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ScienceDirect
International Journal of Pharmaceutics 340 (2007) 92–96
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

An in vitro assessment of bioadhesive zinc/carbomer complexes for antimicrobial therapy within the oral cavity

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Received 26 February 2007; received in revised form 20 March 2007; accepted 21 March 2007
Available online 24 March 2007

Abstract

The effectiveness of antimicrobial agents in the oral cavity is limited by their retention at the site of action. In this work the antimicrobial cation zinc was complexed with the bioadhesive agent Carbopol 971P, in order to allow an extended antimicrobial effect. Zinc ions were shown to form stable complexes with the polymer, and were not released into distilled water. However, in the presence of other cations, it was possible to displace zinc over an extended period. A low pH was seen to enhance zinc release. The complexes were found to have similar bioadhesive properties to the polymer alone when tested using a buccal cell adsorption model and texture probe analysis. It was concluded that this complex shows promise as a means of allowing the extended delivery of zinc ions locally within the oral cavity.

Keywords: Oral cavity; Antimicrobial; Zinc; Carbomer; Poly(acrylic acid); Bioadhesion

1. Introduction

The efficacy of conventional dosage forms for the localised delivery of actives to the oral cavity is often impeded by the residence time of the applied formulation. Salivation, the swallowing reflex, speech, mastication and the passage of food boli may affect displacement or dilution of the dosage form, which may result in reduced bioavailability (Steinberg and Friedman, 1999). The accessibility of the oral cavity however makes it a most attractive route for localised therapy to treat conditions such as periodontal disease, gingivitis and dental caries. Although a number of bioadhesive formulations have been described in the literature (Rathbone et al., 1996; Smart, 2004, 2005), to date clinical applications have been limited. In our previous study, polymers such as Carbopol 974P, polycarbophil and chitosan were found to have the property of adsorbing from aqueous solution onto buccal cell surfaces in vitro and in vivo (in a similar manner to that described for salivary mucins), and being retained for extended periods (Kockisch et al., 2001).

Carbopol™ in particular exhibits good retention in vitro and in vivo (Kockisch et al., 2001). Low toxicity and biocompatibility make this polymer ideal for inclusion in products intended for everyday use. Zinc ions are known to be bactericidal against oral pathogens (Scheie, 1989), and could be used as an alternative to established antimicrobials (such as triclosan) in an oral cavity formulation. The aim of this study was to develop zinc/Carbopol 971P complexes that are not only retained within the oral cavity for extended periods but which also provide sustained release of the antimicrobial zinc salts in order to inhibit pathogen colonisation. This in vitro investigation will consider the stability of zinc–carbomer complexes along with their bioadhesive properties.

2. Materials and methods

2.1. Materials

Carbopol® 971P NF was kindly donated by Noveon Inc. (Cleveland, USA). Polyvinylidene difluoride (PVDF) dialysis membrane, MWCO 250,000 Da 1.8 ml cm^{-1}, was purchased from Spectrum Laboratories (Rancho Dominguez, USA). Zinc sulphate heptahydrate and sodium dihydrogen phosphate

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doi:10.1016/j.ijpharm.2007.03.023

monohydrate were all of ACS standard and purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). All other chemicals used in this investigation were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). All chemicals were used as received without further treatment unless otherwise stated.

2.2. Preparation of aqueous polymer dispersions and zinc ion solutions

Carbopol 971P (0.10 g) was dispersed in 40 ml deionised water under magnetic stirring at 300 rpm for 4 h. The pH of the resultant dispersion was adjusted to 7.0 using 0.1 M sodium hydroxide and the volume made up to 100 ml with deionised water to give a final concentration of 0.10% (w/v). Aqueous polymer dispersions were sealed, stored overnight at 4 °C and used within 1 week of preparation.

Based on the molecular weight of acrylic acid monomer in 0.10% (w/v) Carbopol 971P solution, the initial concentration of the zinc salt solutions was calculated to be twice that of the carboxyl groups. Hence, zinc sulphate was prepared at a concentration of 27.8 mM in deionised water. As necessary, dilutions were prepared in deionised water to obtain the following concentrations of zinc salt: 27.8, 13.9, 6.95, 2.78, 1.39, 0.28 and 0.14 mM.

2.3. Preparation of zinc/Carbopol complexes

0.10% (w/v) aqueous Carbopol 971P (10 ml) was transferred to a 100 ml glass beaker and stirred at approximately 300 rpm with a magnetic stirrer bar. Zinc sulphate solution (10 ml) was added slowly to the aqueous polymer dispersion while stirring and the resultant solution observed for changes in turbidity over 30 min. This was repeated at least three times.

