, which is very desirable having regard to ease of administration.

3.4. Stability

A stability study was performed over 9 months to determine the effect time had on the syringeability and thermoresponsivity of two different systems at two different temperatures (Table 3). The two systems consisted of the final formulation and the delivery vehicle contained in the final formulation. The reason for examining the delivery vehicle alone was that in the event of poor stability results from the final formulation there would be an indication as to whether the system could be formulated as two separate components that could be mixed immediately prior to application. The results for syringeability of each system over time are shown in Fig. 7. It is observed that at time 0, both systems had similar areas under the curve, i.e. similar force of expulsion, but the system containing halloysite had a larger standard deviation than the

Table 2
Syringeability of various systems at 4 and 20 °C

System no.	System	Syringeability at 4 °C (N)	Syringeability at 20 °C (N)
1	*Poloxamer 407* 20% (w/w)	21.68	185.28
2	*Poloxamer 407* 25% (w/w)	41.77	
3	*Poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w)	N/A	94.45
4	*Poloxamer 407* 20% (w/w), PEG 0.5% (w/w), OCA 1% (w/w)	54.31	51.64
5	*Poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w) and 200 mg/ml tetracycline base loaded chitosan-coated halloysite	N/A	45.53

Table 3
Formulations placed on stability study at 4 and 20 °C

System	Formulation
A	200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w), water to 100%, pH 4
B	200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w), water to 100%, pH 4
C	200 mg halloysite double loaded with tetracycline base and coated with chitosan per ml of *poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w), water to 100%, pH 4
D	*Poloxamer 407* 20% (w/w), PEG 20,000 0.5% (w/w), OCA 1% (w/w), water to 100%, pH 4

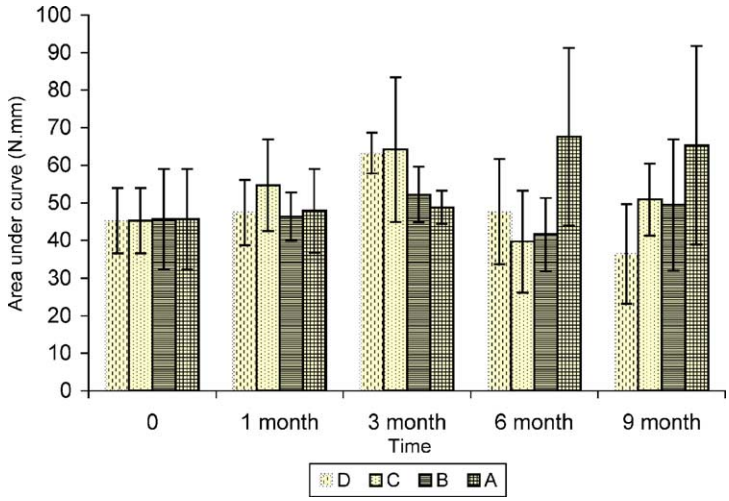

Fig. 7. Changes in syringeability of each system over time.

poloxamer containing system. At the end of 9 months there appears to have been no significant impact on the syringeability of the four different systems, with all showing similar syringeability values as was seen at time 0. While there was a larger increase in system A at 9 months, there was also a significant increase in the associated standard deviation, and when a *t*-test was performed no significant difference was observed between the syringeability of system A at 6 and 9 months ($P = 0.891$). Table 4 shows the changes in gelation temperature of the systems over time. There is no significant change in thermoresponsivity over time, with systems A and B consistently showing gelling temperatures 3–5 °C below those seen for systems C and D.

Table 4
Gelling temperatures for systems over time

Time	System A	System B	System C	System D
0	24.9	24.9	31.8	31.8
1 month	27.1	25.6	27.7	25.2
3 months	26.1	24.2	31.3	34.7
6 months	No sample	25.7	28	31.8
9 months	24.2	28.4	29.9	28.3

3.5. *In vivo dog studies*

The principal information required from the in vivo study was (i) whether there was retention within pockets of the final product for an adequate time period, and (ii) was adequate drug released locally over this extended time to suppress microbial activity with consequent benefit in the treatment of periodontitis.

