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Sodium Alginate-Based Microspheres of Salbutamol Sulphate for Nasal Administration: Formulation and Evaluation

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ABSTRACT

Nasal delivery protects drugs like salbutamol sulphate (SS) from hepatic first-pass metabolism. The study aimed to formulate mucoadhesive sodium alginate (SA) microspheres loaded with (SS) by emulsion cross-linking, with mucoadhesive polymers, for potential nasal delivery by-passing first-pass metabolism. Relevant physichochemical properties, in vitro release and irritation in rabbits were investigated. Stirring rate and cross-linking time affected microsphere parameters. Microspheres were spherical in shape, discrete, with smooth and porous properties favorable for intranasal absorption with high drug-loading of 78.7 %. Mixed-polymers microspheres exhibited higher degree of swelling. Kinetic analysis of release data showed a case II release kinetics for alginate microspheres, and anomalous mechanism for other mixed-polymers formulae. Pronounced sustained drug release over 8 hours was exhibited upon incorporation of Carbopol® 934 and Hydroxypropyl methyl cellulose. The formulated microspheres showed high swelling ability and good mucoadhesion, offering a good potential for future in vivo study to confirm safety and avoidance of first-pass metabolism.

Keywords: Mucoadhesive microspheres; nasal; salbutamol sulphate; sodium alginate

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INTRODUCTION

Carrier technology offers an intelligent approach for drug delivery by coupling the drug to carrier particles, such as microspheres, nanoparticles, liposomes, etc., which modulates the release and absorption characteristics of the drug. Microspheres possess important attributes among the particulate drug delivery systems by virtue of their small size and efficient carrier characteristics; however, the success of these drug delivery systems is limited due to their short residence time at the site of absorption. It would therefore be advantageous to have means for providing an intimate contact of the drug delivery system with absorbing membranes. This can be achieved by coupling mucoadhesion characteristics to microspheres and thereby developing delivery systems referred as "mucoadhesive microspheres"[1]. Coupling of mucoadhesive properties to microspheres has additional advantages, e.g. efficient absorption and enhanced bioavailability of the drugs due to high surface to volume ratio, a much more intimate contact with the mucus layer and specific targeting of drug to the absorption site achieved by anchoring plant lectins[2], bacteria[3] and antibodies[4] on the surface of microspheres.

Microspheres of different mucoadhesive materials were investigated for the purpose of nasal delivery and have been established to increase significantly the systemic absorption of conventional and polypeptides drugs[5-8], as well as vaccines[9-11]. The popularity of alginate is attributed to the inexpensive and renewable, as well as the biocompatible and biodegradable nature associated with mucoadhesive property.

Sodium alginate (SA) is a naturally occurring polysaccharide which can be easily cross-linked into a solid matrix by replacing the sodium ions from the guluronic acids with di- or tri-valent cations and stacking of these guluronic groups to form the characteristic egg-box structure [12].

Salbutamol sulphate (SS) is a direct acting sympathomimetic used for the treatment of acute and chronic asthma, however it suffers from extensive first-pass effect. Therefore, SS was chosen as a model drug in this work to prepare mucoadhesive microspheres for nasal delivery to prolong the mucosal residence time, with expected higher absorption, through by-passing hepatic metabolism. Recently SS loaded alginate microspheres were prepared by ionotropic gelation method for oral controlled release[13,14] and earlier Jain et al[15] have formulated chitosan-based microspheres for nasal delivery of SS and concluded that it could be useful for prophylactic treatment.

The aim of this work was to develop, characterize and in vitro evaluate mucoadhesive SA microspheres loaded with SS in presence of other mucoadhesive polymers; namely

carboxymethyl cellulose sodium (CMC Na), Carbopol® 934P (Cbp) and hydroxylpropyl methyl cellulose (HPMC). In addition, the study aims at in vivo evaluating the nasal irritation after repeated administration of the formulated sodium alginate microspheres to rabbits.

MATERIALS AND METHODS

Materials

Salbutamol sulphate, Hdroxypropylmethyl cellulose 4000 cp, Carbopol 934 were kindly provided by Pharco Group Pharmaceutical Co., Alexandria, Egypt. Sodium alginate (High molecular weight) was obtained from Sisco Research Laboratories, Mumbai, India. Carboxymethylcellulose sodium (High viscosity; 1% aqueous solution 1500±400 cp) and sodium chloride were purchased from BDH Chemical Ltd., Poole, England. Mucin from porcine stomach, Type II was purchased from Sigma, USA. Hexane was obtained from SD Fine-Chem Limited, Mumbai, India. Span 80 was obtained from CRODA Oleochemicals, England. Tween 20 from Fluka AG, CH-9470 Buchus, Switzerland. Isopropyl alcohol, calcium chloride, potassium chloride from ADWIC, El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

Preparation Of Microspheres [16]

Eleven grams of aqueous solution of 4% w/v sodium alginate or SA mixture with HPMC, Cbp or CMC Na in the ratio of 3:1 as mentioned in Table 1 was weighed in a tarred beaker. Polymer solutions were dispersed by a syringe with a needle (0.9 mm diameter) in 26 g of hexane containing 2% w/w Span 80 using a magnetic stirrer (Jenway 1000, England) at 1000 rpm for 10 minutes. Two grams aqueous solution of 1% w/w Tween 20 was then added and the resulting emulsion was stirred for another 10 minutes. Subsequently, 8 g of an aqueous solution of calcium chloride dihydrate (10% w/v) were added drop wise and allowed to react with the aqueous alginate globules for 30 minutes to induce cross-linking and solidify the microspheres. Microspheres were collected by filtration, washed with isopropyl alcohol and finally dried at 40 °C in an incubator (Hereaus, Germany) for 12 hours.