2.4. Stability of the zinc/Carbopol complex

Polyvinylidene difluoride dialysis membrane (approximately 25 cm) was prepared as per the manufacturer's instructions. The dialysis tubing was soaked for 30 min in deionised water and 30 min in 100% ethanol. The dialysis tubing was then washed repeatedly in deionised water and pre-equilibrated overnight in 1.39 mM zinc sulphate solution.

Prior to the experiment, dialysis tubing was rinsed in deionised water. Control experiments determined that at this stage the dialysis tubing was free from bound zinc ions. Zinc/Carbopol 971P solution (20 ml) was prepared 30 min prior to dialysis by adding 1.39 mM zinc sulphate (10 ml) to 0.10% (w/v) aqueous Carbopol 971P (10 ml) and transferred into the dialysis tubing using a bulb pipette (20 ml, Fisherbrand). The dialysis tubing was secured with dialysis clips and subsequently immersed in 480 ml release medium (deionised water) in a covered beaker with constant stirring at 400 rpm at room temperature (20 °C). Aliquots of 20 ml were removed at hourly intervals for 6 h to determine the concentration of zinc within the release medium, and replaced with fresh deionised water. After 6 h, the dialysis tubing was transferred to fresh deionised water (480 ml) under stirring and a final sample was removed after 24 h. Samples were stored for a maximum of 48 h at 4 °C in acid-treated glass bottles (35 ml capacity, Fisherbrand). The concentration of zinc from samples collected during dialysis was determined by means of calibration standards with atomic absorption spectroscopy (Hitachi Z800), using a flame atomiser fuelled with an air–acetylene mixture at a flow rate of 1.5 l min^{-1}. Lamp current and slit width was fixed for all zinc analysis at 5.0 mA and 1.3 nm, respectively. The detection of zinc ions within the release medium was compared to a control composed of 10 ml deionised water and 10 ml zinc salt solution. This procedure was repeated six times for the zinc ion–polymer complex and corresponding experimental controls.

2.5. Release of zinc into simulated saliva

In a further study, a solution of 1.56 mM calcium chloride adjusted to isotonicity with sodium chloride (0.229 g calcium chloride, 8.913 g sodium chloride in 1 l deionised water) was used as the release medium, in order to more closely match the ionic constitution of saliva. The zinc salt Carbopol 971P solution (10 ml) was added to double strength isotonic solution (10 ml) to give a final concentration of 0.695 mM and 0.05% (w/v), respectively, immediately prior to the experiment and this was transferred into the dialysis tubing. This was subsequently immersed in 480 ml of the isotonic solution under constant stirring at 400 rpm. Samples were collected as described previously. The displacement of the zinc ions from Carbopol 971P was compared to a control composed of zinc sulphate solution (5 ml), deionised water (5 ml) and double strength isotonic solution (10 ml).

Each experiment was performed six times at three different pHs: 4.0, 5.5 and 7.0. The pH of the isotonic solution was adjusted with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid (HCl), and the zinc ion concentration evaluated using atomic absorption spectroscopy as before. Three physiologically relevant pHs were selected: pH 5.5, the critical demineralisation pH of enamel (Marsh and Martin, 1999); pH 4.0, the lowest plaque pH measured after the consumption of a 10% sucrose solution following salivary stimulation (Edgar and O'Mullane, 1996) and pH 7.0, the upper range of resting salivary pH.

2.6. In vitro mucoadhesion of polymer dispersions to human buccal cells

The procedure used was based on that described by Kockisch et al. (2001). Buccal cells were collected from healthy individuals 2 h post-prandial by gently scraping the inside of the cheek with a wooden tongue depressor. Collected cells were suspended in 20 ml 0.25 M sucrose solution and stirred using a magnetic stirrer at 500 rpm for 30 min in order to prevent the formation of cell aggregates. Aliquots of cell suspension (5 ml) were dispensed into 15 ml glass test tubes and centrifuged at 200 × g for 15 min (Juoan B4i centrifuge). The supernatant was removed and the resultant cell pellet was re-suspended in 5 ml fresh 0.25 M sucrose solution. This process was repeated three times in order to remove debris and unbound proteins from the cell surface.