3.5.1. *Antibiotic concentration*

The average content of tetracycline base per 100 mg of product was 94.71 μg, with a standard deviation of 19.26 μg. This standard deviation is ∼20% of the content and represents a large spread of varia-

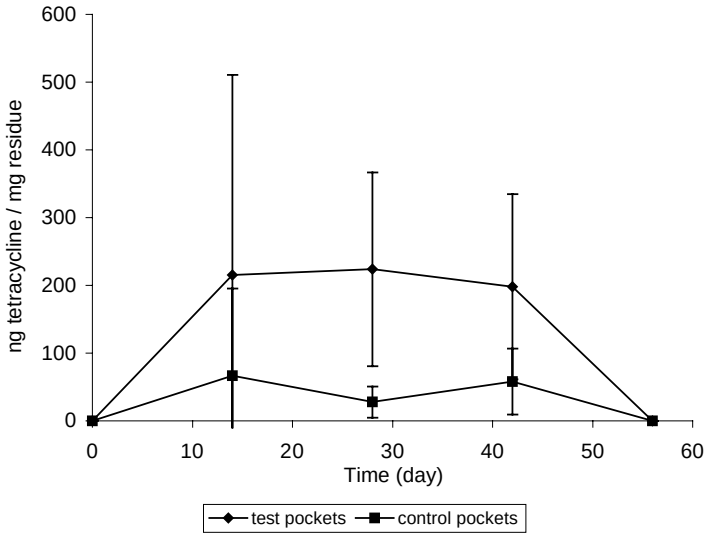

Fig. 8. Concentrations of tetracycline base as ng/mg of residue in pockets.

tion among the samples. The average concentration of tetracycline base (ng) in the pockets per mg of residue is shown in Fig. 8. These concentrations are derived from results of test and control pockets in dogs 1 and 2. At day 14 there is an average concentration of 215.5 ng/mg (21.55 μg/100 mg), with levels rising slightly to 223.69 ng/mg (22.37 μg/100 mg) by day 28. By day 42 concentrations were still at 198.06 ng/mg (19.81 μg/100 mg), however by day 56 there were no detectable levels of tetracycline remaining in the pockets of either dog 1 or dog 2. This gives an average pocket concentration of 21.24 μg/100 mg, over a 6-week period. The control pockets at all days showed detectable levels of tetracycline present, at day 14, 66.79 ng/mg (6.68 μg/100 mg), day 28, 27.82 ng/mg (2.78 μg/100 mg) and at day 42, 57.86 ng/mg (5.79 μg/100 mg). However, these levels are in all cases at least three orders of magnitude smaller than found in the test pockets and presumably arose by cross-diffusion from drug-treated pockets. The presence of antibiotic in control pockets also occurred in a study performed by Needleman et al. (1998), where concentrations of up to 25% of the test pockets were observed, depending on the position of the control pockets in relation to the test pockets.

Variations in concentration of tetracycline in individual pockets are considerable with the test pockets in each dog not showing a consistent release pattern over time (Fig. 9). In 5 of the 12 pockets (3 in dog 1, 2 in dog 2) one type of release pattern was observed, with an increase in tetracycline concentration from day 14 to 28 followed by a subsequent decrease from day 28 to 42. Two pockets in dog 1 showed a continual decrease in tetracycline concentration over time. Three pockets (1 in dog 1 and 2 in dog 2) showed a continual increase in tetracycline concentration over time, while the final 2 pockets in dog 2 showed a sharp decrease from day 14 to 28, followed by a rapid increase in tetracycline concentrations from day 28 to 42. The residue studies clearly indicate that the retention of the product in the pockets was adequate as all pockets showed tetracycline base present for a 6-week period, except for one pocket at day 42.