Drug loading

SS was loaded into the microspheres by two methods; a) Simultaneous method (Method I), where the drug was mixed with SA solution prior to cross-linking and formation of microspheres and b) Sequential method (Method II), where hydroalcoholic solution of the drug was incubated with the plain microspheres for 48 hours. The loaded microspheres were then separated by filtration and allowed to dry in an incubator at 40°C.

CHARACTERIZATION OF MICROSPHERES

Particle size analysis [17]

Particle size analysis was performed by laser diffraction particle size analyzer (Cilas, model 1064 liquid). Microspheres were suspended in isopropyl alcohol and sonicated for 10 minutes. The size distribution (polydispersity) was evaluated in terms of a SPAN factor, expressed as $\frac{D90\%-D10\%}{D50\%}$ where D90%, D10% and D50% are the diameters where the given percentage of particles is smaller than that size.

Drug Content

Ten mg of the loaded microspheres were dissolved in 10 ml 2% sodium carbonate solution and SS content was determined spectrophotometricaly at 245 nm (Pharmacia LKB Ultrospec III double beam, England).

Swelling ability [15]

Swelling ability of the blank and loaded microspheres was determined by swelling to equilibrium in simulated nasal fluid (SNF)[18]. Twenty mg of either blank or loaded microspheres were immersed in 5 ml of SNF placed in stainless steel cup (16 mm diameter and 3 mm depth) covered with cellulose nitrate membrane (0.45 µm) through specially designed stainless steel ring at 37 °C for 24 hours. Swelling ratio was calculated from the following formula:

$Qw = \frac{Ws-Wo}{Wo}$ where Q_w is the degree of swelling, W_s is the weight of microspheres after swelling and W_o is the initial weight of microspheres.

Viscometric determination

Viscosity measurements of 20 ml aliquot samples of polymer solutions were carried out using Brookfield viscometer (Model DV-II$^+$ pro, Brookfield, Middleboro, Massachusetts, USA) at a single angular velocity of 3 rpm using spindle number 7 at 25° C. Average of two readings was used to calculate the viscosity in centipoises.

In vitro drug release study

An accurate weight (20 - 25 mg) of the loaded microspheres equivalent to 4 mg of SS was placed in specially designed diffusion cell made of stainless steel cup covered with cellulose nitrate membrane through a specially designed stainless steel ring[19]. The cup was placed in the bottom of a 25-ml beaker containing 5 ml of SNF as the release medium, the beaker was sealed with parafilm to avoid evaporation and to guarantee a constant relative humidity simulating nasal humidity [19]. The beaker was placed in a thermostatically controlled water bath (Type 1083 GFL Gesellschaft fuer Labortechnik m.b. H. & Co., Burgwedel, Germany) adjusted at 37°C and

shaken horizontally at 75 strokes/min [20]. At specified time intervals, the 5 ml of the release medium was taken and replaced by pre-warmed fresh 5 ml of SNF at 37 °C [20, 21]. SS content of the samples was analyzed by UV spectrophotometry at 276 nm after appropriate dilution. Blank microspheres were also subjected to the release test to quantify the contribution of polymer, if any, to UV absorption. Complete release was experimentally verified by dissolving the loaded microspheres in 2% Na_2CO_3 solution at the end of experiments. All measurements were performed in triplicate and the values are presented as the mean ±SD.

Release kinetic analysis

The in vitro release data was fitted to the Korsmeyer–Peppas power law equation [22] (equations 1 and 2) and the Heuristic model proposed by Peppas and Sahlin [23] (equation 3) for quantifying the parameters controlling the release from swellable matrix. The goodness of fit was evaluated by calculation of the r (correlation coefficient) values.

$$Mt = M\infty\, Kt^n \qquad \text{equation (1)}$$

This in logarithmic form is $\log\frac{Mt}{M\infty} = \log K + n\log t$ equation (2), where M_t is the amount of drug dissolved after time t, M_∞ is the amount of drug dissolved after infinite time (all the drug content in the formulation), M_t/M_∞ is the fractional release of the drug in time t, K is a constant incorporating the structural and geometric characteristics of the dosage form, n is the release (diffusion) exponent, which depends on the release mechanism and the shape of the matrix tested, and t is the release time.

Heuristic model $\frac{Mt}{M\infty} = K_1\sqrt{t} + k_2\, t$ equation (3), where M_t is the amount of drug dissolved in time t, M_∞ is the amount of drug dissolved after infinite time (all the drug content in the formulation), M_t/M_∞ is the fractional release of the drug in time t and K_1 and K_2 are the diffusion and erosion terms, respectively.

Ex vivo mucoadhesion testing [24]

Mucoadhesive properties of loaded microspheres were tested by determination of the quantity of microspheres sticking to a filter paper saturated with mucin after applying an air load simulating in and out breathing. This would reflect the expected effect of air flow on nasal clearance of microparticulate formulations after their administration.

A filter paper (diameter=30 mm) was soaked in a mucin solution (2% w/v in SNF) in a chamber with 79% relative humidity (saturated ammonium chloride solution) at room temperature for 10 minutes. Ten mg of the microspheres were spread out onto the filter paper and a stream of hot air

was blown over the microspheres for 30 seconds. The amount of microspheres remain sticking to the filter paper was determined by weighing the remaining microspheres after drying. The in vitro mucoadhesion property of the microspheres was expressed as follows:

Percentage mucoadhesion = $\frac{Wa - Wl}{Wa}$ X 100 , where Wa :weight of microspheres applied, and Wl : weight of microspheres leached out.