After the final wash, the cell pellet was re-suspended with gentle vortexing in 5 ml 0.1% (w/v) aqueous polymer dispersion or 5 ml 0.25 M sucrose solution (negative control) and incubated at 32 °C for 30 min. A temperature of 32 °C was chosen as being between the likely temperature of mouthwash solution prior to application (20 °C) and body temperature (37 °C), and is also the experimental temperature used frequently for the surface of the skin. Cells were separated from excess polymer by centrifuging at 400 × *g* for 15 min. The supernatant was removed and the resultant cell pellet washed five times in 0.25 M sucrose solution. Cells were then stained by adding 5 ml 0.05% (w/v) Alcian Blue 8GX in 0.16 M sucrose acetate buffer (pH 5.8 using 0.1 M hydrochloric acid) for 30 min at 32 °C. Uncomplexed dye was removed by washing the cells with 0.25 M sucrose solution, until the supernatant was clear.

One to two drops of the remaining cell suspension were transferred to a microscope slide and examined using light microscopy (Nikon eclipse E600). Images of 20 different cells chosen randomly from the centre of the slide were captured using a digital camera (Leica DC 200) at ×200 magnification and converted to greyscale using Image Converter and Editor software (v1.87, Copyright © 2001–2003 Global General Computer Inc.). Image analysis was carried out using Scion Image (vBeta 4.0.2, Copyright © 2000 Scion Corporation). An area within the cell containing 10,000 pixels was measured and analysed according to the average pixel intensity based on a scale (white = 0, black = 255). The experiment was repeated three times.

In order to investigate the effect of simulated in vivo conditions on binding, buccal cells were collected as before and suspended in 20 ml simulated saliva (5 mM sodium bicarbonate, 7.36 mM sodium chloride, 20 mM potassium chloride, 6.6 mM sodium dihydrogen phosphate monohydrate, 1.5 mM calcium chloride dihydrate) under constant stirring with a magnetic stirrer at 500 rpm for 30 min to avoid cell aggregation. The composition of the simulated saliva was based on the ionic component of submandibular saliva (excluding magnesium) (Lentner, 1981). Aliquots of cell suspension (5 ml) were dispensed into 15 ml glass test tubes and centrifuged at 200 × *g* for 15 min. The supernatant was removed and replaced with fresh simulated saliva and this washing procedure was repeated as above.

The resultant cell pellet was incubated at 32 °C for 15 min with zinc/Carbopol 971P solution (5 ml) and compared with cells incubated with a negative control (simulated saliva), and a positive control, 0.05% (w/v) Carbopol 971P (pH 7.0). Cells were centrifuged at 400 × *g* for 15 min. The cell pellet was washed once in 0.25 M sucrose solution, three times in simulated saliva and once more in 0.25 M sucrose solution. Sucrose was used for the initial wash as large polymer precipitates would form following an initial simulated saliva wash. Simulated saliva also had to be avoided for the final wash due to the interference of cell staining by the presence of free ions. The cells were stained and evaluated as described previously.

2.7. Texture probe analysis

Polymer disks were prepared by accurately weighing 30 mg of Carbopol 971P and applying 2 tonnes of pressure for 10 s using a KBr press (Specac) and a KBr disk assembly unit (8 mm diameter). The resultant polymer disks were stored in a desiccator before use. A non-adhesive material, ethylcellulose, was prepared for use as a negative control.

Porcine oesophagi were excised from freshly slaughtered animals (P.C. Turner, Farnborough, UK) and stored in saline solution (0.9%, w/v, sodium chloride) during transportation. Using dissecting equipment, oesophageal epithelial tissue was separated from the surrounding smooth muscle within 2 h of collection and flash frozen in liquid nitrogen to prevent the formation of large ice crystals. Frozen oesophageal tissue was then stored at −40 °C and defrosted in saline solution at room temperature prior to use. Tissue sections (3 cm × 6 cm) were cut and fixed to a Plexiglas support (Stable Micro Systems, Surrey, UK) covered with black electrical tape, using cyanoacrylate glue (Loctite III). Oesophageal epithelium was moistened with simulated saliva (1 ml) prior to testing. The polymer disk to be tested was fixed onto a movable probe using a circle of double-sided tape (4 mm diameter).

Maximum detachment force and work of adhesion were determined using a TA.XTplus Texture Analyser with a 5 kg load cell (Stable Micro Systems, Haslemere, Surrey, UK). The test conditions were as follows: pre-test probe lowering speed, 0.6 mm s^{-1}; test speed, 0.1 mm s^{-1}; applied force, 0.2 N; contact time, 300 s; post-test speed, 0.1 mm s^{-1}; return distance, 5.0 mm; trigger force, 0.05 N. Data were recorded on a force–distance graph using texture exponent software (v.2.0.0.3). Maximum detachment force (mN) and work of adhesion (mN mm^{-1}) were recorded for six samples of each polymer.

