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A flow system for the on-line quantitative measurement of the retention of dosage forms on biological surfaces using spectroscopy and image analysis

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ABSTRACT

Measuring the retention, or residence time, of dosage forms to biological tissue is commonly a qualitative measurement, where no real values to describe the retention can be recorded. The result of this is an assessment that is dependent upon a user's interpretation of visual observation. This research paper outlines the development of a methodology to quantitatively measure, both by image analysis and by spectrophotometric techniques, the retention of material to biological tissues, using the retention of polymer solutions to ocular tissue as an example. Both methods have been shown to be repeatable, with the spectrophotometric measurement generating data reliably and quickly for further analysis.

1. Introduction

Bioadhesion is defined as the attachment of synthetic or natural materials to biological surfaces. In pharmaceutics this phenomenon has often been exploited by using the ability of different polymers to adhere to mucosal surfaces and provide prolonged retention of dosage forms, resulting in improved drug bioavailability (Peppas and Sahlin, 1996; Smart, 2005; Bernkop-Schnurch, 2005; Serra et al., 2009; Andrews et al., 2009; Khutoryanskiy, 2011). The excellent potential of mucoadhesive dosage forms has been recognized in buccal, nasal, ocular and vaginal drug delivery and a number of novel pharmaceutical technologies utilizing mucoadhesion have been commercialized. Recent reports have suggested that the market for mucoadhesive drug delivery systems is expanding rapidly (Andrews et al., 2009).

Mucoadhesive dosage forms can be formulated as tablets, films, powders, pellets, solutions and gels. A number of methods have been developed for evaluating the mucoadhesive properties of various formulations. The most common in vitro methods include tensile studies, visual detachment/dissolution time determination, rheological and flow retention techniques (Peppas and Sahlin, 1996; Khutoryanskiy, 2011).

The flow retention technique first described by Rango Rao and Buri (1989) is often used for estimating the mucoadhesive properties of dosage forms administered in regions of the human body where formulations are affected by the flow of biological fluids. For example, it can be used for testing gastrointestinal, ocular, nasal or vaginal formulations and it involves simulation of a biological flow which washes off a dosage form from the surface of a mucosal tissue. This technique can typically be used for evaluating mucoadhesive properties of micro-particles and nano-particles as well as liquid and semi-solid formulations (e.g., syrups, gels and creams).

Different variants of this technique have been successfully used and described in pharmaceutical literature. In the original publication, Rango Rao and Buri (1989) used this technique with 0.45–0.50 mm glass beads coated with various mucoadhesive polymers. They estimated the retention potential of different polymers by measuring the mass of beads washed off a mucosal surface by simulated gastric juice or phosphate buffer solution. Mikos and Peppas (1990) proposed the use of a video camera to monitor the retention of a single polymer microparticle on mucosal tissue in a flow channel and discussed the theoretical basis of flow phenomena in this system. Nielsen et al. (1998) have applied the flow method to evaluate the adhesion of glyceryl monooleate (GMO) and glyceryl monolinoleate (GML) cubic phase gel formulations to rabbit stomach, buccal and intestinal mucosal tissues. They used high performance liquid chromatography (HPLC) to determine the concentration of GMO/GML in the effluent collected after 30 min, and used this to estimate the amount of gel remaining on the surface of the mucosal tissue. Belgamwar and Surana (2010) used a similar technique for evaluating the retention potential of

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calcium alginate microspheres loaded with various mucoadhesive polymers (hydroxypropylmethylcellulose and Carbopol 971P) and atenolol as an active ingredient. 25 microspheres were placed on the mucosal surface of rat intestinal tissues, left for 20 min to establish mucoadhesive contact and then washed with phosphate buffer for 20 min. The retention of microspheres on the mucosal surface was quantified by manually counting at the end of each experiment. Irmukhametova et al. (2011) investigated the retention of fluorescently labelled thiolated and PEGylated organosilica nanoparticles on bovine corneal surfaces using fluorescence microscopy. The nanoparticles were washed off the mucosal surface with 20, 40, 60 and 100 mL of artificial tear fluid and the proportion of nanoparticles remaining on the mucosa was quantified by image analysis of fluorescent microphotographs.

