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Evaluation of the bioadhesive properties of hyaluronan derivatives: detachment weight and mucociliary transport rate studies.

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Abstract

Hyaluronan of various molecular weights, and microspheres made from several of its esters, were assessed for adhesiveness in vitro by means of detachment weight and mucociliary transport rate. Microspheres made from esters of alginic acid and gellan gum were also evaluated. The results were compared with those obtained from Carbopol 974 which was used as a positive control. Hyaluronan and its autocross-linked esters displayed comparable adhesion to Carbopol in both studies. All microsphere preparations were less adhesive than Carbopol ($p < 0.05$, Mann-Whitney *U*-test) when tested for detachment weight (using mucosal epithelium) and mucociliary transport rate. Adhesion to a mucus gel was similar for most preparations. Hyaluronan has been shown to possess excellent adhesion in vitro. Although formulation of hyaluronan into microspheres tends to reduce its inherent adhesive properties, the microspheres formed displayed significantly decreased mucociliary clearance. The inclusion of drug into such a biodegradable and biocompatible dosage form is an attractive prospect for transmucosal delivery.

Keywords: Bioadhesion; Carbopol; Detachment force; Epithelia; Hyaluronan; Microsphere; Mucoadhesion; Mucociliary transport rate; Mucus

1. Introduction

Bioadhesives can be defined as natural or synthetic materials capable of adhering to a biological substrate for an 'extended' period of time (Gu et al., 1988). This 'extended' period of time should be sufficiently long to allow for a reduction in dosage frequency compared to conventional, non-adhesive dosage forms (Helliwell, 1993). The potential for bioadhesives to enhance drug bioavailability via a prolonged and intimate contact with the absorbing membrane has long been recognised. Indeed, bioadhesives have been studied in almost all accessible routes of drug absorption, including the eye (Middleton et al., 1990), nose (Illum et al., 1987), mouth (Nagai and Kon-

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ishi, 1987), rectum (Helliwell and Sektere, 1993) and vagina (Richardson et al., 1992).

Over the last decade, the bioadhesive properties of a wide range of materials have been evaluated and it is generally accepted that the synthetic polymers Carbopol and polycarbophil, both of which are cross-linked derivatives of poly(acrylic acid), display excellent adhesion when tested in vitro (Leung and Robinson, 1988; Smart, 1991). However, such performance cannot be duplicated in vivo (Khoshla and Davis, 1987) which explains why few, if any, bioadhesive delivery systems have become commercially available. In addition, bioadhesives may have the potential to induce toxicity, mainly as a result of local irritation (Bottenberg et al., 1991).

Over the last 5 years, interest has been focused on naturally-occurring bioadhesives such as lectins (Lehr et al., 1991a) and fimbrial proteins (Caston et al., 1990), which are able to adhere to the mucosal cell surface via receptor-mediated processes. However, access to the mucosal lining will be impeded by the overlying mucus layer, to which the adhesives may also bind. Hence, the ultimate success of such adhesives, as with the synthetic adhesives, will be determined to a large extent by the rate of mucus turnover.

Another naturally occurring material with bioadhesive potential is hyaluronic acid, or hyaluronan (HA) as it is now called (Saettone et al., 1989; Morimoto et al., 1991; Richardson et al., 1993). HA is a mucopolysaccharide found in the extracellular tissue matrix of vertebrates, including connective tissue, synovial fluid, vitreous humour and aqueous humour (Rastrelli et al., 1990). HA functions to maintain the unique rheological properties of several body fluids, in addition to being involved in tissue hydration and repair, lubrication, cell function and differentiation.

HA is water-soluble which limits the variety of dosage forms in which it can be used. The physical properties of HA can be altered by controlled esterification with alcohols to produce a variety of dosage forms such as fibres, films, gels, sponges, gauzes and pellets (Barbucci et al., 1993, Benedetti, 1994). In addition, microspheres can be made from esters of HA by spray drying (Kyyronen et al., 1992) and solvent evaporation (Benedetti et al., 1990). Vaginal absorption of salmon calcitonin was found to be greater from microspheres made of HA esters than from a simple solution and histological examination showed the microspheres to be closely attached to the vaginal epithelium some 6 h after administration (Richardson et al., 1993).

