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In situ evaluation of drug-loaded microspheres on a mucosal surface under dynamic test conditions

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Abstract

The ability of polymeric microspheres fabricated from Carbopol, polycarbophil, chitosan or Gantrez to retain a model hydrophilic drug (sodium fluorescein) was evaluated in situ, using a dynamic test system and image analysis. This technique used oesophageal tissues and simulated the physiological conditions within the oral cavity in terms of temperature, humidity and saliva flow. The point of sample application was observed over a 2 h period by means of a digital camera. No significant differences in fluorescein colour intensity was obtained for the Gantrez and chitosan particles over 100 min, indicate that these two polymers provide the possibility of prolonged action. Carbopol and polycarbophil particles became rapidly swollen and released the sodium fluorescein completely within 20 min. It was concluded that the test system allowed the evaluation of the in situ behaviour of test particles, in terms of their ability to retain a water-soluble, coloured marker in 'dynamic' test conditions, and that chitosan and Gantrez were promising candidates for the production of mucoadhesive, sustained-release microspheres for water-soluble materials.

Keywords: Bioadhesion; Mucoadhesion; Oral cavity; Chitosan; Poly(acrylic acid); Maleic anhydride copolymers

1. Introduction

The efficacy of conventional dosage forms for the 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 local and systemic drug delivery. Although a number of bioadhesive formulations have been described in the literature (Gurny and Peppas, 1990; Nagai et al., 1990; Rathbone et al., 1996; Smart, 1993), to date clinical applications have been limited. In our previous study, polymers such as Carbopol 974, polycarbophil, chitosan and Gantrez, identified as being retentive in vivo (Kockisch et al., 2001), were

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formulated into microspheres using a "water-in-oil emulsification" solvent evaporation method (Kochisch et al., 2003). All these microspheres were found to adhere to porcine oesophageal mucosa although in a preliminary elution experiments involving washing with artificial saliva, particles of chitosan or Gantrez were seen to be retained for longer time periods relative to those assembled from the poly(acrylic acid)s.

In vitro test systems have been developed that are performed under 'dynamic' conditions (Abd El-Hameed et al., 1999; Batchelor et al., 2002; Gaserod et al., 1998; Riley et al., 2002, 2003; Young and Smart, 1998). Among these, the flow channel method (Mikos and Peppas, 1990) and the falling liquid film method (Teng and Ho, 1987) have received most attention. Such tests attempt to mimic in vivo conditions in terms of temperature, humidity and liquid flow and aim to extract information about the influence of a range of parameters on the mucoadhesion process. Dynamic techniques are typically based on the assessment of the percentage retention of an applied sample on a mucosal surface over time. However, it is important for the prediction of their eventual in vivo behaviour to investigate these formulations in situ, in terms of their ability to gel, spread, erode and become dislodged from the site of application. In our previous study (Kockisch et al., 2003), the time taken for all the test particles to move from the point of application to two points along a section of oesophageal mucosa was evaluated in a 'dynamic flow' test system (Riley et al., 2002, 2003). In this further study the ability of test microspheres to retain an included model water-soluble drug (sodium fluorescein) will be evaluated by image analysis, allowing the behaviour of these particles (in terms of retention, swelling and release rate) on the mucosal surface to be observed.

2. Materials and methods

2.1. Materials

Carbopol® 974P NF and polycarbophil (Noveon® AA-1) were supplied by BF Goodrich, Cleveland, Ohio. Gantrez® MS-955 was purchased from ISP Europe, Guildford. Chitosan (150,000 MW grade) was purchased from Fluka Chemicals, Gillingham. Mucin (type III, partially purified from porcine stomach) were purchased from Sigma Chemicals, Poole and sodium fluorescein from BDH Chemicals Ltd., Poole. Porcine oesophagi were obtained from a local abattoir on the day of slaughter.