3.5.2. Microbiological counts

Both control and test pockets showed similar low aerobic and anaerobic counts at day zero. This is as would be expected, as there is no significant difference between the pockets at this time point, due to the fact that they had been surgically created just prior to having the test product delivered into them. By day 14, there had been a three-fold increase in the average number of aerobic organisms and a 1.3-fold increase in the average number of anaerobic organisms in the test pockets. However, in the control pockets there had been a 10.7-fold increase in aerobic organisms and a

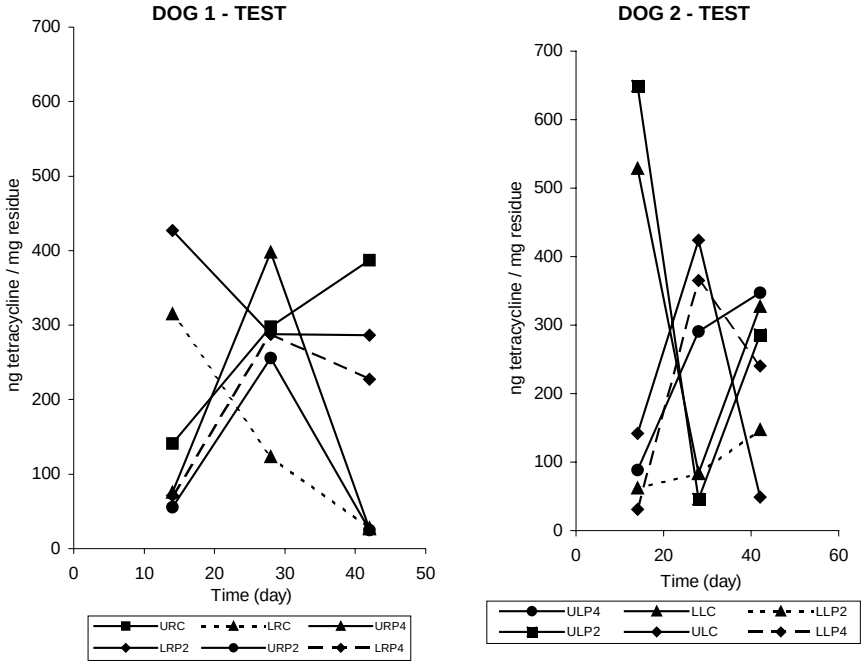

Fig. 9. Level of tetracycline base over time in individual pockets.

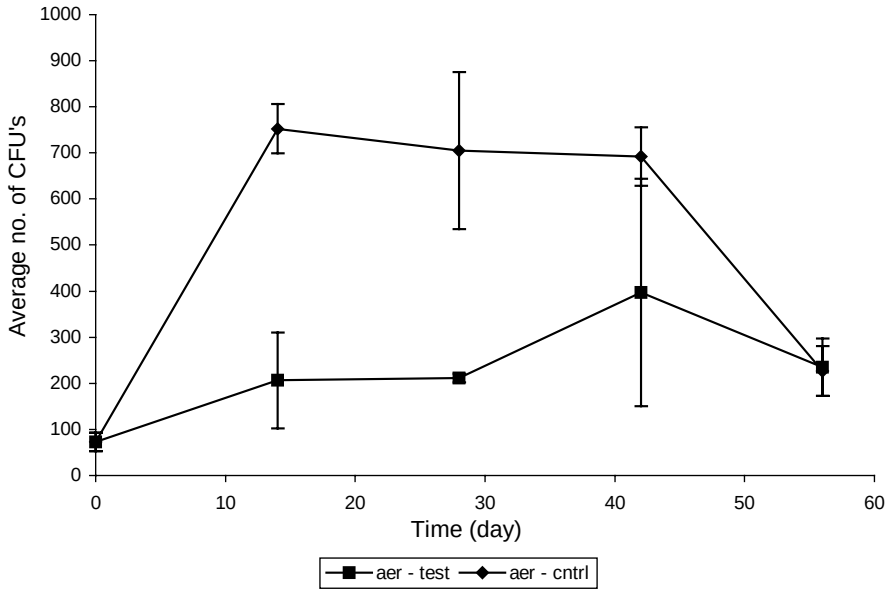

Fig. 10. Average aerobic counts for test and control pockets (dogs 1 and 2).