Since HA has been shown to be bioadhesive both in vivo and in vitro, the aim of the present study was to compare the in vitro adhesive performance of various esters of HA with Carbopol. The use of the relatively simple and inexpensive detachment weight test was considered appropriate since a wide range of formulations can be evaluated quickly (Helliwell, 1993). In addition, mucociliary transport rate studies were performed and results were compared with the detachment weight technique.

2. Materials and methods

2.1. Materials

The following preparations were supplied by Fidia Advanced Biopolymers (Abano Terme, Italy): hyaluronan, Mol. wt. 170000 (HA108); autocross-linked hyaluronan, synthesized as described by della Valle and Romeo (1989) (ACP); hyaluronan, Mol. wt. 4000000 (HAM03); microspheres of the ethyl ester of HA108 (HYAFF7); microspheres of the benzyl ester of HA108 (HYAFF11); microspheres of the benzyl ester of HA108 in which only 75% of the carboxyl groups of HA108 had been esterified (HYAFF11p75); microspheres of the benzyl ester of alginic acid (ALAFF11); and microspheres of the benzyl ester of gellan gum (GEFF11). Carbopol 974 was kindly supplied by B F Goodrich (Hounslow, UK).

Protease-inhibiting solution comprised 200 mM sodium chloride, 0.02% w/v sodium azide, 5 mM EDTA and 1 mM phenylmethylsulphonyl chloride (all from Sigma Chemical Co., Poole, UK). Hepes buffer (pH 7.4) comprised 0.182% w/v Hepes sodium salt (Aldrich Chemical Company, Gillingham, UK), 0.07% w/v Hepes free acid

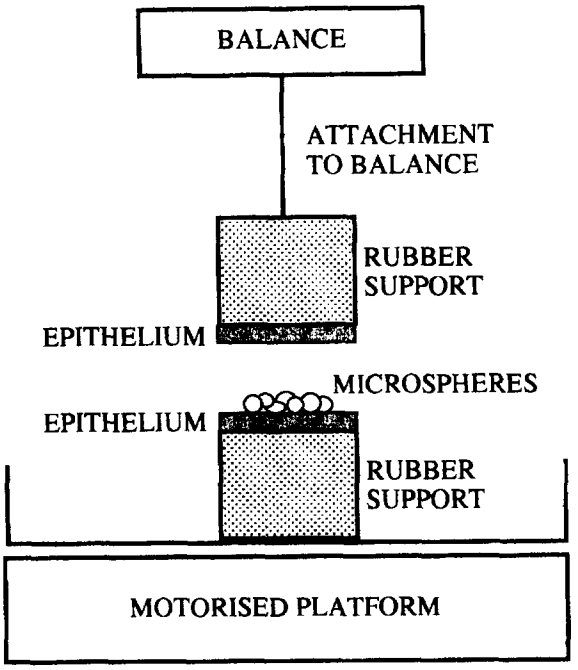

Fig. 1. Diagram of apparatus used in detachment weight studies.

(Aldrich Chemical Company, Gillingham, UK) and 0.818% w/v sodium chloride (BDH, Poole, UK).

2.2. Substrate preparation

The adhesiveness of the test bioadhesives was assessed using two types of biological substrate: Rat epithelia (from the small and large intestines, stomach, rectum and vagina) and pig gastric mucus. The appropriate tissue sections from the Sprague–Dawley rat (approximately 200 g in weight) were stored at −16°C until required. Before use, the tissue samples were allowed to thaw at room temperature.

Mucus samples were obtained by gently scraping the stomachs of freshly slaughtered pigs with a wooden spatula and washing the resultant gel mixture with an equal volume of protease-inhibiting solution. The mucus suspension was then centrifuged at 20000 × *g* for 90 min (4°C) and the decanted supernatant exhaustively dialysed (in excess of 12 h) at 4°C in distilled water (Visking tubing; Medicell Int. Ltd., London, UK). The mucus sample was then concentrated in a Filtron ultrafiltration cell (fitted with a 100-kDa cut-off Minisett cartridge) followed by an Amicon ultrafiltration cell (fitted with a 30-kDa cut-off membrane), and stirred continuously overnight. After this, stirring was stopped and the mucus was allowed to gel for 2 h on the filter membrane from which it was eventually removed and stored (4°C) until required. The dry weight of the mucus gel was determined by drying a known weight at 100°C for 30 min and then reweighing.