3. Results

The aim of the initial study was to evaluate the concentration of zinc sulphate solution that still allowed the formation of a water dispersible complex. At 1.39 mM zinc sulphate caused no visible changes to the Carbopol 971P aqueous dispersion (Table 1). Based on the molecular weight of acrylic acid, this corresponds to an estimated 1:10 ratio between the metal and carboxyl group concentration, leaving the majority of carboxyl groups free.

Table 1
Visual inspection of 0.10% (w/v) Carbopol 971P aqueous dispersion following addition of various concentrations of zinc ions in solution

Concentration (mM)	Estimated metal:carboxyl group ratio	Observed precipitation after 30 min by zinc sulphate
27.80	2:1	***
13.90	1:1	**
6.95	1:2	**
2.78	1:5	*
1.39	1:10	–
0.28	1:50	–
0.13	1:100	–

The grades allocated were large aggregates (***), small aggregates (**), no precipitation accompanied by an increase in polymer turbidity (*) and no visible changes (–).

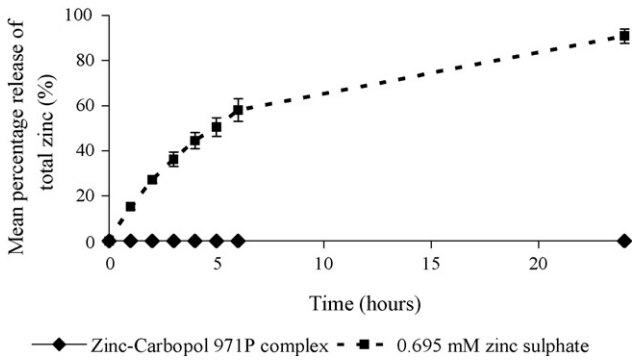

Fig. 1. Cumulative release of zinc detected in the release medium when dialysing 0.05% zinc–Carbopol 971P (1.10) complexes and 0.695 mM zinc sulphate solutions in deionised water ($n = 6$, ±S.D.).

Zinc remained undetectable in the dialysate when the complex was dialysed against deionised water (Fig. 1) indicating that an association had formed between Carbopol 971P and zinc ions. When the complex was dialysed against an isotonic solution of calcium and sodium chloride, zinc was displaced from the Carbopol and detected within the dialysate but at a rate less than the zinc sulphate control (Fig. 2). The release of zinc from the zinc sulphate solution control into the receptor phase was the same at all pH's, being initially very high, with 42.9 ± 2.3% of total zinc detected within the release medium after 1 h. After 24 h 89.2 ± 4.6% of total zinc was released from within the dialysis tubing and equilibrium had been reached. At pH 7.0 the rate of release of zinc from the polymer complex was significantly lower than that from the control, with 56.4 ± 5.7% of total zinc released after 6 h and 77.2 ± 5.6% after 24 h. At pH 5.5 the rate of release of zinc was significantly slower than the zinc sulphate control, with only 56.4 ± 5.7% of total zinc released after the first 6 h. After 24 h an average of 77.2 ± 5.6% had been displaced from the zinc/Carbopol 971P complex, which was 12% less than the zinc sulphate control. At pH 4.0 the rate of release of zinc from the polymer complex had increased from that observed at higher pHs, with 77.9 ± 8.0% of total zinc released after 6 h, similar to the zinc sulphate control rates.

An increase in stain intensity was observed for buccal cells exposed to the positive control and the zinc/Carbopol 971P complex when compared to the untreated control regardless of the solution used to collect and wash the cells, i.e. 0.25 M sucrose or simulated saliva (data shown for simulated saliva; Fig. 3) However, the polymer was not evenly deposited across the buccal

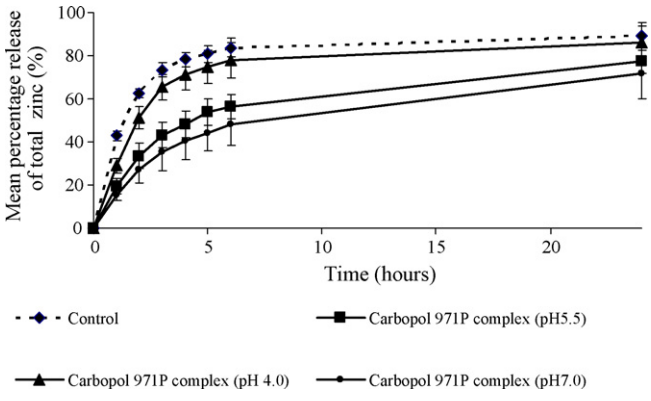

Fig. 2. Cumulative release of zinc from the 0.05% zinc sulphate–Carbopol 971P (1:10) solution compared to a 0.695 mM zinc sulphate solution control in simulated saliva at various pHs ($n = 6$, ±S.D.).