2.2. Preparation of the mucosal surface

Pig oesophagi were rinsed gently with water to remove foodstuff. Strips, 4 cm × 15 cm, were cut from the middle of the oesophagus and the underlying muscle carefully removed. The strips were gently rinsed with a non-ionic isotonic aqueous solution (0.25 M sucrose), then flash-frozen in liquid nitrogen and stored at −20 °C. Preliminary studies revealed that freezing was essential to inactivate the muscle tissue within the mucosae and therefore provide the flat surface essential for this study, and to minimise the effects of enzymatic and bacterial degradation. Flash-freezing has been shown in previous work to minimise tissue damage (Young and Smart, 1998). Prior to use, the tissue was defrosted by placing into an isotonic aqueous solution (0.25 M sucrose). For each experiment, a new porcine oesophageal tissue was used.

2.3. Preparation of fluorescein-containing polymer microspheres

Carbopol or polycarbophil (0.50 g) was dispersed in ca. 40 ml of de-ionised water by rapid vortexing (magnetic stirrer) for 4 h. The pH of this dispersion was adjusted to 7.0 using aqueous sodium hydroxide (1 M). De-ionised water was added to the resulting hydrogel to give a total mass of 50.0 g then 20 mg of sodium chloride with stirring. An appropriate mass of chitosan was added gradually to a solution of aqueous acetic acid (1.0% w/w) to yield a concentration of 2.0% (w/w). The mixture was stirred until a viscous gel was obtained. Gantrez was dissolved under vortexing in de-ionised water to give a 2.0% (pH 6.5) solution.

An appropriate amount of sodium fluorescein was dissolved directly into these aqueous polymer dispersions (prior to making to the final concentration) to give a ratio of 1:10 w/w relative to the polymer and subsequently stored in hermetically sealed containers at 4 °C for 24 h prior to use.

Microspheres were prepared by a water-in-oil emulsification solvent evaporation method utilising a

U-shaped beaker and a two-blade paddle stirrer. By means of a syringe, 25 ml of each polymer dispersion was added dropwise, at a speed of 3 ml/min, to 250 ml of light mineral oil containing 1.0% (v/v) Span 80 (a surfactant for the emulsification process). Complete dispersion of the aqueous phase into the oil and the facilitation of microsphere formation were ensured by continuous stirring of the emulsions using an overhead stirrer with calibrated speed control. The stirring speeds were set at 500 rpm for chitosan and Gantrez and at 600 rpm for Carbopol and polycarbophil.

Whilst stirring, the samples were heated to 60 °C in a waterbath. This temperature was maintained for 24 h to promote the evaporation of water. The remaining oil phase was then centrifuged at 200 × *g* for 5 min. The oil was decanted and the particles collected into a vial and washed several times with hexane. Subsequently, the microspheres were dried and stored in a vacuum desiccator at ambient temperature.

2.4. Analysis of model drug

For the quantification of the sodium fluorescein content in microspheres, polymer microspheres containing the hydrophilic model drug (ca. 1 mg) were weighed accurately into a 1 l flask, which was filled with de-ionised water (pH 7). The flasks were shaken well, and the mixtures were allowed to stand overnight to facilitate the formation of a solution of the polymer and/or the release of sodium fluorescein. Subsequently, the solution was centrifuged to remove undissolved polymer. By means of a pipette, the clear supernatant solution was transferred into a cuvette and its fluorescence determined against that of a blank (de-ionised water at pH 7) (Perkin-Elmer LS-5B). The optima for the excitation and emission were identified at 490 and 512 nm, respectively. The fluorescence intensity values obtained for the sodium fluorescein solutions (1×10^{-5}% w/v) containing polymer were compared with those of a polymer-free solution of equivalent fluorescein content. To determine the sodium fluorescein content in the microspheres, the concentration values obtained were compared with a calibration curve. For each polymer, this analysis was performed three times. A preliminary study confirmed that the presence of polymers in solution did not quench the fluorescence of the sodium fluorescein.

2.5. In vitro testing

The dynamic test system design (Fig. 1) was adapted for the testing of polymer microspheres (Riley et al., 2002, 2003). Placing two inserts along the long sides of the test cell which resulted in the reduction of the width of the available test surface (from 15 to 10 mm) and in the formation of a channel, which ensured that saliva flows through the centre of the tissue.