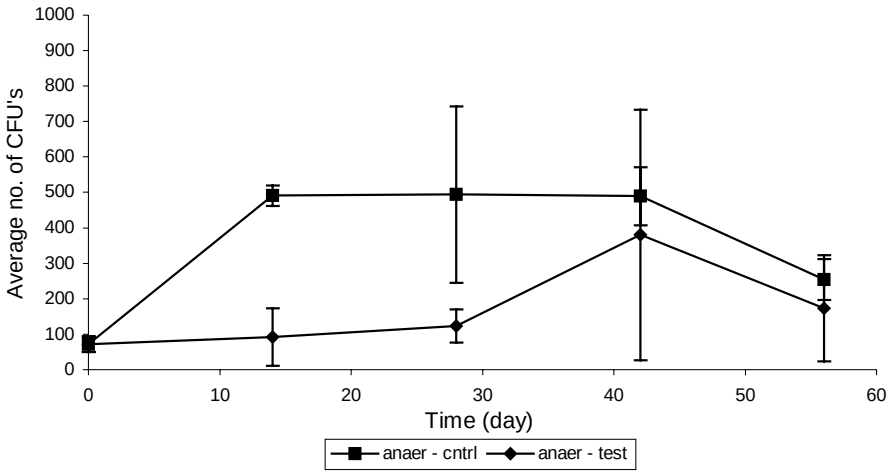

Fig. 11. Average anaerobic counts for control and test pockets (dogs 1 and 2).

7-fold increase in anaerobic organisms. The increase in CFU is to be expected as the pocket has experienced a surgical trauma and opportunistic bacteria would take advantage of this situation (Renvert et al., 1996). The reduction in total number of CFU seen in the test pockets compared to the control pockets, is due to the antimicrobial activity of the locally liberated tetracycline base, which is active against both aerobic and anaerobic microorganisms. Figs. 10 and 11 highlight the significant differences in aerobic and anaerobic counts between the test and control pockets over time. The suppression that occurs in the test pockets over the first 28 days can be clearly seen with the subsequent return to baseline that follows. However, the counts seen for the control pockets could be artificially low due to the low levels of tetracycline found in the control pockets as well as the test pockets due to a crossover effect. This may lead to suppression of CFU in the control pockets to a certain extent. As a result, values seen here may not necessarily represent the full magnitude of the difference between test and control pockets in other

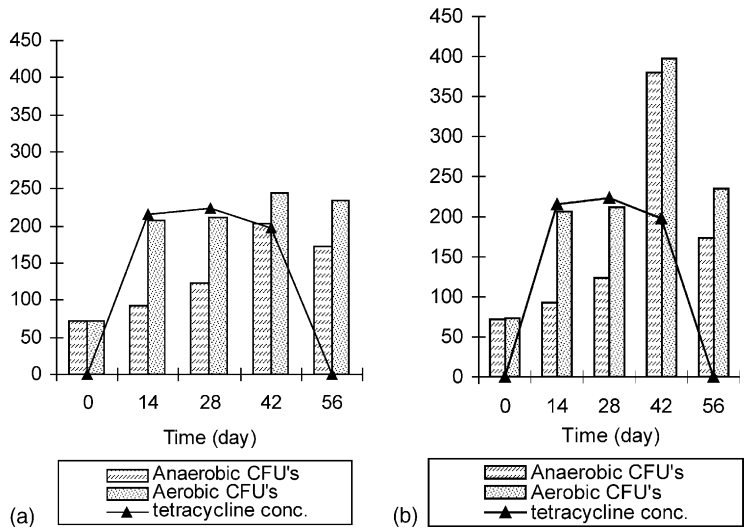

Fig. 12. (a). Effect on data distribution of excluding two outliers at day 42. (b) Effect on data distribution of including two outliers.