The analysis of existing literature on the use of flow retention techniques demonstrates that the majority of studies have utilized a number of analytical techniques (gravimetric analysis, video recording, spectrophotometry, HPLC, fluorescent microscopy, etc.) to quantify the retention potential of bioadhesive dosage forms and this quantification was usually conducted at the end of each experiment. Only in a few studies, the evaluation of mucoadhesiveness was performed under dynamic test conditions. Kockisch et al. (2004) studied the retention ability of mucoadhesive microspheres loaded with a fluorescent dye on the surface of oesophageal tissues by means of a digital camera with subsequent image analysis. Batchelor et al. (2002) studied the adhesion of fluorescently labelled alginate solutions to porcine oesophageal tissues using a flow retention technique with manual collection of eluted material at different time intervals and its analysis using fluorescent spectroscopy. The analysis of dosage form retention on mucosal surface under dynamic conditions is particularly important for formulations that provide a multistage retention and release profiles, for example, when mucoadhesive micro- or nano-particles are dispersed in an adhesive gel.

Here we report a modification of a flow retention technique that enables on-line quantitative monitoring of the retention of a dosage form on the surface of a biological tissue using a combination of spectrophotometric and image analysis methods. These methods were found to be highly complementary and their combination gives an opportunity to simultaneously monitor both the retention of the dosage form on mucosal surface and drug release from the formulation.

2. Materials and methods

2.1. Materials

The polymers used in the following experiments were Carbopol 971 PNF (C971) and Carbopol 974 PNF (C974) (Surfachem, Leeds), 6 MDa polyacrylamide (PAAm) (Sigma–Aldrich, UK), standard carboxymethylcellulose (CMC) (Sigma–Aldrich, UK) and a formulation of CMC optimized for superabsorbency resulting in high viscosity solutions (HV-CMC) (Aquasorb A500, Aqualon). All polymers were dissolved at concentrations between 1 and 2 wt% (w/v) in deionized water with 0.1 wt% (w/v) of Water Blue (aniline blue) dye (Fluka, UK).

Three sets of experiments are described in this paper. Set A consisted of various concentrations, 1.0, 1.5 and 2.0 wt%, of C971. Set B consisted of C974, CMC, HV-CMC and PAAm, all dissolved at 2.0 wt%. Set C consisted of 1.5 wt% C971 neutralized with the addition of 0, 2 and 4% (v/v) 0.5 M NaOH (Fisher Scientific, UK). These sample sets allowed the comparison of the same polymer at different concentrations, and so viscosities (Set A), different polymers at the same concentration (Set B), and a neutralized Carbopol polymer, with different viscosities due to gelling at higher pH values (Set C).

All of the experiments used bovine corneal tissue and attempted to simulate the conditions on the surface of the eye, and so the temperature of the system was set to 32 °C (Hillery et al., 2001). Cornea were extracted from bovine eyes obtained from a local abattoir, and stored in a frozen state (−18 °C) until use. The animal tissues were typically used within 1–2 weeks after freezing. These ocular tissue storage conditions are in line with the literature reporting the evaluation of mucoadhesive properties of dosage forms for ophthalmic applications (Sandri et al., 2006). The cornea were defrosted at room temperature for 4 h before use, and then placed on the channel for 15 min prior to a sample being run, in order to allow it to reach 32 °C.

Two flow eluents were used throughout the experiments, deionized water and simulated tear fluid (STF). The simulated tear fluid was made (pH 7.60), as described by Srividya et al. (2001), with 0.67 wt% (w/v) sodium chloride (NaCl), 0.2 wt% (w/v) sodium bicarbonate ($NaHCO_3$) and 0.008 wt% (w/v) calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) (all laboratory reagent grade, Fisher Scientific, UK).