Thawed mucosal tissue was fixed onto the test cell and pulled flat by the application of a moderate vacuum through small holes at the base plate of the cell. The cell surface was inclined at an angle of 30°. The tissue was allowed to stand for a period of 1 h at 37 °C (maintained by circulation of a water jacket through the body of the cell) and ca. 90% relative humidity (achieved by passing warm, humidified air over the tissue surface). A transparent cover was placed on the cell, which ensured that the desired humidity and temperature could be maintained in the chamber whilst allowing the visualisation of the mucosa. During this 1 h period, artificial saliva (0.5% partially purified porcine gastric mucin in pH 7 isotonic phosphate buffer) was circulated over the tissue at a rate of 1 ml/min by means of a peristaltic pump. This ensured that the tissue was sufficiently hydrated and that mucins from artificial saliva could adhere onto the mucosal surface. After this time, the flow of saliva was interrupted and the inclination angle set back to 0° to facilitate sample application. The test microspheres (1 mg) were gently placed onto the mucosal surface by means of a small glass funnel (final diameter ca. 3 mm). The point of sample application was a circle of 3 mm diameter that was located in the centre of the tissue. No force of application, other than gravity, was employed. After a 1 min contact time, the cell was brought back to 30° and saliva flow resumed.

The retention of sodium fluorescein was assessed at the point of sample application. For this purpose, the applied microspheres were observed by means of a digital video camera (Panasonic NV-DS15, 20× zoom) that was positioned on a tripod immediately above the test cell. At set times points and over a 2 h period, the particles were recorded for some seconds under both visible and UV-light. The former allows the identification of the distribution of fluorescein on the tissue surface, while the latter indicates the diffusion of water into the particles, as well as the concentration