2.3. Detachment weight studies

The weight required to detach two tissue sections with the test bioadhesive sandwiched between them was determined using the apparatus shown in Fig. 1. Briefly, a section of tissue was attached, mucosal side up, to a polythene strip with the aid of a cyanoacrylate resin (Superglue,

Bostik Ltd., Leicester, UK). The polythene strip had previously been fixed to a large rubber support which itself had been secured and positioned centrally at the bottom of a glass beaker. Another tissue section was attached, by the serosal side, to the lower surface of a rubber support (6 mm in diameter) which had a glass rod attached vertically to its upper surface. A small plastic washer (7.5 mm internal diameter with a depth of 1.5 mm) was placed over the mucosal surface on the polythene strip to allow a fixed volume of the test bioadhesive to be applied. The upper mucosal section was then placed on the lower mucosal section, thus sandwiching the test bioadhesive, and held in position with an arbitrary load of 47 g for one min. The whole apparatus was then placed on a platform and the glass rod was attached to an analytical balance by means of a hook. The motorised platform was lowered at a controlled rate of 136 mm/min until complete detachment of the tissue sections occurred. The maximum weight required for detachment was measured by the balance and recorded on a printer. A fresh piece of tissue was used for each run and a total of six determinations were carried out for each test bioadhesive.

A similar method to the one described above was employed when mucus was used as the substrate for adhesion. This time, the test bioadhesive was pressed, with the same load of 47 g, onto the lower surface of the rubber support (with the glass rod attached vertically to the upper surface), which had previously been coated with a thin layer of cyanoacrylate resin. After the resin had dried, the rubber support was gently tapped to remove excess bioadhesive. The mucus gel (which was allowed to equilibrate to room temperature) was introduced into a glass beaker at a depth of 1 cm and the coated bioadhesive surface of the rubber support was placed in contact with the gel with a load of 47 g for one min. After this time, the apparatus was placed on the platform, the glass rod was connected to the balance and the weight required to detach the test bioadhesive from the mucus gel was determined as before. The results were analysed for significance using the non-parametric Mann-Whitney two-tailed *U*-test.

2.4. Mucociliary transport rate studies

The transport of the microspheres and other test bioadhesives along part of an excised upper palate from the frog (*Rana pipiens*) was assessed. The palate was washed in Hepes buffer and placed on a cork tile in a perspex box through which water was circulated at 25°C. After 5 min, excess buffer was removed from the palate using a Pasteur pipette and graphite particles were tapped onto the front of the palate using a blunt seeker. Care was taken at all times not to touch the palate. The movement of six particles towards the back of the palate (approximately 15 mm) was monitored through a stereomicroscope (Watson Barnett, UK), illuminated with a fibre optic light source, and the rate of movement was measured with the aid of a calibrated eyepiece and a stopwatch (a total of three measurements per palate).

The palate was then washed in Hepes buffer to remove the graphite particles and left for a further 5 min. The test bioadhesive was then applied in an identical manner to the graphite particles and the transport rate recorded. A total of six determinations were carried out for each bioadhesive, using a fresh palate each time. The results were analysed for significance using the non-parametric Mann-Whitney two-tailed *U*-test.

3. Results and discussion

3.1. Detachment weight studies

Detachment weight studies, which are based on surface tension measurements, are among the most commonly employed in vitro adhesion test (Smart et al., 1984). In addition to being quick, inexpensive and easy to perform (Helliwell, 1993), a wide range of dosage forms, e.g., tablets, suppositories, hydrogels, pastes, microparticles, can be screened for adhesiveness to a variety of biological substrates. The technique is mainly used to rank adhesives and it is assumed that the force required to detach a material from a biological substrate is directly proportional to the adhesive strength of that same material.