test situations. The sharp increase in counts, seen at day 42 for both aerobic and anaerobic test pockets warranted further investigation. It is clear that the standard deviations in the test pockets for both aerobic and anaerobic counts at day 42 are much larger than at any other time point for the test pockets (Figs. 10 and 11). When aerobic and anaerobic counts (test pockets) for individual dogs were examined, it was observed that a sharp increase in both counts was observed in dog 2 at day 42. This was due to two pockets that were showing extremely high counts, compared with all other pockets. If these two outliers were excluded from the data (Fig. 12a) it caused a visible difference to the data distribution that is seen when the two outliers are included (Fig. 12b). Fig. 12 also shows the average concentration of tetracycline in the test pockets overlaid onto the average aerobic and anaerobic counts in the test pockets at the different time points. At day 42, including the two outliers, there is a large increase in aerobic and anaerobic counts even though there is little difference in the tetracycline levels present in the test pockets. If the two outliers are excluded from the data a clear difference can be seen with aerobic and anaerobic counts being reduced to levels only slightly above day 28 levels. This result agrees well with the tetracycline levels detected.

4. Discussion

The aim of this work was to develop a localized drug delivery system for the treatment of periodontitis. The product was developed using the novel excipient halloysite, which was drug loaded using tetracycline base. Drug loading occurs through two mechanisms, firstly and most importantly the entrapment of the drug in the hollow tubules and secondly, adsorption of cationic drug onto the surface of the tubules. Release of the active agent is probably initially mainly from the exterior surfaces of the halloysite by desorption, if binding is reversible, followed by a more prolonged phase of release, mainly by diffusion from the ends of the microtubules (Price et al., 2001). The halloysite as can be seen from the SEM (Fig. 1) has a wide range of tubular sizes, with much of the tubular material being below 1 μm particle size. The smaller particulate material is less useful for drug loading as it is fragmented and not able to take up significant amount of drug, due to its lack of tubular space. It would be preferable if halloysite samples containing larger proportions of long tubules could be obtained, as it would give greater drug loading and also more uniform and consistent drug loading. The encapsulation efficiencies of the drug loading procedure are relatively low as a certain amount of the drug will be lost through the drug loading process itself and unavailable through irreversible binding onto anionic sites on the halloysite. The level of irreversible binding will depend on the nature of the drug, as a cationic drug will bind strongly to the halloysite resulting in a larger reduction of drug available for release. When coating with chitosan is performed, the level of drug available for release is significantly reduced due to a number of factors. Firstly, the chitosan binding is performed by centrifugation of the halloysite sample in an acetate buffer containing chitosan. A certain amount of tetracycline will dissolve into the buffer during the process. Secondly, the chitosan will cause a further reduction in the amount available for sustained release, as the chitosan will compete with the tetracycline for binding sites on the halloysite. Also the chitosan will cause an increase in the overall weight of the halloysite, reducing the tetracycline content in terms of mg tetracycline/g of coated halloysite. Initially both tetracycline base and tetracycline HCl was examined. The release of drug is the same over the first 48–72 h for both tetracycline base and tetracycline HCl. This drug release is most likely the portion of the drug that is loaded into the intra-tubular spaces, found in the halloysite aggregates. This will go into solution more rapidly as the aggregate breaks up in the buffer medium. This portion of the drug does not have to diffuse through the lumens of the halloysite tubules and so is not retarded to the same extent, therefore the differing solubility of the tetracycline base and tetracycline HCl will not have as significant an impact. The reduction in burst release between the coated halloysite and uncoated halloysite would point to the chitosan having an effect on either the intra-tubular drug or the drug found on the surface of the halloysite tubules. It is more likely that it is the drug on the surface of the tubules that is affected as the chitosan coating may bind around the tetracycline resulting in the presence of a complex layer. The drug in the lumen of the halloysite will be released more slowly due to the fact that it must diffuse out through