2.2. System design

The retention testing system consists of an adjustable angled Perspex® channel (inclination angle is 30°), upon which the biological tissue sample is placed, inside a controlled temperature cabinet (Stuart+BioCote SI60D, UK). A schematic of the flow is shown in Fig. 1. Flow over the biological tissue originates from a reservoir of simulated physiological fluid or water and the flow is generated through a capillary, connected to a peristaltic pump (HR Flow Inducer Pump, Watson-Marlow Ltd., UK), which is positioned to flow over the biological tissue. The flow down the channel passes into a funnel containing a small volume of the initial eluent for mixing, which is then pumped into a cuvette inside a UV–vis spectrophotometer. The flow rate was constant for all runs, 4 mL/min.

A Jasco V-530 UV/vis (Jasco, UK) spectrophotometer was used for the acquisition of the UV/vis data using the Time Course Measurement function. This measures the absorbance over a set period of time, at set intervals. For all of the experiments carried out in this paper, the time interval was set at 2 s, with the maximum time set to 4000 s, to exceed the predicted maximum for loss of material to reach 100%. The absorbance that is measured will be proportional to the concentration of the polymer in solution, below a maximum absorbance of 1.

Video capture is obtained using an Advent A20BAWC09 (Advent) webcam set to a resolution of 640 × 480 pixels, recording in MPG format with a frame rate of 30 fps. Recording was started simultaneously with the fluid flow and the UV/vis detection.

2.3. Repeatability testing

In order to ensure that the data obtained using the UV/vis detector is repeatable; three runs using four different materials were carried out on the channel without corneal tissue present. The results were analysed as they would be when biological tissue was used, and compared in order to assess the repeatability of the two measurement methods.

2.4. Analysis of visual data

Video files were filtered using VirtualDub, with a threshold filter set to 50, in order to view only the darker elements, which in the case of these experiments would be the dye (Water Blue). The frame rate was also decimated by a factor of 150 in order to create an image every 5 s. Images were exported in JPEG format, at a quality of 100%.

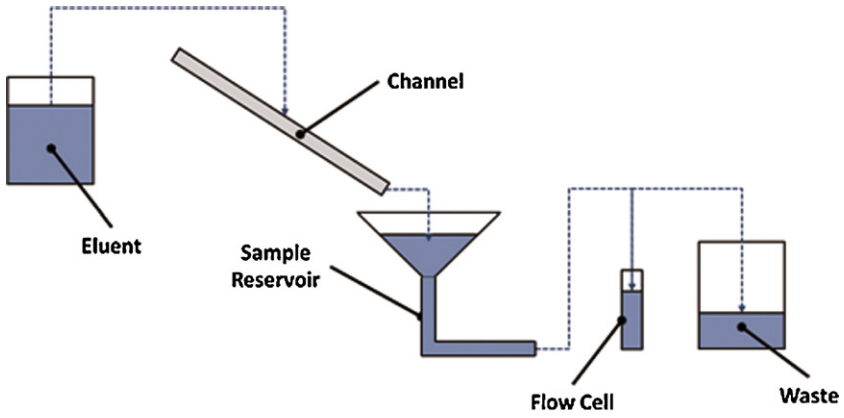

Fig. 1. Schematic diagram showing the flow in the retention testing setup. Flow is controlled by a single peristaltic pump attached to all three lengths of tubing: eluent source to channel, sample reservoir to flow cell and flow cell to waste.

Analysis of the coverage was carried out using ImageJ, with a Macro (Supplementary Information) to automatically convert the image to black and white, using the "Threshold" function to enable the subsequent measurement of the coverage using the "Analyze Particles" function.

The Macro is designed to then open the next file in sequence for the process to be repeated. Once all of the data for the run has been obtained, the material lost can be calculated using the following equation:

Loss calculation for image analysis data :

$$\text{Loss}/\% = \left(1 - \frac{C_t - C_{min}}{C_{max} - C_{min}}\right) \times 100 \quad (1)$$

where C_t is the covered area (that is, the area covered by dark pixels) at the time the image was taken (time t), C_{max} is the initial starting value, when 100% of the dye was present, and C_{min} is the minimum value once the dye has been removed, in order to baseline-correct for the presence of the flow tubing, the outer edge of the cornea and any remaining gel which was outside of the flow stream. The values of loss can then be plotted against time.