Table 1
Mean forces of detachment of the test bioadhesives from rat intestinal epithelium and pig gastric mucus

Test bioadhesive	Rat small intestinal epithelium		Pig gastric mucus (washed)	
	Mean detachment force ($N\ m^{-2} \times 100$) ($n = 6$)	Standard deviation ($N\ m^{-2} \times 100$)	Mean detachment force ($N\ m^{-2} \times 100$) ($n = 6$)	Standard deviation ($N\ m^{-2} \times 100$)
HA108	102.9	36.4	26.8	6.2
ACP	135.5	65.2	39.4	3.5
HAM03	37.2*	34.3	17.3*	3.5
HYAFF7	16.9*	10.7	33.8	3.5
HYAFF11	49.3*	20.5	13.6*	1.4
HYAFF11 p75	12.8*	3.1	33.0	5.2
ALAFF11	36.4*	16.6	33.5	4.9
GEFF11	20.1*	17.7	37.1	2.8
Carbopol 974	115.9	55.1	29.3	12.1

*Indicates a result significantly different from that of Carbopol 974 ($p < 0.05$).

The conditions of each test must be identical in every way if reproducible results are to be obtained. For instance, it was originally reported (Ch'ng et al., 1985) that the adhesion of polycarbophil to rabbit gastric epithelia increased from pH 1 to 6. Later in the same year, however, the opposite was reported to occur (Park and Robinson, 1985). It became apparent that the initial load weight and detachment rate had not been controlled in the original study of Ch'ng et al. (1985).

The force per unit area required to detach the test bioadhesives from the biological substrates in the present study was calculated from the equation:

$$F = \frac{W \cdot G}{A}$$

where: F = detachment force (Nm^{-2}); W = maximum detachment weight recorded by the balance (kg); G = acceleration due to gravity ($m\ s^{-2}$); A = tissue-tissue or bioadhesive-tissue contact area (m^2).

The results of the detachment weight studies on rat small intestine and pig gastric mucus are summarised in Table 1. When small intestinal epithelium was used to assess adhesion, only HA108 (hyaluronan, Mol. wt. 170000) and ACP displayed comparable adhesion to that observed with Carbopol. All the other formulations displayed significantly lower adhesive forces compared with Carbopol ($p < 0.05$).

Different trends in adhesive performance were obtained when the biological substrate was pig gastric mucus. The dry weight of the mucus samples was determined to be 17% w/w. Only HAM03 (hyaluronan, Mol. wt. 4000000) and HYAFF11 (microspheres of the benzyl ester of HA108) displayed significantly lower adhesive forces than Carbopol ($p < 0.05$); all the other preparations displayed similar, if not higher detachment forces compared with Carbopol.

Regardless of the differences observed in adhesive performance between the test bioadhesives, results obtained from the biological substrates differed in two respects. First, detachment forces were, on the whole, larger when small intestinal epithelium was used compared to when the mucus gel was used (four times higher in the case of Carbopol). Although an identical load of 47 g was used to initiate contact between the test bioadhesive and both substrates, a greater 'sandwiching' effect or spread of bioadhesive would have been obtained using the tissue, simply because of its greater rigidity. This would tend to favour adhesion and similar findings have been reported elsewhere (Saettone et al., 1989). It is also likely that a greater amount of test bioadhesive was used when tissue was employed as the substrate. Finally, the detachment forces obtained from using

Table 2
Mean forces of detachment for selected test bioadhesives on epithelia obtained from different regions of the rat

Test bioadhesive	Mean detachment force ± standard deviation (N m^{-2} × 100) (n = 6)				
	Small intestinal epithelium	Colonic epithelium	Gastric epithelium	Rectal epithelium	Vaginal epithelium
Carbopol 974	115.9 ± 55.1	177.0 ± 71.1	217.0 ± 85.6	200.1 ± 45.1	202.8 ± 42.6
HYAFF7*	16.9 ± 10.7	38.2 ± 19.1	45.2 ± 27.4	36.1 ± 8.7	37.8 ± 21.1
HYAFF11*	49.3 ± 20.5	34.5 ± 9.7	35.4 ± 20.5	30.9 ± 17.7	15.6 ± 5.2
HYAFF11p75*	12.8 ± 3.1	58.6 ± 57.5	15.6 ± 6.6	19.8 ± 11.1	24.3 ± 12.1

*Indicates that all results differ significantly from Carbopol 974 ($p < 0.05$).

mucus as the substrate for adhesion will be influenced not only by the inherent stickiness of the test material, but also by the cohesive nature of the mucus gel itself. If cohesive forces within the mucus gel are generally stronger than the adhesive forces between bioadhesive and mucus, then fracture will tend to occur at the adhesion interface. However, fracture may occur within the mucus gel itself if cohesive forces are weaker than the bioadhesive forces (Ponchel et al., 1987; Helliwell, 1991). This latter situation would tend to give rise to artificially low detachment forces.