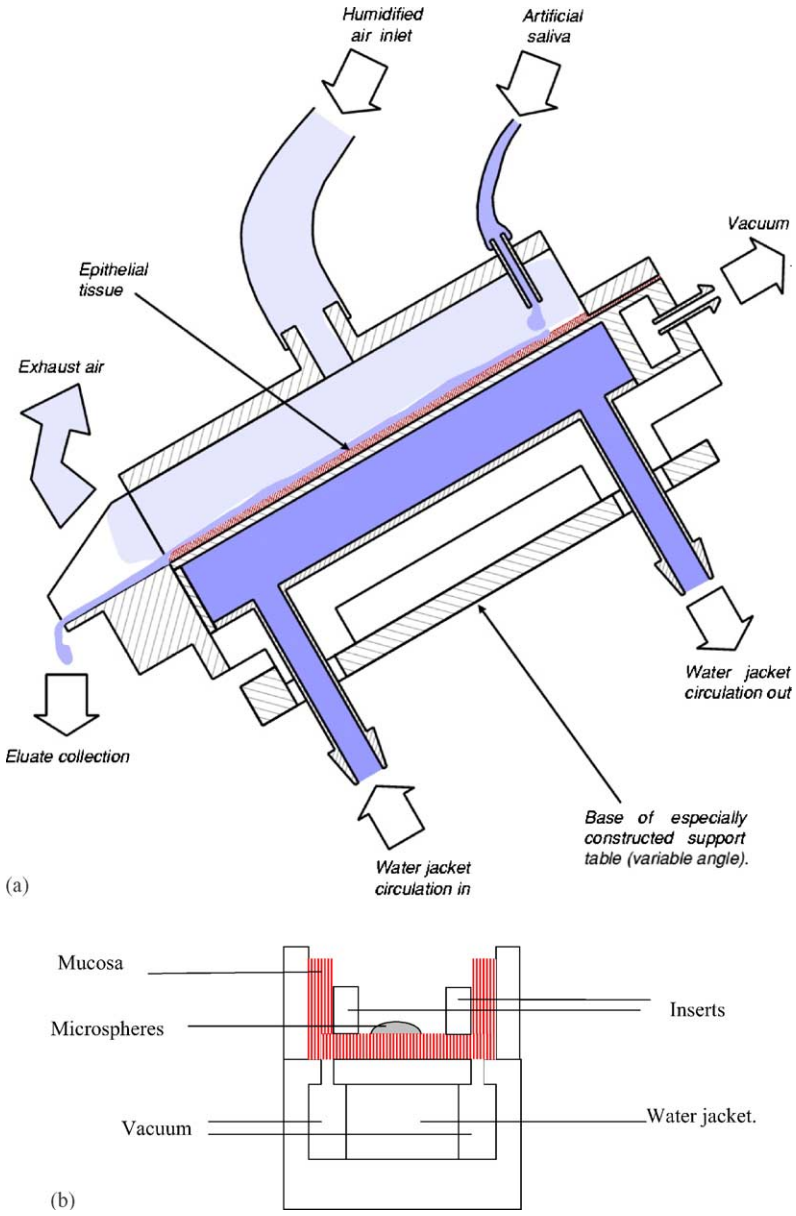

Fig. 1. Basic design of the porcine oesophageal mucoadhesion test system in viewed from the side (a) and in cross-section (b).

of the marker in the field of vision. The field of vision and the magnification (20×) of the camera along with the position of the light source were not altered during the experiment to allow direct comparison of images from the same run. From the short movies, single frames were extracted for analysis by means of the software Adobe Premiere 5. These images were then analysed in terms of tissue area covered by polymer particles and the intensity of sodium fluorescein using the image analysis program ScionImage. The intensity measurements were performed on the whole area covered by the microspheres using a greyscale image, on the basis that white corresponds to zero whereas black (the maximum count) corresponds to a value of

255. The area covered by microspheres and the pixel intensity were calculated as a percentage of the initial values (time zero) at which both parameters were assigned a value of 100%. The two parameters that were extracted from the frames gave an image of the distribution of drug on the mucosa and provided an indication of its elution from microspheres. The experiment was performed five times for all microsphere samples.

3. Results

The results for the analyses of the sodium fluorescein content in polymer microspheres are summarised in Table 1.

The concentration of sodium fluorescein in microspheres ranged between 5 and 9% (w/w) (see Table 1). During microsphere manufacture, some sodium fluorescein migrated into the oil phase, which was evidenced by the orange colour that developed following centrifugation and separation of the microspheres.

Typical examples of the resulting images are presented in Figs. 2 and 3. These illustrate the distribution and elution of sodium fluorescein from Carbopol and chitosan microspheres at discrete time points, ranging from 0 to 20 min for Carbopol and from 0 to 120 min for chitosan. These time periods (20 and 120 min, respectively) were consistent with previously made observations for the retention of polymer microspheres on mucosal tissue at the same flow rate (Kockisch et al., 2003).

For microspheres consisting of Carbopol and polycarbophil, following application to the tissue, this colour changed from orange to yellowish (consistent with dilution of sodium fluorescein) and faded within 20 min (Fig. 2). Both poly(acrylic acid) polymers behaved similarly in terms of the observed changes in

Table 1
Sodium fluorescein content in polymer microspheres (mean ± S.D.; $n = 3$) determined by fluorescence spectroscopy (490 nm excitation; 512 nm emission)

Sample	Sodium fluorescein in microspheres (% w/w)
Carbopol	6.5 ± 0.3
Polycarbophil	4.9 ± 0.3
Chitosan	5.4 ± 0.5
Gantrez	8.8 ± 0.3