the pores of the halloysite, first travelling through the narrow lumen to reach the open ends of the halloysite. The release of the drug contained in the lumen of the halloysite is further retarded by the presence of the chitosan coating on the surface of the halloysite, which provides an additional barrier through which the drug must diffuse. Chitosan is particularly effective at retarding drug release at neutral pH, which these dissolution tests were performed at (Singh and Ray, 1999 and Ganza-González et al., 1999). This is due to the insoluble nature of chitosan at neutral pH, which results in excellent retention of the chitosan structure and so an enhanced ability to provide sustained release.

The delivery system for the drug loaded halloysite is based on *poloxamer 407*, however this alone did not provide a suitable delivery system with modification of the thermoresponsivity of the gel being achieved through the use of polyethylene glycol (PEG), which acted to increase the gelling temperature of the system. The PEG causes an increase in the gelation temperature by modifying the micellar formation of the *poloxamer* molecules and possibly also by forming mixed micelles with the *poloxamer* (Gilbert et al., 1987). This disruption to the structure of the *poloxamer* micellar structure results in a lower storage modulus, as the PEG reduces the ability of the *poloxamer* molecules to associate with one another, so weakening the structural integrity of the system. This disruption to the structure is clearly concentration related, with the 1% PEG 20,000 causing a further decrease in the storage modulus when compared to the 0.5% PEG 20,000. At 37 °C the storage modulus of the system containing 20% (w/w) *poloxamer 407* and 0.5% (w/w) PEG 20,000 was only 10,000 Pa. This is a further 45% reduction in the storage modulus of the *poloxamer 407* 20% (w/w) system alone, which is undesirable as it indicates a weaker gel forming which may reduce the ability of the product to be retained in the periodontal pocket. In order to improve the retention of the product in the periodontal pocket, OCA was incorporated into the formulation. Its presence affected gelation temperature, having the effect of slightly increasing the value for the system, implying that OCA further disrupted association of the *poloxamer* micelles.

Tetracycline has the effect of further decreasing the gelation temperature, which is similar to the result reported by Esposito et al. (1996). This effect could possibly be related to the presence of the tetracycline on the surface of the halloysite, which could further disrupt the micellar structure, increasing entanglement and further reducing the gelation temperature. The effect of the broader transition is significant as the storage modulus of the *poloxamer 407* 20% (w/w), OCA 1% (w/w) water to 100%, system reaches 6288 Pa at 21.9 °C, whereas the storage modulus of the *poloxamer* 20% (w/w), octyl CA 1% (w/w), PEG 20,000 0.5% (w/w), water to 100%, only reaches 5117 Pa at 25.2 °C. This should allow the syringeability to be sufficient up to a temperature of 25.2 °C. At 37 °C the storage modulus of this system is 14,480 Pa, which is significantly higher than the system less the drug component. This is advantageous, as it should aid better retention in the periodontal pocket.

The stability results showed a larger standard deviation in syringeability results when stored at 4 °C, which could be due to uneven mixing as a result of sedimentation of the halloysite fraction within the product. The sedimentation is aided due to the aggregated nature of halloysite. At low temperatures the *poloxamer 407* system has a low viscosity, due to its thermoresponsive nature, and the aggregated halloysite particles tend not to remain evenly suspended. It was found that it was difficult to achieve uniform mixing of the sample after sedimentation had occurred, despite vigorous agitation.