Scanning electron microscopy (SEM)

Surface morophology of the microspheres was studied by scanning electron microscope after gold vacuum coating (JEOL JSM-5300, Japan).

Differential scanning calorimeter (DSC)

Differential scanning calorimetry was carried out on pure drug, polymer, blank microspheres and drug loaded microspheres. Samples (3 mg each) were accurately weighed into aluminum pans and then sealed. DSC runs were conducted over a temperature range 50-400 °C at a heating rate of 10 °C / min under nitrogen (Perkin Elmer, Germany).

Mucosal irritation testing on rabbits

The study was approved by Pharos Institutional animal ethics committee and was done according to the method described by Callens et al [25], where 10 mg of the sodium alginate microspheres (F.1) was sprayed into the right nostril of New Zealand white rabbits (n = 3, 2.5 ± 0.5 kg) over 6 consecutive days. The microspheres were administered using an administration device and 1 h after administration, the right nostril was washed with 500 µl saline solution (0.9%) using a micropipette to quantify the amount of proteins, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) released from the mucosa into the nasal cavity. During these manipulations, the rabbits were in supine position, their back lifted to an angle of 45° and their head was kept horizontally to prevent drainage of the saline solution into the nasopharynx. After 5 sec, saline solution was collected in a test tube by returning the rabbits to their normal upright position. The administration device comprised a conical tube with an outlet aperture of 1 mm in diameter and was connected via polyethylene tubing to a 20 ml syringe to aerosolize the formulation. The microspheres were delivered as a single bolus by pushing 20 cm^3 of air through the device where the rabbit was restrained by trained personnel. Total protein concentration present in the saline perfusate was determined by using a colorimetric method (Pyrogallol-red molybdate complex) using Micrototal Protein (MT-P) kit (Spectrum, Egypt) at room temperature and expressed as mg/dL[26]. Lactate dehydrogenase (LDH) activity was measured colorimetery at 37°C with an enzyme kit (BioSystems, Spain) and expressed as U/L and Alkaline phosphatase (ALP) activity

was measured colorimetery at 37°C with an enzyme kit (VitroScient, Egypt) and expressed as U/L

RESULTS AND DISCUSSION

The formulated alginate microspheres were prepared by an emulsification method adapted from the method of Wan et al. [16], using the appropriate surfactants to produce the required emulsion type [27] . The target emulsion is a water-in-oil system, since SS is water-soluble and intended to be dispersed in the internal phase, hence surfactants mixture with overall low HLB will be optimal [11]. Therefore, a blend of 2% Span 80 and 1% Tween 20 were selected after preliminary trials to produce the target microspheres. At concentration of SA lower than 4% w/v, the resultant particles were a loose network which collapsed during drying. On the other hand, an alginate concentration of 4% w/v under high shearing rate caused the breakdown of the viscous aqueous alginate solution to fine globules resulting in small microspheres with dense matrix structure. Higher polymer solution > 4% was viscous and did not flow easily during manufacturing process. Therefore, 4% w/v aqueous SA was found optimal in preparation of the mucoadhesive microspheres. Isopropyl alcohol was used previously for hardening and washing the gelled microspheres to prevent its aggregation after drying[28].

Alginate gelation takes place when divalent cations (usually Ca^{+2}) interact with blocks of guluronic acid residues of the alginate, resulting in the formation of three-dimensional network, which is usually described by ‘egg-box' model [29]. Application of 2.5% and 5% w/v aqueous solution of $CaCl_2$ resulted in formation of low-density spherical microspheres which turned into clumps and collapsed after drug loading. Increasing concentration of $CaCl_2$ to 10% w/v and curing time to 30 minutes were found to be satisfactory for the production of discrete small microspheres of particle size suitable for nasal delivery.

It has been reported that gel matrix of calcium alginate microspheres is usually very permeable and having a low retention capacity of the encapsulated molecules due to its pH dependent solubility [30]. There have been made numerous efforts to control the erosion of alginate microspheres and extend drug release, e.g. blending alginate with other polymers, such as cellulosic derivatives [31], pectin [32] and chitosan [33].

In the present study, anionic polyelectrolytes; CMC Na and Cbp were selected as copolymers with SA (Table 1). Since the chosen copolymers carry negative charges as alginate, this will prevent the interaction between the polymers [17]. Furthermore, the possible occurrence of an interaction between the negatively charged alginate and the positively charged salbutamol is very

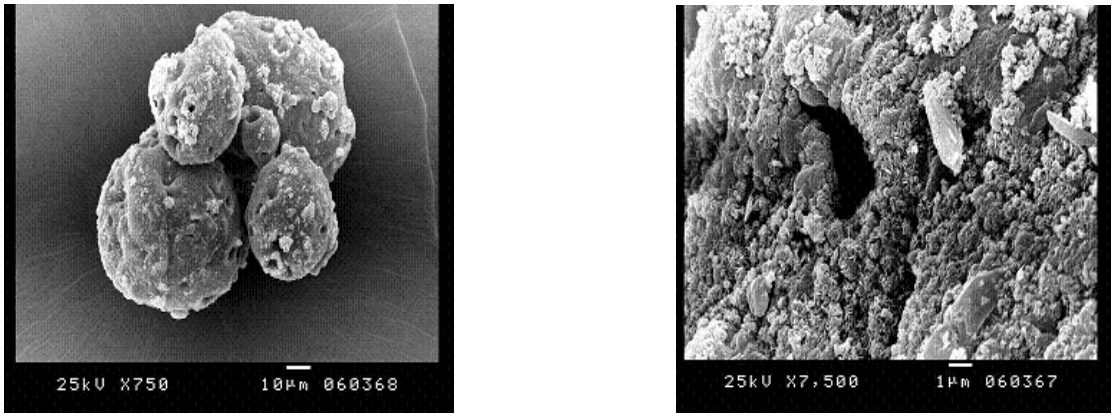
important to assure high loading efficiency.