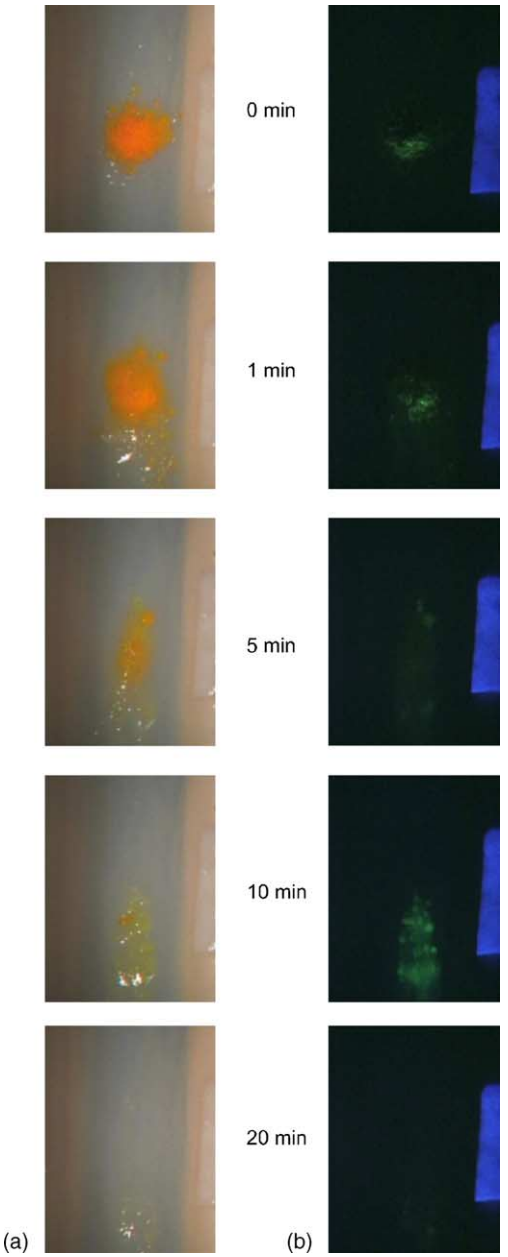

Fig. 2. Carbopol 974 microspheres containing sodium fluorescein on porcine oesophageal mucosa (washed with artificial saliva at 0.5 ml/min flow rate; recorded with a digital camera 20×). (a) Under visible light, (b) under UV-light.

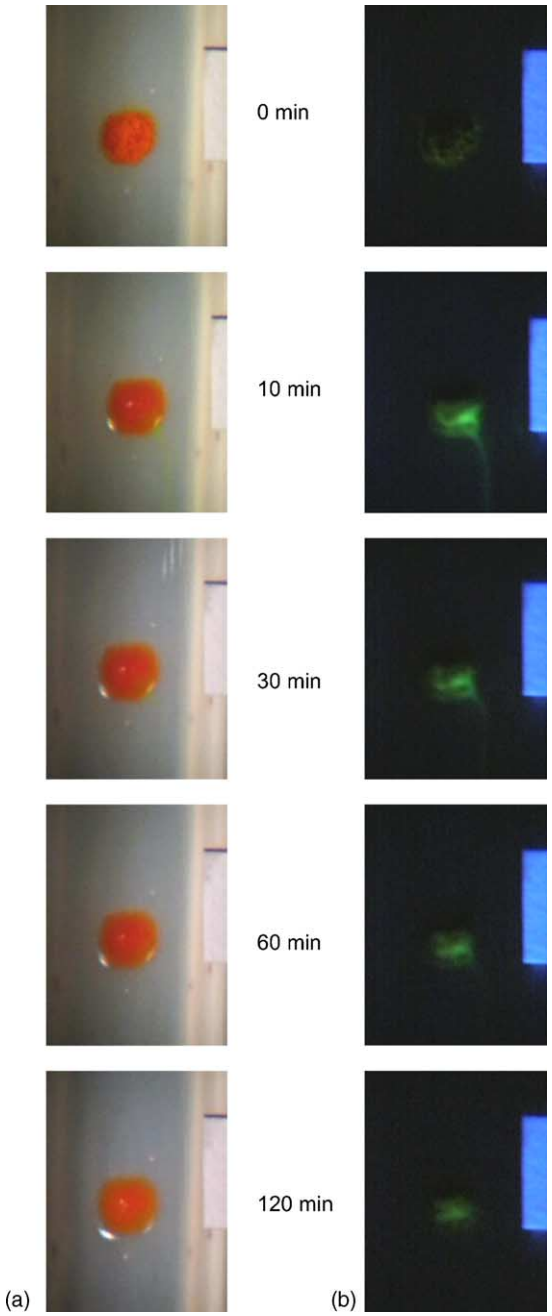

Fig. 3. Chitosan microspheres containing sodium fluorescein on porcine oesophageal mucosa (washed with artificial saliva at 0.5 ml/min flow rate; recorded with a digital camera 20×): (a) under visible light, (b) under UV-light.