2.5. Analysis of UV/vis data

The loss of polymer was calculated using the following equation:

Loss calculation for spectrophotometry data :

$$\text{Loss}/\% = \left(\frac{\sum_{t=0}^{t=t} A_t}{\sum_{t=0}^{t=t_{max}} A_t}\right) \times 100 \quad (2)$$

where A_t is the absorbance at time t, and t_{max} is the maximum or final time at which the absorbance is measured. The numerator of Eq. (2) measures the cumulative absorbance, and the denominator normalizes the signal. The wavelength was set to 580 nm, this being the maximum absorbance wavelength of the Water Blue (Aniline Blue) dye used to colour the polymer solutions.

It should be noted that whilst the video data shows instantaneous changes to the loss of material from the corneal tissue, the sample travelling to the UV/vis instrument must first travel through the tubing. The delay was calculated as being approximately 25 s from leaving the eye to reaching the flow cell cuvette and so this amount of time was subtracted from the start of the run.

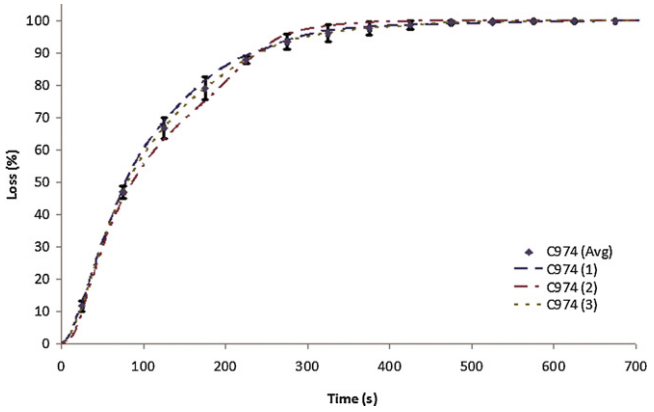

Fig. 2. Repeatability testing of UV/vis measurements of the retention of 2 wt% of Carbopol 974 PNF (C974) placed directly onto the channel with deionized water as the eluent. Selected points show the error present between runs.

3. Results and discussion

3.1. Repeatability testing

Several tests were carried out with different materials in order to test the repeatability of the results when measuring the retention of materials. In order to remove the inherent variability produced when using biological tissue, these experiments were carried out by placing the material directly onto the channel.

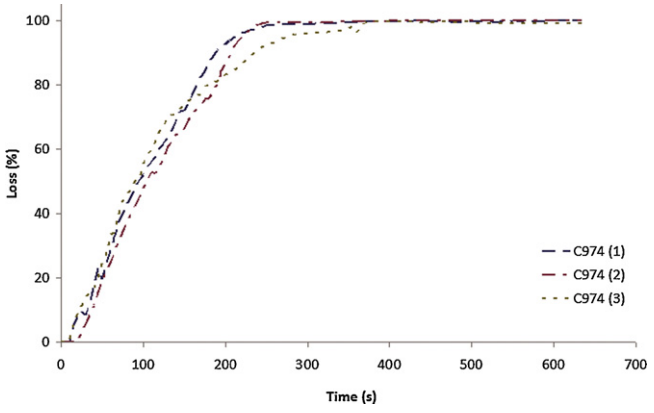

Fig. 3. Repeatability testing of image analysis measurements of the retention of 2 wt% of Carbopol 974 PNF (C974) placed directly onto the channel with deionized water as the eluent.

Fig. 4. A comparison of images of gelled Carbopol 971 PNF neutralized with 4% (v/v) of 0.5 M NaOH before (left) and after (right) thresholding.

Figs. 2 and 3 show one example of the repeatability results, using 2 wt% (w/v) C974 with 0.1 wt% (w/v) aniline blue and deionized water as the eluent. The results obtained using UV/vis measurement (Fig. 2) show good repeatability with the standard error of the mean not exceeding 10%. The image analysis results (Fig. 3) also show relatively good repeatability (standard error of the mean < 15%), despite the variability introduced by the measurement using image conversion and analysis.