Table 1: Polymer composition and physical characteristics of the formulated microspheres

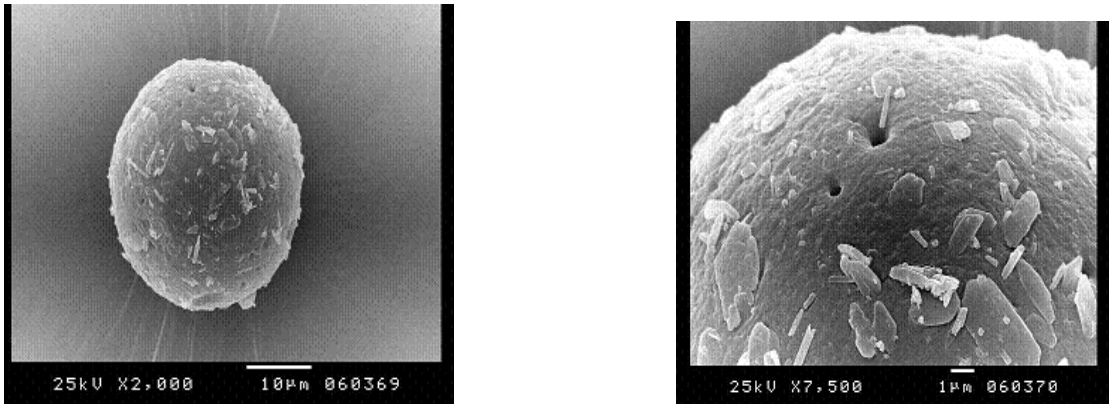
Formulation Code	Polymer(4%w/v)	Mean Particle Size(µm)	SPAN Factor*	% Bioadhesion
F.1	SA	30.20	3.617	82 ± 7.5
F.2	SA : CMC Na	18.9	1.188	86 ± 6.8
F.3	SA : Cbp	40.54	4.698	86 ± 6.0
F.4	SA : HPMC	28.64	1.130	96 ± 4.0

*****SPAN Factor is a measure of size distribution and its determination is illustrated in the experimental part.

The microspheres were found to be discrete and spherical in shape (Figure 1). SEM examination of the surface structure of formulated SA microspheres (F.1) revealed appearance of pores with diameter of a few microns in both blank and loaded microspheres, in addition to the appearance of drug crystals on the surface of the drug-loaded microspheres (Figure 1).



(a)



(b)

Figure 1: SEM of the blank microspheres (a) and loaded microspheres (b) of formula F.1 (SA).

Particle size analysis

Microspheres obtained from pure alginate (F.1) showed a unimodal size distribution with a mean diameter value of 30.2 µm and a SPAN factor of 3.617 (Table 1). When alginate was blended with HPMC (F.4), no significant variation was detected concerning the mean diameter of microspheres, whereas the addition Cbp (F.3) caused slight increase of mean diameter of the microspheres which could be due to aggregation of small particles in the presence of carbopol as a result of the high viscosity of the blend (26666 cP).

Mean diameter of F.2 (SA: CMC Na; 3:1) was smaller than F.1 (SA). This could be due to lower viscosity of blend polymer solution, as evidenced by viscosity measurement; namely 333.3 cP for the blend compared to 3333 for SA. It has been reported that size distribution of microspheres prepared by emulsification is related to the internal phase viscosity [34].

SPAN factor which is an indication of polydispersity of the formulated microspheres ranged between 1.130 to 4.698 (Table 1**),** which could be an indication of narrow size distribution [17]. SPAN factor increases with the mean diameter of microspheres. As reported in literature, particles smaller than 2 µm will continue to move towards the lungs after nasal administration and particles larger than 10 µm are generally filtered by the vibrissae at the nostrils[35]. The prepared particles are thus most likely being retained into the nasal cavity where they can be effective in enhancing the absorption of drugs through the nasal respiratory mucosa by prolonging the nasal residential time of the drug. Therefore, the size of the formulated microspheres in the range of 18.9 to 40.54 µm, could be favorable for intranasal absorption.

Drug Loading

Encapsulation efficiency was found to be only 5% (w/w) when SS was added to the polymer solution prior to cross-linking (Method I; Simultaneous method). This could probably be attributed to a significant loss of the highly water-soluble drug (250 mg/ml) [36] upon expulsion of water during cross-linking induced by microparticle shrinking in addition to drug loss during the washing step. Moreover, the high porosity of alginate microspheres as shown by SEM examination (Figure 1**),** could be partially responsible for the loss of drug due to its migration from the gelled microspheres to the external medium through the pores [37]. It has been reported that low encapsulation efficiencies for pure alginate microparticles were generally observed with low molecular weight drugs and was found to be 4.0% for nitrofurantoin [38] and 32% for indomethacin [39]. Therefore, in order to avoid such drug loss, blank alginate microspheres were prepared using the emulsion-cross linking technique and subsequently soaked in hydroalcoholic solutions of the drug in different concentrations (Method II; Sequential method). A possible reason for better loading efficiency is the electrostatic interaction of the positively charged amino

group of SS and the negatively charged carboxylic group of SA. This result is in agreement with the findings of Moebus et al about hydrogel-based microparticles containing thermo-gelling Poloxamers and cross-linked alginate containing terbutaline sulphate as a model drug [40].