area and intensity: the differences at each time point (Figs. 4 and 5) were found to be insignificant ($P > 0.05$, two-sample *t*-test). Immediately after sample application, the area covered by microspheres was seen to increase, but this was statistically significant only for Carbopol (one-way analysis of variance; Tukey's multiple comparison test; 95% confidence level). This expansion was only detected for up to 2.5 min from the onset of saliva flow, after which time a decrease in area was observed. In parallel, the colour intensity of the microspheres was seen to decrease: the value determined after 1 min was significantly different to the initial intensity at time zero. A 50% reduction in colour intensity was witnessed within ca. 5–10 min. The area covered by chitosan microspheres, and the colour intensity, was not seen to change greatly over the 2 h time period (Fig. 3): only for times of 100 min or greater were the observed differences in area of statistical significance (one-way analysis of variance; Tukey's multiple comparison test; 95% confidence level). Notably, over 80% of the initial coverage was still present after 2 h.

In the case of Gantrez, the orange particles were seen to fade gradually throughout the recording period of 2 h (Fig. 5). An initially significant reduction in covered area (up to 2.5 min) was followed by insignificant further changes (one-way analysis of variance; Tukey's multiple comparison test; 95% confidence level). However, at 120 min, for two of the conducted five runs, no particles remained attached to the tissue area under consideration.

When using UV-light, little fluorescence was observed for the poly(acrylic acid) containing particles (Fig. 2), while fluorescence was observed for Gantrez at time zero, which gradually increased with time. With chitosan, the fluorescence reached a maximum at 10 min and declined after this (Fig. 3).

4. Discussion

The procedure used allowed the investigation of the retention of a model drug incorporated into a microsphere on a model mucosal surface analogous to the conditions within the oral cavity. Saliva flow and the presence of mucin were modelled, but not the presence of food, or abrasion caused by mouth movements during mastication. The image analysis considered colour

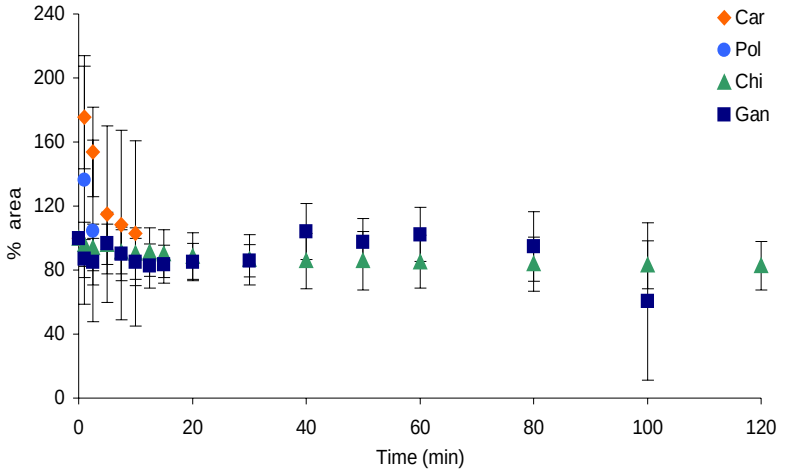

Fig. 4. Changes in area covered by microspheres containing sodium fluorescein (S.D. bars; $n = 5$). Area at time 0 is 100%.

intensity as a measure of drug content, but this (measured as the 'darkness' in a greyscale image) is clearly not linearly related to the actual drug concentration. However, it does allow a semi-quantitative indication of the presence of model drug (agreeing with the visually observed colouration), and therefore the retention on a mucous membrane under simulated in vivo conditions.