The repeatability of the results supports the potential usefulness of this apparatus as a retention measurement system, particularly the use of UV/vis spectroscopy to measure the behaviour of the dosage forms in contact with biological tissues.

3.2. Image analysis

Fig. 4 shows a comparison between the images obtained directly from the video with the image files after thresholding. The thresholding process will convert the colour images to greyscale and then to a binary black and white image based on the brightness. From a comparison of the images, it is clear that the areas which are covered by coloured polymer solution are included in the image

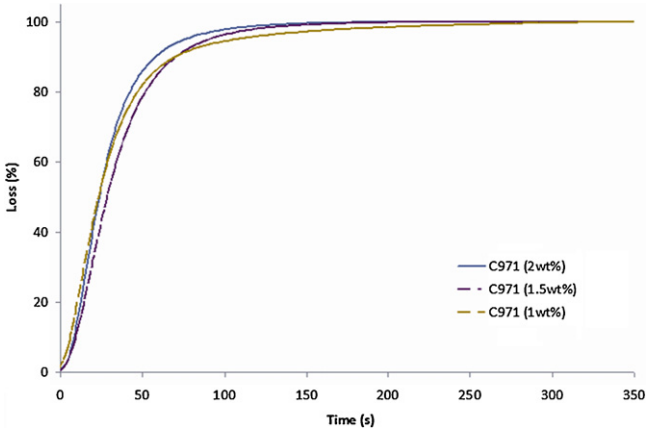

Fig. 5. UV/vis measurements of the retention of Carbopol 971 PNF (C971) at varying wt% with STF as the eluent.

after thresholding, but so are the outer circumference of the cornea and the tubing that delivers the flow of STF. As the presence of the extra dark areas of the cornea and the tubing will remain constant throughout the recording (because they are not removed), these areas determine the minimum value of the covered area, C_{min}, that is subtracted when calculating the loss (Eq. (1)). Taking this into account, this method of image processing allows a good estimation of the coverage of a formulation on the corneal tissue and, therefore, a good measurement of the loss of material over time.

3.3. Retention behaviour of C971

In order to assess the suitability of the instrument to measure the retention behaviour of a potentially mucoadhesive formulation, various concentrations (%, w/v) of C971 were applied to corneal tissue and the retention measured.

As can be seen from the UV/vis and image analysis data shown in Figs. 5 and 6, respectively, the retention behaviour is almost identical for all of the samples at the different concentrations. The Carbopol solutions increase in viscosity with increasing concentration (Lubrizol, 2010), and this increase in viscosity is having no effect upon the retention behaviour of the polymers. The presence of the salts in the STF, which according to the manufacturer's data will reduce the viscosity (Lubrizol, 2010), is most likely responsible for the lack of retention of the formulations as solutions in water only.

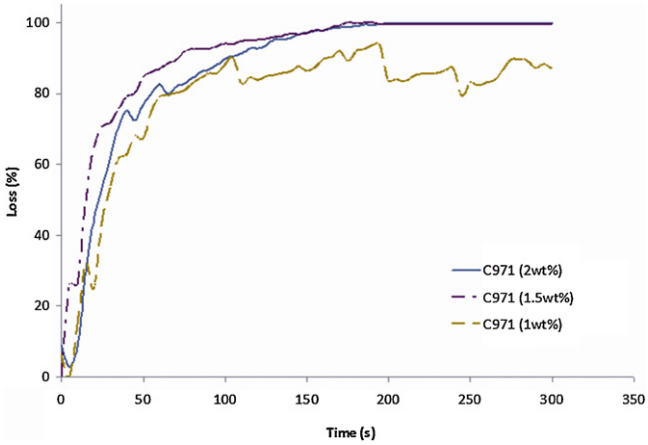

Fig. 6. Image analysis measurements of the retention of Carbopol 971 PNF (C971) at varying wt% with STF as the eluent.