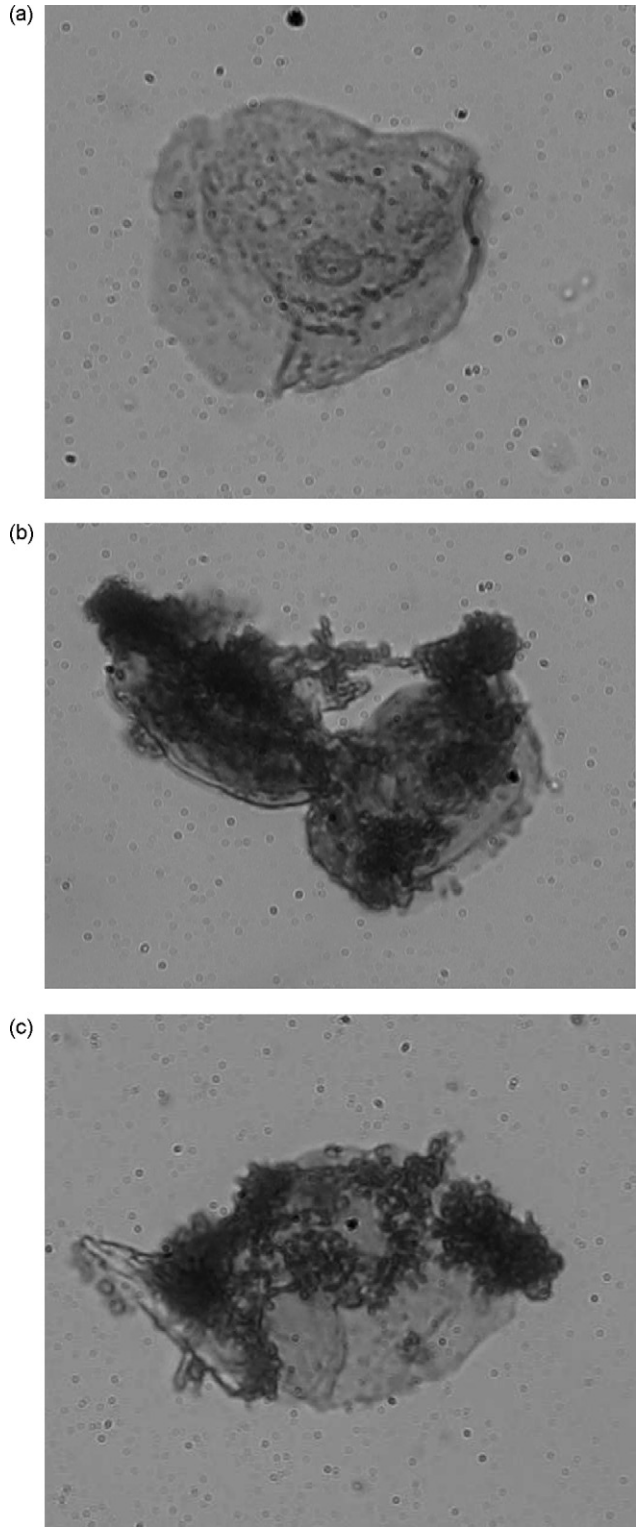

Fig. 3. Light microscopy images of the adsorption of polymer onto buccal cell surfaces detected by Alcian Blue staining in the presence of simulated saliva; untreated control (a), 0.05% (w/v) Carbopol 971P aqueous dispersion (b) and 0.05% zinc/Carbopol 971P (1:10) complex (c); ×200 magnification.