Swelling Study

The swelling ability of the formulated microspheres is expressed as the swelling ratio (Q_w) (Figure 2**).** The drug-loaded microspheres exhibited higher degree of swelling, compared to the corresponding blank microspheres. This finding may be due to the hydrophilic nature of SS, which dissolves quickly upon contact with the SNF and in turn facilitating water penetration. All microspheres prepared by blending SA polymer with other mucoadhesive hydrogels; CMC Na (F.2), Cbp (F.3) or HPMC (F.4) exhibited higher degree of swelling than SA based-microspheres (F.1). This could be due to the high hydrophilicity and swellability of the added copolymers [20]. It has been suggested that upon administration of mucoadhesive microspheres, the microspheres hydrate by withdrawing water from the secretions of the nasal mucosa, resulting in alterations of the viscoelasticity of the mucous gel and hence changes in the mucociliary clearance [35].

It has been reported that the degree of swelling is governed by the degree of cross-linking of the polymer network. Thus, swelling can be controlled over a wide range by modifying the degree of cross-linking of the microspheres. On the other hand, cross-linking of a polymer generally leads to a reduction in the permeability of solutes. Consequently, by proper modification of the structural and molecular characteristics of the microgel carriers, control of the release rate of encapsulated drug can be achieved [41]. Alginate, being a polyelectrolyte, exhibits swelling properties that are sensitive to pH, ionic strength and ionic composition of the medium [42]. The microspheres exhibited significant degree of swelling (Figure 2) when exposed to pH 6.8 of SNF[18]. The swelling mechanism could be related to Ca^{+2}, Na^{+} and/or K^{+} exchange, which is in agreement with Bajpai and Sharma study [43], where they concluded that calcium alginate beads swelled through ion-exchange process which was confirmed by monitoring the Ca^{2+} release from the calcium alginate beads.

Figure 3 presents the release profiles of SS from the formulated mucoadhesive microspheres in SNF (pH 6.8)**.** During the first hour, all formulations showed the same behavior of release, where nearly 28% of the drug was released from the microspheres without any lag time. After 1 h, marked difference of release pattern was observed in the rank order: SA (F.1) > SA: CMC Na (F.2) > SA: Cbp (F.3) > SA: HPMC (F.4). Pronounced sustained effect over 8 hours was exhibited upon incorporation of Cbp (F.3) and HPMC (F.4) with SA.

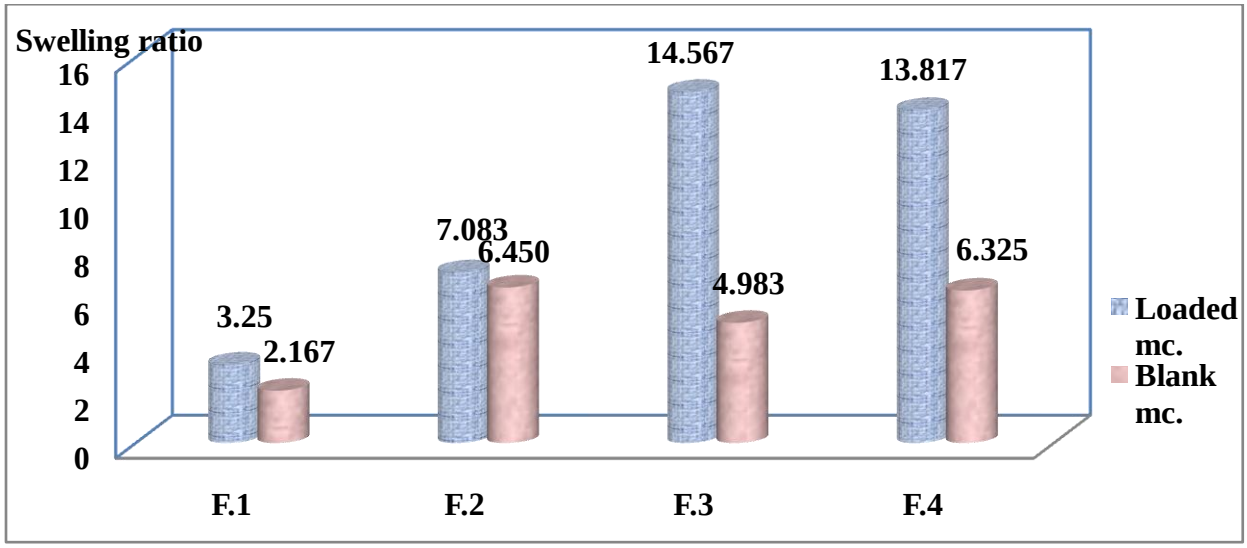


Figure 2: Swelling ratio profiles of the formulated microspheres in SNF at 37°C.