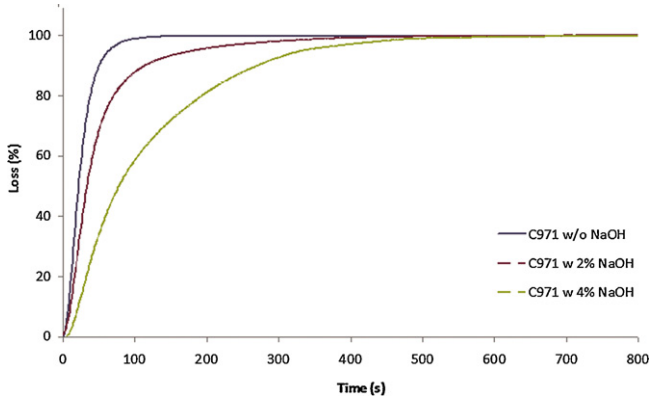

Fig. 7. UV/vis measurements of the retention of 1.5 wt% Carbopol 971 PNF (C971) neutralized with increasing concentrations of NaOH solution with STF as the eluent.

It should be noted that an increase in polymer concentration does not always improve corneal retention. For example, Edsman et al. (1996) have studied corneal retention of different Carbopols® at different concentrations (0.1–2.0%) in human volunteers. They demonstrated that the effect of polymer concentration on the retention time is dependent on Carbopol® grades. An increase in Carbopol 1342NF concentration from 0.1% to 1.0% increases the contact time from 5 to 70 min, whereas for solutions of 1.5% and 2.0% the residence time was found to be identical (150 min).

Experiments were then carried out with the 1.5 wt% Carbopol 971 PNF, neutralized with 0, 2 and 4% (v/v) of 0.5 M NaOH in deionized water. The UV/vis and image analysis results are shown in Figs. 7 and 8, and there is clearly an improvement in the retention of the material as the concentration of the NaOH solution added is increased. Both the UV/vis and image analysis results show the same trend, but with the UV/vis data giving a clearer view of the difference in the retention behaviour.

Neutralization of the Carbopol solutions will give an increase in viscosity (Lubrizol, 2010). This is because the repulsion of the ionized carboxylic acid groups results in a conformation change of the polymers in solution. We have already demonstrated that the concentration, and so viscosity, of the C971 solutions does not affect the retention behaviour. Therefore, the improved retention of the neutralized Carbopol solutions must be due to a chemical, or mucoadhesive, effect, and not the result of the change in viscosity. This improved mucoadhesion must result from the change in the polymer behaviour in solution, with the presence of the NaOH and the resultant increase in the pH. The specific mucoadhesion effects

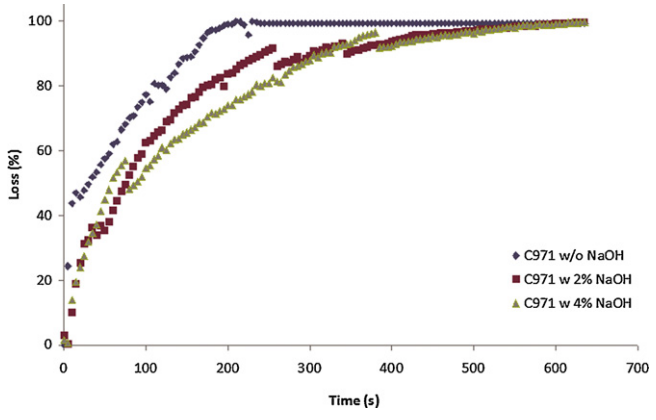

Fig. 8. Image analysis measurements of the retention of 1.5 wt% Carbopol 971 PNF (C971) neutralized with increasing concentrations of NaOH solution with STF as the eluent.

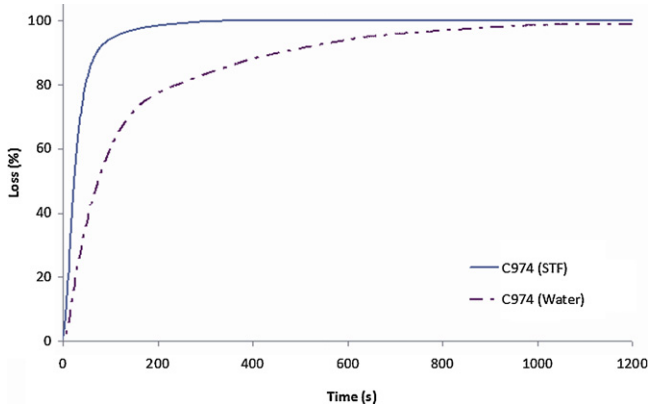

Fig. 9. UV/vis measurements of the retention of 2 wt% Carbopol 974 PNF (C974) with either STF or deionized water as the eluent.