With the poly(acrylic acid)s, the initial increase in area can be attributed to both the swelling of polymer microspheres in contact with artificial saliva and the displacement and spreading of the particles, caused by saliva flow (Fig. 4). Particles that could not make successful contact with the tissue and mucoadhere will be washed off. Later, particle removal would result from either overhydration and erosion of the polymer gel or by the weakening of the adhesive forces. It was observed that all fluorescein had been removed from the Carbopol and polycarbophil particles within 20 min of the onset of saliva flow, and no visual evidence of particle retention was apparent. The decrease in colouration observed will be caused by particle dislodgement, spreading, along with the elution of sodium fluorescein from the hydrating polymer mi-

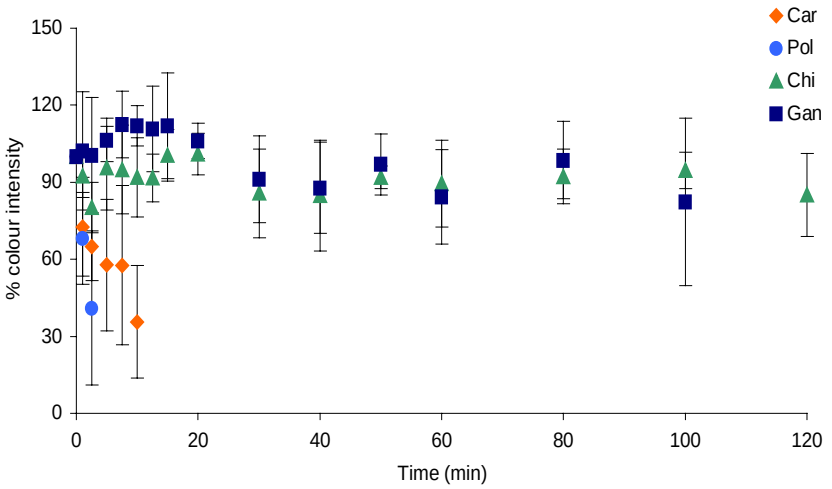

Fig. 5. Changes in colour intensity for microspheres containing sodium fluorescein (S.D. bars; $n = 5$). Intensity at time 0 is 100%.

crospheres. The fact that both the drug and polymer carry the same charge would also promote elution.

The limited swelling of chitosan may be the reason for the persistence of these microspheres. However, a cationic polymer is also capable of interacting with an anionic drug and this could also affect retention.

In previous studies, Gantrez was seen to be retained on the tissue for extended periods and to show lower swelling relative to the poly(acrylic acid)s (Kockisch et al., 2003), while the high efficiency achieved for the loading of Gantrez microspheres with sodium fluorescein (98%) may be taken as evidence for the high affinity of this model drug for the polymer.

Under UV-light, fluorescence is minimal in dry particles when the molecule is not in solution, but became more apparent as the system hydrated, and then declined as the particles erode and the fluorescein washed away. This was seen to occur with the chitosan and Gantrez particles (Fig. 3), but surprisingly little fluorescence was evident with both of the poly(acrylic acid)s containing particles (e.g., Fig. 2), indicating that the flourescein was rapidly washed away and the remaining areas of weak fluorescence were not detected by the camera.

This study describes a ‘dynamic’ in vitro test system that can be used to evaluate in situ the behaviour of bioadhesive microspheres. It was demonstrated that it is possible to formulate a microsphere that will retain a water-soluble drug on a mucosal surface for extended periods. Chitosan and Gantrez were the most promising candidates, being adhesive, retentive and allowing prolonged drug release, and should be investigated with other drug molecules (e.g., cationic or larger molecular weight) in vitro prior to in vivo evaluation. The poly(acrylic acid) containing microspheres appeared to offer few advantages as drug delivery systems in the conditions used in this study, and their properties would clearly need modifying to reduce the rate of swelling and drug release.

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