In vitro drug release

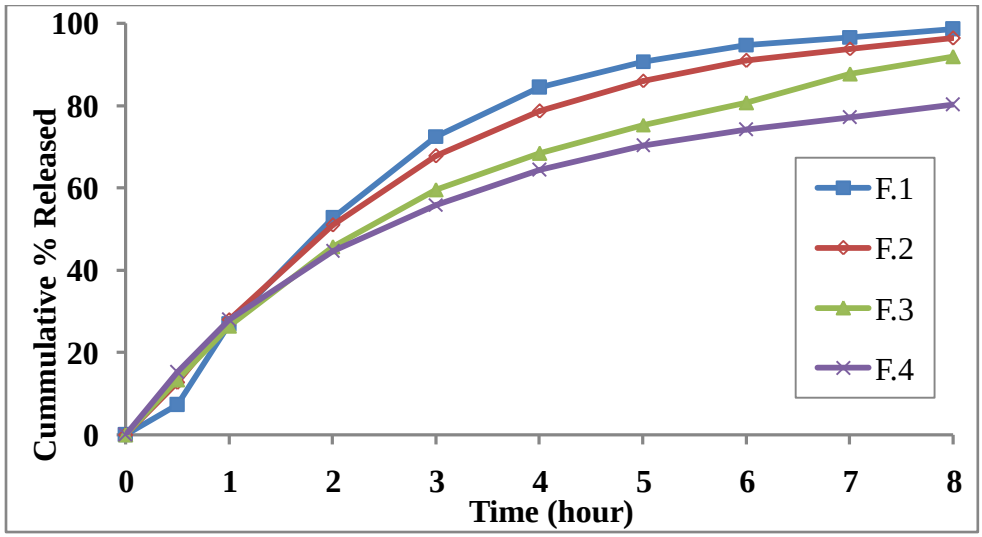


Figure 3*:* In vitro release profiles of salbutamol sulphate from the formulated microspheres in SNF at 37°C.

When microspheres of hydrophilic polymers come into contact with the dissolution medium, it is generally observed that they could swell due to the absorption of water by the matrix, forming a gel diffusion layer. This layer would hinder the outward transport of the drug, producing sustained release effect [44]. However, the release of drug from the microspheres might occur due to: a) release from surface of particles, b) diffusion through swollen rubbery matrix, and c) release due to polymer degradation or erosion.

As could be expected, the drug loading in this study was externally on the surface of microspheres due to the possible electrostatic interaction of the cationic drug with the anionic polymer. In spite of the presence of this electrostatic interaction, it did not prohibit the release of

the drug upon contact with the release medium, which is in agreement with the work of Tafaghodi et al [10]. It has been previously reported that sodium alginate beads are eroded at alkaline pH and contents are released in a sustained manner by both diffusion and slow erosion of the polymer matrix [45].

The results of the in vitro sustained release of the drug from the formulated microspheres may be explained on the basis of the swelling ability of the microspheres. As shown earlier in Figure 2, the swelling ratio of each of formula F.3 and F.4 (SA: Cbp or HPMC; respectively) was higher than that of F.2 (SA: CMC Na) and F.1 (pure SA). This relative higher sustained release effect from F.3 and F.4 could be due to the excessive swelling of the microspheres upon contact with release medium resulting in a dense gel diffusion layer with an increase in diffusional path length that drug molecules have to traverse.

Release kinetic analysis

To determine the mechanism of drug release, % drug release versus time profiles have been fitted to the empirical equation proposed by Korsmeyer - Peppas [22]; $\frac{Mt}{M\infty} = Kt^n$, where M_t/M_∞ is the fraction of drug released at time t, K is a kinetic rate constant and n is diffusional exponent characterizing the mechanism of drug release. For spheres, when *n* approximates to 0.43, a Fickian diffusion controlled release is implied. Values of *n* between 0.43 and 0.85 refers to both diffusion controlled release and swelling controlled drug release (anomalous transport). Values above 0.85 indicate case-II transport which relate to polymer relaxation during gel swelling [46]. The estimated values of n along with the correlation coefficient (r) values are presented in Table 2. The value of n for the release of SS from alginate microspheres F.1 was 0.99, indicating case II release kinetics (zero order). Values between 0.63 to 0.79 for other formulae indicate that drug release from these microspheres followed anomalous or non-Fickian mechanism.

Table 2: Mathematical modeling and drug release kinetics parameters of salbutamol sulphate from the formulated microspheres

	Peppas-Korsymer model			**Peppas- Sahlin model**					
Formula	r	**n**	**Mechanism**	**r**	**Time(h)**	**K_1**	**K_2**	**K_1/K_2**	**Mechanism**
F.1	0.9942	0.99	Case II	0.994	0.5	-2.227	25.232	-0.088	erosion
					3	-1.1611	25.232	-0.046	erosion
F.2	0.9963	0.79	Anomalous	0.9947	0.5	5.899	22.801	0.259	erosion
			Transport		3	3.076	22.801	0.134	erosion
F.3	0.9956	0.72	Anomalous	0.9847	0.5	25.162	15.67	1.605	diffusion
			Transport		3	13.119	15.67	0.837	erosion
F.4	0.9981	0.63	Anomalous	0.967	0.5	24.733	13.6133	1.816	diffusion
			transport		3	12.895	13.613	0.947	erosion

In order to determine whether the drug release was due to erosion or diffusion, the release data of the formulations were fitted to the Peppas-Sahlin equation [23] and the constants K_1 (diffusion constant) and K_2 (erosion constant) were determined at different time intervals (Table 2). Accordingly if the diffusion to erosion ratio $K_1/K_2 = 1$, then the release mechanism involves diffusion and erosion equally. If $K_1/K_2 > 1$, then diffusion prevails, while erosion predominates when $K_1/K_2 < 1$.