The second difference between the biological substrates employed is that detachment forces obtained from mucus were more reproducible than those obtained from tissue. This is presumably because the contact surface of the mucus gel was almost identical, i.e., level and even, in each determination. Samples of tissue from the same anatomical region may not necessarily have identical surface morphology and so identical contact between the tissue layers cannot be guaranteed with each determination.

As far as the authors are aware, this is the first study to compare the adhesion of the test materials to such a wide range of substrates. Table 2 shows the effect of tissue type on the detachment forces obtained with Carbopol and the hyaluronan ester microspheres. In each case, Carbopol was significantly more adhesive than the microspheres ($p < 0.05$). With the exception of HYAFF11, adhesion appeared to be greater when tissues from the rat colon, stomach, rectum and vagina were employed compared to when tissue from the small intestine was used. Indeed, detachment forces of Carbopol from gastric epithelium were almost twice as great as those obtained from the small intestinal epithelium. Factors such as physical state and surface morphology of the epithelium, and the presence of mucus, will all influence adhesive force. This may account for the variability of the data obtained and the subsequent difficulty encountered in identifying any trends within the results. The presence of mucus will probably have the greatest influence on measured adhesiveness; mucus can reduce the measured adhesiveness, especially if it is only loosely associated with the epithelium. The inherent fragility of the rat small intestinal epithelium prompted Saettone et al. (1989) to use moist tissue paper coated with a thin layer of porcine gastric mucin as the 'biological' substrate for adhesion studies.

3.2. Mucociliary transport rate studies

The rationale for carrying out these studies was that the greater the adhesiveness of the test material, the greater the reduction in transport rate along the frog palate compared to non-adhesive graphite particles. The mean transport rate of the test bioadhesive was compared with the mean transport rate of the carbon particles on the same palate and the mean change in transport rate (%) was calculated. A negative value for the mean change in transport rate indicates that the test bioadhesive was transported more slowly than the carbon particles. Table 3 summarises the data obtained from the frog palate. Carbopol, HA108, ACP and HAM03 were not transported along the

palate and hence could be said to be the most adhesive formulations. All the remaining microsphere preparations were transported to varying degrees and hence displayed less adhesiveness than Carbopol. However, with the exception of ALAFF11, the transport rate of all the microspheres was significantly slower than the transport rate of the graphite particles; HYAFF11 ($p = 0.009$), GEFF11 ($p = 0.007$), HYAFF11p75 and HYAFF7 ($p < 0.05$).

Results from the mucociliary transport rate test were, in general, in close agreement with results from the detachment weight studies (employing rat small intestinal epithelium), with the possible exception of HAM03 which performed better in the former test. The mucociliary transport rate method has thus emerged to be a useful and quick screening method for bioadhesives, although materials which remain firmly attached to the palate can only be classified as being of equal adhesiveness. The mucociliary transport rate test would be an ideal method to investigate the adhesive potential of dosage forms intended for delivery via the nose and lung.

Biological factors such as ciliary beat frequency, together with physical factors such as particle size, density, degree of clumping and water solubility of the test material, will all influence transport rate. No significant decline in the ability of the palate to transport the graphite particles was seen throughout the study (results not shown). With the formulations showing no transport, cilia could still be seen to beat, indicating that the lack of movement of the formulations was not due to any detrimental effect on the cilia. Hence, differences observed between formulations are likely to be due to differences in inherent stickiness and differences in the physical properties mentioned above.

Table 3
Mean changes in mucociliary transport rates of test bioadhesives, compared with graphite particles, on the frog palate

Test bioadhesive	Mean change in transport rate (%)	Standard deviation (%)
Carbon particles	0*	6.27
HA108	−99.8	0.4
ACP	−100.0	0.0
HAM03	−100.0	0.0
HYAFF7	−25.8*	17.3
HYAFF11	−37.5*	7.3
HYAFF11p75	−30.8*	8.5
ALAFF11	−14.4*	27.8
GEFF11	−45.5*	12.8
Carbopol 974	−100.0	0.0

*Indicates a result significantly different from that of Carbopol 974 ($p < 0.05$).