The release profile seen in the test pockets is clearly different than that seen with in vitro testing, where there was non-linear release characteristics observed in the dissolution tests performed. In the in vivo studies, when results from the test pockets in dogs 1 and 2 were averaged, the delivery system released an apparently constant concentration of tetracycline, independent of time, from days 14 to 42, i.e. zero-order release. In order to confirm that there was no significant difference in concentrations between days 14 and 42 analysis of variance (ANOVA) was performed on the data. The results show that there was no difference between the average release over the 6-week period and the average release at days 14, 28 and 42, i.e. the release is time independent, verifying that the system shows apparent zero-order release. This is similar to the release profile observed by Tonetti et al. (1994) using ethyl vinylacetate fibres loaded

with tetracycline HCl. However, their average concentration was 1590 μg/ml over the 10-day period. It is difficult to draw an exact comparison with concentrations achieved in this in vivo study, as their method of sampling was different, with gingival fluid samples being taken. However, it is clear that the concentrations achieved using the tetracycline fibre were higher than with the product currently being tested. There is little significance in these high concentrations as concentrations achieved need only be higher than the minimum inhibitory concentration ($MIC_{90\%}$ of *P. gingivalis* and *P. intermedia* is 0.5 and 0.25 μg/ml, respectively) of periodontopathic bacteria. The high burst and unnecessarily high concentrations achieved with the tetracycline fibre are a result of the design of the product. The in vitro release rate of the fibre is estimated to be 35 μg/cm h for the first 2 h and 2 μg/cm h thereafter, with the result that the concentration achieved in the pocket is dependent on the amount of fibre placed in the pocket and also the sampling time used.

When the average release of tetracycline in the test pockets was examined in the two dogs separately, an inverse relationship was observed, however this difference was shown not to be statistically significant when examined using a paired *t*-test (95% confidence interval, $P < 0.05$). The problem observed with variation in levels and release patterns within the pockets could be due to issues surrounding the sampling method used. There are two concerns with this method. Firstly the amount of residue taken can vary greatly from pocket to pocket and there is no way to standardise this to ensure that the same amounts are taken from each pocket. The second difficulty concerns the non-specific nature of the sample obtained. The residue sampled could consist of three different components, gingival crevicular fluid, blood and product residue. Depending on the amount of each component in the residue obtained, the levels of tetracycline base detected will differ significantly. If there is mainly product residue, tetracycline levels could be high whereas with samples containing mainly blood and gingival crevicular fluid, levels of tetracycline base would be expected to be lower. The more common method for sampling concentrations of antibiotic in gingival pockets is using gingival fluid volume. The gingival crevicular fluid is obtained by inserting filter paper strips into the pocket until 80% wet and then determining the volume of fluid obtained by using a gingival crevicular fluid meter (*Periotron*) (Goodson et al., 1991; Killoy, 1998). However, this method is affected by many of the same problems; it is difficult to ensure that the strips do not get contaminated with saliva, blood and plaque residue, and if they do, biased readings will be obtained. Overall the gingival pocket is a difficult site to properly sample and studies involving sampling the pocket will always be subject to significant amounts of variation as a result. It is important for this reason, that large-scale studies are performed in order to allow for the variability. The problems associated with a small-scale study are emphasised with the skewed results in this study seen at day 42, where two apparently anomalous results distorted the whole data set significantly.

5. Conclusions

The aim of this work was to formulate a localized drug delivery system for the treatment of periodontitis. The product was developed using the novel excipient halloysite, which was drug loaded using tetracycline base. This alone did not offer sufficient extended drug release and therefore chitosan was used as a coating on the halloysite to further retard release of drug. This resulted in a drug release over a satisfactory time period of 60 days approximately.

A delivery system was required to allow for application of the halloysite to the periodontal pocket. A thermoresponsive delivery system was chosen as it offered ease of application to the periodontal pocket, with subsequent good retention at body temperature (37 °C). The sol–gel transition of the thermoresponsive polymer was manipulated, using PEG 20,000 to give a suitable transition temperature. In order to improve the retention of the product further, a tissue adhesive was incorporated into the formulation. Syringeability of the product was also examined and it was seen to be acceptable offering ease of application. Studies showed that the final formulation was stable for up to 9 months when stored at 20 °C.

The final formulation was tested in vivo in dogs using a wound pocket creation model and it showed good retention, release and microbiological activity over a 6-week period.

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