F.1 microspheres (pure SA) showed prevalence of K_2 over K_1, revealing that drug release mechanism was controlled mainly by the erosion of the matrix. It is important to notice the negative values for K_1, calculated from drug release data, suggesting that diffusion process was insignificant compared to the relaxation mechanism [46]. F.2 microspheres (SA : CMC Na), exhibited K_1/K_2 ratio < 1, indicating predominance of erosion mechanism over diffusion. This observation is in agreement with Moebus et al findings[40]. Incorporation of the mucoadhesive polymers Cbp and HPMC with SA (F.3 and F.4; respectively) resulted in drug release mechanism controlled by diffusion mechanism for the first 2 hours followed by polymer erosion. These kinetic analysis results could explain the above mentioned rank order of drug release from the formulated microspheres.

Ex vivo mucoadhesion

The results of the ex vivo mucoadhesion revealed that all SS microspheres showed good mucoadhesivity ranging from 82% to 96% (Table 1), which may be explained on the basis of swelling study. It could be observed that there is a direct relationship between degree of swelling and mucoadhesion. High swelling (sufficient chain flexibility), leads to enhanced physical entanglement and interpenetration of the polymer chains with those of the mucin, in addition to possibility of formation of secondary chemical bonds, hydrogen bonding and van der Waals' forces, thus contributing to the mucoadhesion [47]. This observation could explain the higher mucoadhesion of microspheres prepared of polymer blends with SA (F.2, F.3 and F.4) due to their higher degree of swelling compared to microspheres formed from pure SA (F.1).

The results of this ex vivo mucoadhesion testing show that the produced microspheres have good mucoadhesive properties which could potentially improve the residence time of SS in the nasal cavity allowing a better sustained release.

Differential scanning calorimetry (DSC)

DSC is a useful method to assess the physical state of the drug and the interaction between the drug and polymer used. Figures 4 (a-d) display the DSC thermograms of the blank microspheres, drug loaded microspheres, and the drug. SS crystals exhibited a sharp endothermic peak at

202.719°C, which is in agreement with a previous study[48]. Blank pure 4% w/v alginate microspheres (F.1, Figure 4 a) showed two peaks, one broad peak at 98.219°C and a second very small peak at 160°C. Similar thermograms were observed for the other blank microspheres composed of polymer blends (F.2, F.3 and F.4; Figures 4 b-d). Regarding drug-loaded microspheres, drug's peak was shifted to a lower temperature at about 75°C. This shift indicates possible interaction between the cationic drug SS and the anionic SA polymer. The low intensity and broadness of such peak could also indicate partial loss of crystallinity of the drug in the loaded microspheres. It has been reported in the literature that the incorporation of a cationic drug into alginates, whether before or after gel formation, causes an interaction between alginate and cationic drugs such as propranolol[49] and imipramine[50].