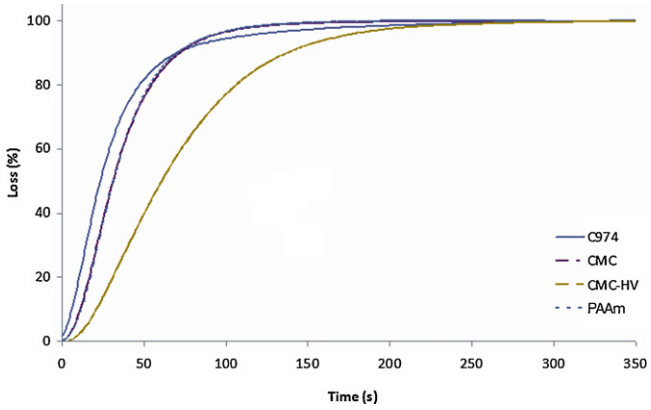

Fig. 11. Comparison of the retention of 2 wt% solutions of various polymers as measured by UV/vis detection, with STF as the eluent. Polymers: Carbopol 974 PNF (C974), carboxymethylcellulose (CMC), high viscosity carboxymethylcellulose (CMC-HV) and polyacrylamide (PAAm).

were reported previously by Davies et al. (1991) when comparing the precorneal retention of equiviscous solutions of Carbopol 934P and poly(vinyl alcohol) in vivo in rabbit model.

As discussed previously, the viscosity of Carbopols is shown to decrease when in the presence of increasing concentrations of sodium chloride, NaCl, which is one of the primary constituents of the STF. This indicates that the coulombic repulsion of the ionized carboxylic acid groups is reduced due to charge screening by the ions in the salts. In order to observe this effect using the retention testing rig, 250 μL samples of 2 wt% C974 (not neutralized) were applied to corneal tissue and two different flow eluents were used, STF and deionized water. As can be clearly seen from the data obtained (Figs. 9 and 10) the gels are better retained when deionized water is used instead of STF.

3.4. Comparison of various polymers

Various polymers at the same concentration of 2 wt%, but inherently different viscosities, were also tested, including C974, PAAm, CMC and HV-CMC. The differences in the retention behaviour can be clearly seen (Figs. 11 and 12) between HV-CMC and CMC, C974 and PAAm. In this case, it appears as though the HV-CMC-based formulation is retained well, most likely due to a significantly higher viscosity, which is not affected by the presence of the salts in the STF. The other polymers are removed more rapidly, with the C974 being removed at the same rate as both PAAm and CMC. As demonstrated previously, the viscosity of the C974 is inhibited by the presence of the salts in the STF.

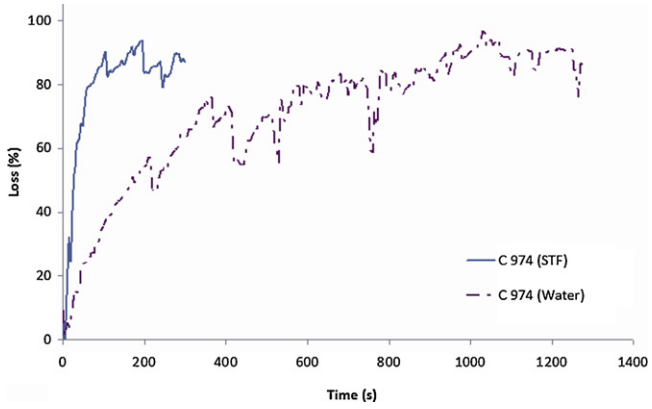

Fig. 10. Image analysis measurements of the retention of 2 wt% Carbopol 974 PNF (C974) with either STF or deionized water as the eluent.