3.3. Adhesive differences between formulations

Hyaluronan (HA) consists of alternate residues of *N*-acetyl-D-glucosamine and D-glucuronic acid. The abundance of COOH groups will promote adhesion through hydrogen bond formation with components of the biological substrate (Gu et al., 1988). As with Carbopol, the presence of unionised COOH groups will tend to favour adhesion (Park and Robinson, 1985; Park, 1986; Helliwell et al., 1991).

HA exists as a water-soluble polymer and cannot, as yet, be formulated directly into microspheres. Reaction of the COOH groups with alcohols in the presence of an aprotic solvent yields esters of HA with different physical properties to HA itself. It is these esters, namely the ethyl ester (HYAFF7) and the benzyl esters (HYAFF11 and HYAFF11p75) that can be formulated into microspheres and loaded with drug (Benedetti et al., 1990).

Esterification of the COOH groups reduces the bioadhesiveness of HA, as can be seen in Tables 1–3. This may be expected since the esters will have a reduced tendency to form hydrogen bonds with the biological substrate. However, if a reduction in hydrogen bond formation was the only reason for a reduction in bioadhesion, then HYAFF11p75 may have been expected to display greater adhesiveness than HYAFF11, since only 75% of the COOH groups in the former are esterified. Results obtained from the detachment weight technique are conflicting; HYAFF11 performed better on intestinal tissue than HYAFF11p75 but worse on the mucus gel. Both formulations performed similarly in the mucociliary transport rate studies.

Differences observed between HYAFF7, HYAFF11 and HYAFF11p75 in the detachment weight studies may be a result of differences in hydrophilicity and degree of swelling between the three esters. HYAFF11 is the most hydrophobic of the three and increases in weight by only 48% when placed in contact with water, compared with HYAFF7 which can increase in weight by as much as 259% (Hunt et al., 1990). Hence, HYAFF7 (and HYAFF11p75) would have been likely to hydrate faster than HYAFF11 when placed in contact with the mucus gel, which may have resulted in a faster initiation of the bioadhesion process. This effect was not so readily apparent when the substrate used for adhesion was mucosal epithelium, presumably due to the lower amount of water available. The picture becomes more complicated when one considers the contribution that hydrophobic bonding can make to the bioadhesion process. Contact angle measurements (Lehr et al., 1991b) have suggested that both mucus and mucosal tissue display an appreciable hydrophobicity which may have important implications for bioadhesion.

An increase in adhesiveness between HA preparations of increasing molecular weights was not observed. The lower molecular weight preparations (ACP, Mol. wt. 200000 and HA108, Mol. wt. 170000) performed better than the higher molecular weight preparation (HAM03, Mol. wt. 4000000) in detachment studies although their effect on mucociliary transport was similar. This is in disaggrement with the study of Saettone et al. (1989) which showed that HA with a molecular weight of 620000 was more adhesive than HA with a molecular weight of 130000, when tested in vitro using a detachment weight technique. Differences may have resulted from differences in hydration rates of the substances studied and it seems reasonable to assume that a cut off point exists, beyond which a further increase in molecular weight does not produce a corresponding increase in adhesiveness. The influence of molecular weight on adhesion depends on the material being studied; Smart et al. (1984) found that the molecular weight of sodium carboxymethylcellulose should exceed 78000 in order for it to display significant adhesion.

To summarise, HA is an excellent adhesive with characteristics comparable to, and possibly better than, Carbopol. Esterification of HA, a prerequisite for microsphere production, results in some reduction of bioadhesive properties. HYAFF7, HYAFF11 and HYAFF11p75 did not appear to be particularly effective as mucoadhesives as determined from detachment weight studies, but they were all able to significantly reduce mucociliary transport rate. Because of the difficulty in extrapolating results obtained in vitro to the situation in vivo, it is essential that further work is carried out on the esterifed HA microspheres to establish their in vivo bioadhesiveness — some promising results have already been obtained following vaginal administration of HA-ester microspheres (Richardson et al., 1993). Since HA is a naturally occurring material, products made from it are likely to be both biodegradable and biocompatible; microspheres made from HA are thus attractive pharmaceutical dosage forms. In conclusion, HA and microspheres made from HA derivatives show promise for further investigation as mucoadhesive delivery platforms.

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