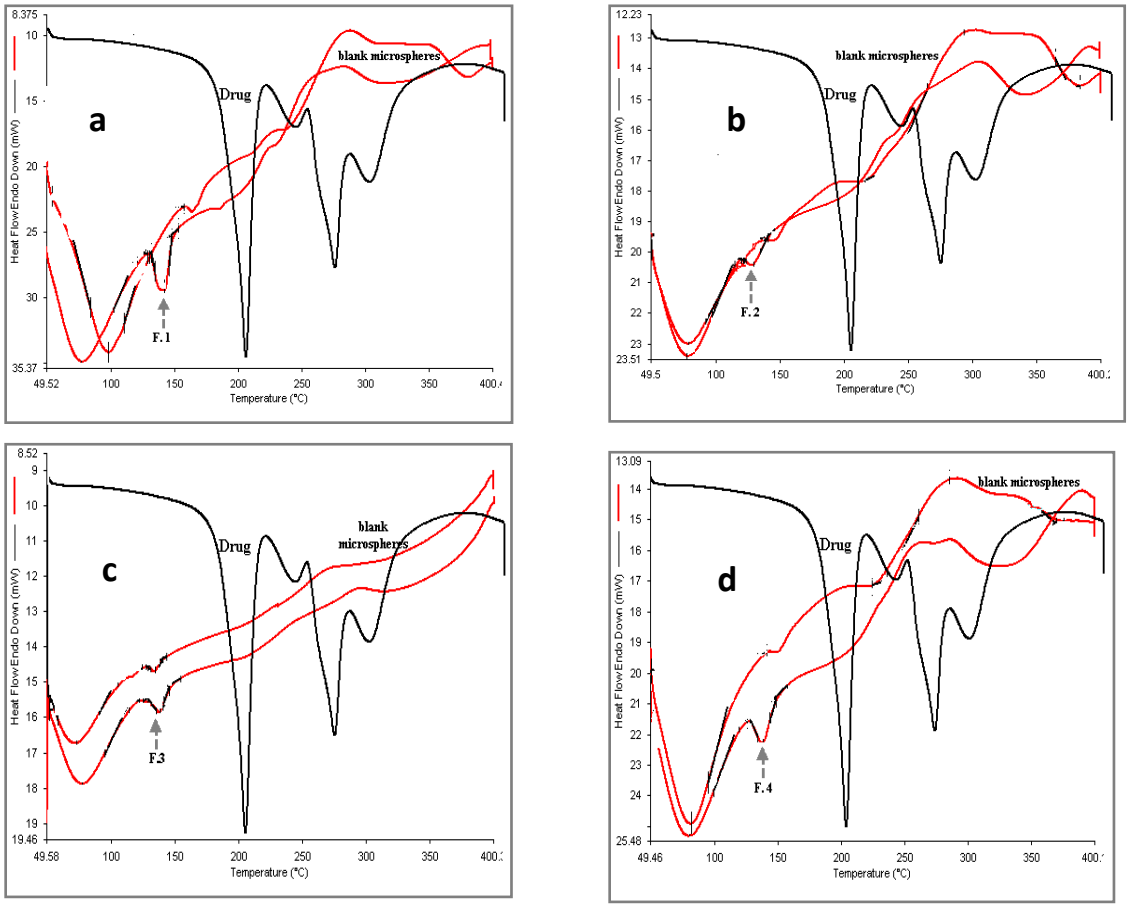


Figure 4: DSC thermograms of salbutamol sulphate, blank microspheres and drug-loaded microspheres of F.1; SA (a), F.2; SA and CMC Na; 3:1 (b), F.3; SA and Cbp; 3:1 (c) and F.4; SA and HPMC; 3:1 (d)

Nasal irritation study

Generally, for nasal delivery four areas of toxicity can be distinguished: local irritation of the mucosa, effect on mucociliary clearance, epithelial damage and rate of recovery of the damaged

epithelium [51]. In this study we examined the mucosal toxicity of the formulated mucoadhesive F.1 microspheres intended for nasal delivery after multiple administrations, using a non-invasive in vivo method in rabbits [25]. The advantages of this method are the possibility of using non-anaesthetized and non-sedated animals and the possibility of repeated determination of the release of marker molecules on the same animal during a long-term experiment.

One hour after administration of the formulation, the nasal cavity was washed with saline solution (0.9%) to determine the release of proteins, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), which are biochemical markers for membrane damage. The protein concentration and enzyme activity measured before administration were used as basal levels [25]. The results were compared to untreated rabbits (negative control group) and to rabbits treated with alginate microspheres mixed with benzalkonium chloride (in the ratio 3:2) over 6 consecutive days (positive control group) (Table 3).

Table 3: Protein concentration, ALP and LDH after repeated administration of sodium alginate microspheres (F.1) to the right nostrils of rabbits

Days		Formula F.1	Negative control	Positive control
Basal line	P	8.33±14.4	0	25± 10.6
	ALP	0	0	1.1± 8.5
	LDH	0	0	0
Day 2	P	n.d	n.d	n.d
	ALP	4.87 ± 8.4	n.d	6.5± 11
	LDH	0	n.d	32± 9.8
Day 4	P	n.d	n.d	n.d
	ALP	8.90 ±15.9	n.d	1.1± 4.5
	LDH	0	n.d	8.1± 6.5
Day 6	P	58.33 ± 28	25± 17.5	25± 22.4
	ALP	11.67 ±14.8	5.1± 7.6	0.9± 5.7
	LDH	0	0	0
Day 9	P	33.33 ± 28	25± 18.7	30± 23
	ALP	2.97 ±3.3	5.4± 4.8	7.6± 6.2
	LDH	0	0	0

P: total protein in mg/dl.
ALP: alkaline phosphatase enzyme in IU/L.
LDH: lactate dehydrogenase enzyme in IU/L.
Basal line: one hour before administration.
n.d: not detected.

Benzalkonium chloride was incorporated with the formulation because of its well known damaging effects to different cell types and its frequent use as a positive control [25]. Three days after the last administration (i.e. day 9), an additional washing was performed to evaluate if the release of protein, LDH and ALP had returned to their basal levels. Repeated administration of

the formulated alginate microspheres for 6 successive days resulted in an increase in the level of total protein and ALP enzyme compared with the basal levels, indicating an irritation to the nasal mucosa. However, on day 9 (3 days after last administration) there was a decrease of the protein and ALP levels, indicating that the irritation to the nasal epithelium was reversible [52].

Furthermore, the absence of the intracellular marker enzyme LDH in the treated group, the same as the untreated rabbits, confirms the absence of appreciable damage to the nasal epithelium after repeated administration of the formulated microspheres.

In the positive control group, LDH enzyme was detected after 2 days of administration with high level (32 IU/ L) indicating membrane damage of the cells that could be due to benzalkonium chloride. The decrease in LDH activity observed in this positive control group towards the end of treatment could be due to the adaptation of nasal mucosa to the administered mixture of microspheres and benzalkonium chloride after multiple exposures [52].

Tafaghodi et al (2006) have demonstrated that nasal application of blank alginate microspheres to four human volunteers did not cause any local irritation. In addition, when different concentrations of blank alginate microspheres were incubated with human RBCs, no hemolysis was observed and this could be interpreted as an additional safety issue for alginate microspheres.

Therefore, it can be concluded that SS-loaded alginate microspheres prepared by emulsion cross-linking method are potentially safe with no severe damage of nasal epithelium. In addition, the nasal mucosa irritation, indicated by the increased levels of protein and ALP marker enzyme compared to the basal level, was found to be reversible.

CONCLUSION

The developed microspheres are characterized by high swelling and good mucoadhesion properties, suggesting them as suitable carrier systems for nasal delivery of the model drug salbutamol sulphate with sustained release possibilities as a potential alternative to conventional dosage form. The formulated alginate microspheres were safe and showed only transit change of nasal epithelium after repeated administration to rabbits. Further in vivo study for bioavailability assessment for intranasal administration of salbutamol sulphate would be useful for ultimate evaluation of the microspheres as drug delivery system.

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