It should be noted that we did not aim to evaluate and compare the retention potential of these polymers on the ocular tissues in this study. We used different polymer solutions to demonstrate that our flow system with on-line quantitative measurement allows a comparison of different formulations.

3.5. Comparison of UV/vis and image analysis data

In this work we have demonstrated that both the UV/vis and image analysis data can be used to give comparative results as to the retention behaviour of different polymers and compositions under simulated physiological conditions. Whilst flow apparatuses similar to this are already being used by various research groups around the world (Rango Rao and Buri, 1989; Mikos and Peppas, 1990; Nielsen et al., 1998; Belgamwar and Surana, 2010), the data obtained using the set up reported here provides better quantification of dosage form retention potential because of its on-line measurement principle.

There are also three distinct advantages to using UV/vis detection as opposed to image analysis. Firstly, the data obtained is much more repeatable than that obtained by image analysis. This can be considered primarily due to the fact that the image analysis process, including the thresholding and coverage measurement, which is an essential step in converting the data from image to number, introduces errors of its own into the results. The mixing of the sample coming from the biological tissue with the sample reservoir in

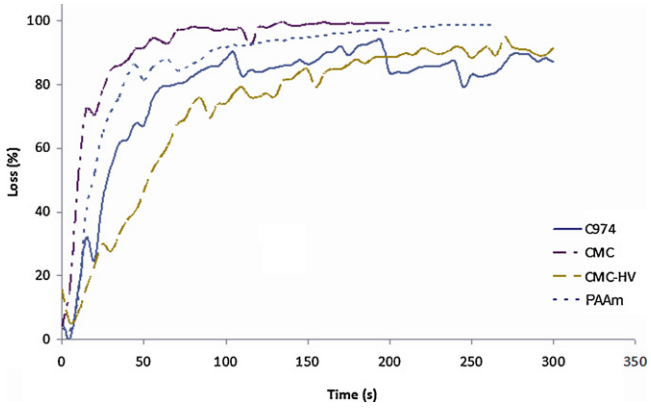

Fig. 12. Comparison of the retention of 2 wt% solutions of various polymers as measured by image analysis, with STF as the eluent. Polymers: Carbopol 974 PNF (C974), carboxymethylcellulose (CMC), high viscosity carboxymethylcellulose (CMC-HV) and polyacrylamide (PAAm).

the funnel will also produce a cleaner trend than the image analysis as it is not an immediate measurement.

Secondly, acquisition of data using UV/vis is a much simpler process, with only the extraction of ASCII data and the conversion to loss values being required, whilst with image analysis, several steps are involved, with the video first being thresholded, then converted to a string of images and finally analysed step-by-step using image analysis software. Whilst more programming expertise could potentially speed this process up, it would always remain a longer process.

Thirdly, UV/vis spectroscopy can be used to detect polymers or drug that cannot be seen visually with a camera. This allows the apparatus to be used for a variety of substances that could not normally be observed. The UV/vis detector used in these experiments is a conventional spectrophotometer, and a spectrofluorophotometer could also be used in the same manner. It is the modification to the cuvette, and the utilization of flow that allows the instruments to be used in this manner. In principle, the retention of any species that either absorb in the UV/vis range or has detectable fluorescence could be measured using this system.

4. Conclusion

This work demonstrates a simple modification to a long established technique, which allows quantitative measurement of the retention of bioadhesive formulations to biological tissue with greater ease, accuracy and reliability. In this work we have used a series of model liquid formulations where a model dye was directly dissolved in an aqueous solution of a mucoadhesive polymer. The experiments with the flow cell in this case give an indication of the retention of an active ingredient on the biological surface, which is mediated by a mucoadhesive polymer present in the formulation. Potentially our flow system can also be used to evaluate the retention of various dosage forms including solutions, semi-solid gels, and some solid formulations such as films.

The simple modification of a cuvette to measure concentration via UV/vis, fluorescence or refractive index, will enable many more drugs and polymers to be assessed, which will be particularly useful for those that are not visible under normal light conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2012.02.047.

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