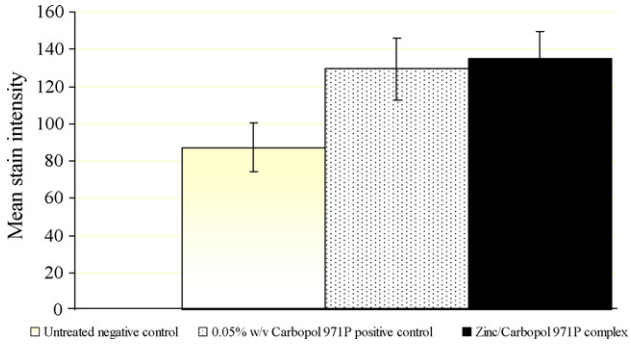

Fig. 4. Mean stain intensities from image analysis of buccal cells incubated with simulated saliva; untreated control, 0.05% (w/v) Carbopol 971P aqueous dispersion and 0.05% zinc/Carbopol 971P (1:10) complex ($n = 60$, ±S.D.).

Table 2
The adhesive bond strength exhibited by polymer samples to a model mucosal surface using texture probe analysis ($n = 6$)

Material	Maximum detachment force (mN)		Work of adhesion (mN mm^{-1})	
	Mean	S.D.	Mean	S.D.
Negative control (ethylcellulose)	0	0	0	0
Positive control Carbopol 971P	102	54	26	7.9
Zinc/Carbopol 971P complex	101	57.9	19.2	6.3

cells. The mean stain intensity of cells exposed to the aqueous dispersion of Carbopol 971P or zinc/Carbopol complex (Fig. 4) were significantly greater than the control ($P < 0.05$), but not significantly different to each other ($P > 0.05$, ANOVA Tukey's multiple comparison). Texture probe analysis of the complex suggests that the presence of zinc at these levels does not affect the bioadhesion of Carbopol 971P to the model mucosal surface in the presence of simulated saliva (Table 2). Both the positive control and the zinc/Carbopol 971P complex produced adhesion profiles that were different to the negative control. The complex and the polymer alone were not significantly different from each other ($P > 0.05$, unpaired Student's t-test).

4. Discussion

The aim of this work was to produce complexes of zinc and the bioadhesive polymer that will allow the release of zinc ions in vivo, to produce a local antimicrobial effect. In the hydrated state, Carbopol 971P particles uncoil to a limited extent due to extensive intra and inter-chain hydrogen bonding between associated carboxyl groups (R-COOH) on the polymer backbone (Dittgen et al., 1997). At higher pH's, carboxyl groups dissociate (R-COO$^-$) and the negative charges cause chain repulsion, fully uncoiling the polymer and allowing ionic interactions with divalent cations. The compatibility of an aqueous Carbopol 971P dispersion with cations is concentration dependent, with high concentrations resulting in the collapse of the uncoiled polymer network and the precipitation of tightly coiled polymer particles. A divalent cation like zinc can also cause cross-linking of the polymer by ionic bridging between two carboxyl groups. The highest concentration of metal salt that could be added to 0.10% (w/v) Carbopol 971P aqueous dispersion in a 1:1 (v/v) was investigated visually and a concentration a little below that found to produce turbidity used.

The positively charged divalent zinc ions were found to be retained with the negatively charged carboxyl groups of the partially ionised Carbopol 971P dispersion and when exposed to deionised water, were not released. However, in the presence of other ions such as those present in the oral cavity, zinc is released over a period of time, presumably by an ion exchange-like displacement reaction with other cations. At lower pHs the carboxyl groups on Carbopol 971 are predominantly unionised, meaning that the ability to retain cations by ionic interactions is reduced and the zinc is released. This leads to the intriguing possibility of targeting the release of the antimicrobial agent to areas of low pH, such as those where cariogenic bacterial are present.

Bioadhesive polymers will increase the substantivity of the antimicrobial at a site often affected by poor retention times via mucosal adhesion. The levels of zinc binding in the complex produced adhesive properties similar to that of the polymer alone when using both the buccal cell adsorption model and the tensiometer adhesive testing system. A previous in vivo study using a somewhat insensitive detection procedure indicated that bioadhesive polymers can be retained in the oral cavity for at least an hour (Kockisch et al., 2001). Although it is recognised that the 'payload' of zinc carried by the polymer is relatively low, it is envisaged that in local areas of the oral cavity, e.g. on gingival and buccal mucosa, the local concentration of zinc would be sufficiently high to have an antimicrobial effect, and this will be investigated in further in vitro and in vivo work. These complexes therefore have the potential to form a bioresponsive antimicrobial delivery system that will preferentially release the antimicrobial agent in regions with a lower pH such as those generated in the oral cavity by cariogenic microorganisms.

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