Evaluation of Mucoadhesive Properties of Chitosan Microspheres Prepared by Different Methods

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ABSTRACT

The mucoadhesive properties of chitosan microspheres prepared by different methods were evaluated by studying the interaction between mucin and microspheres in aqueous solution. The interaction was determined by the measurement of mucin adsorbed on the microspheres. A strong interaction between chitosan microspheres and mucin was detected. The intensity of the interaction was dependent upon the method of preparation of chitosan microspheres and the amount of mucin added. The extent of mucus adsorption was proportional to the absolute values of the positive zeta potential of chitosan microspheres. The zeta potential in turn was found to be dependent upon the method of preparation of microspheres. The adsorption of type III mucin (1% sialic acid content) was interpreted using Freundlich or Langmuir adsorption isotherms. The values of r^2 were greater for Langmuir isotherm as compared with Freundlich isotherm. The adsorption of a suspension of chitosan microspheres in the rat small intestine indicated that chitosan microspheres prepared by tripolyphosphate cross-linking and emulsification ionotropic gelation can be used as an excellent mucoadhesive delivery system. The microspheres prepared by glutaraldehyde and thermal cross-linking showed good stability in HCl as compared with microspheres prepared by tripolyphosphate and emulsification ionotropic gelation.

KEYWORDS: chitosan microspheres, emulsification ionotropic gelation, glutaraldehyde cross-linking, mucoadhesion

INTRODUCTION

There has been considerable interest in developing biodegradable, injectable microspheres for the controlled release of proteins and peptides.[1-6] Mucoadhesive polymers may fulfill the desirable features of a prolonged residence time at the site of drug absorption owing to increased contact with the absorbing mucosa, resulting in a steep concentration gradient to favor drug absorption, and localization in specified regions to improve and enhance the bioavailability of the drug.[7] Factors such as cross-linking status, ionic modification, and salt formation can significantly influence the ability of a material to show substantial mucoadhesion in an in vitro system.[8,9] Contrary to these factors, it has been postulated that positively charged polymeric hydrogels develop additional molecular forces by electrostatic interaction with negatively charged sugar moieties of the mucosal surface.[10] The mucoadhesive polymer should have a strong hydrogen bond–forming group, such as carboxylate or hydroxyl; carry a strong anionic charge; have a high molecular weight; possess sufficient chain flexibility; have surface energy property favoring spreading onto the mucus; and be nontoxic, nonabsorbable, and noninteracting with the drug.

Chitosan obtained by deacetylation of chitin (a naturally occurring polymer) has been shown to possess mucoadhesive properties owing to the molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces.[11] Chitosan has 1 primary amino and 2 free hydroxyl groups for each C_6 building unit. Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus, in turn, reacts with many negatively charged surfaces/polymers.[12]

Chitosan microspheres are used to provide controlled release of many drugs and to improve the bioavailability of degradable substances such as protein, as well as to improve the uptake of hydrophilic substances across the epithelial layers. These microspheres are being investigated both for parenteral and oral drug delivery.[13] Chitosan microspheres can be prepared by reacting chitosan with controlled amounts of multivalent anion resulting in cross-linking between chitosan molecules. The cross-linking may be achieved in acidic, neutral, or basic environments depending on the method applied. Chitosan microspheres can be prepared by various methods such as cross-linking with anions,[14] precipitation,[15] complex-coacervation,[16] modified emulsification and ionotropic gelation,[17] precipitation-chemical cross-linking,[18] glutaraldehyde cross-linking,[19] thermal cross-linking,[20] and more. The cross-linking of polymers affects the mucoadhesive strength of the microspheres.

The in vitro evaluation of the mucoadhesive properties of polymeric microspheres is a basic step in the development of a mucoadhesive microparticle drug delivery system. As the process of mucoadhesion is a consequence of interaction between the mucus layer on mucosa and mucoadhesive polymer, it is greatly dependent upon mucus and polymer structure including their charges.[21] He et al[12] had proposed a salt

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bridge effect for the interaction of positively charged chitosan microspheres with the negatively charged mucus glycoprotein, but subsequently it was demonstrated that positive charge on the surface of chitosan could give rise to a strong electrostatic interaction with mucus or with a negatively charged mucosal surface.[22,23] Therefore, by measuring the zeta potential of chitosan microspheres, an insight into electrostatic interaction during mucoadhesion can be obtained. Hence, in this study, the zeta potential and mucoadhesive properties of chitosan microspheres prepared by different methods were evaluated. Using a biological approach, the adhesion of chitosan microspheres to mucosal tissue (rat small intestine) was also evaluated.

Materials and Methods

Chitosan (molecular weight [MW] 600 000, viscosity of 1% solution in 1% acetic acid at 20°C = 400 mPa/s) was obtained from Fluka (Fluka Chemie AG, Buchs**,** Switzerland); Span 85 from Koch-Light laboratories Ltd (Colnbrook Berks, UK); ethylcellulose (EC) (14 cp) from BDH Chemicals Ltd (Poole, UK); fuchsin (pararosaniline) (NB) from Merck AG (Darmstadt, Germany); heavy liquid paraffin and periodic acid from SD Fine Chemicals Ltd (Boisar, India); glutaraldehyde from E. Merck India Ltd (Mumbai, India); mucin (type III, partially purified from porcine stomach, bound sialic acids ~1%) from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany); and polyvinyl alcohol from SD Fine Chemicals Ltd (Poicha, India). Hexane, petroleum ether, NaOH, and all other reagents were of analytical grade.

Preparation of Microspheres by Thermal Cross-linking

The microspheres were prepared by modifying the method used by Orienti et al.[24] Citric acid, as a cross-linking agent was added to 30 mL of an aqueous acetic acid solution of chitosan (2.5% wt/vol) maintaining a constant molar ratio between chitosan and citric acid (6.90×10^{-3} mol chitosan: 1 mol citric acid). The chitosan cross-linker solution was cooled to 0°C and then added to 25 mL of corn oil previously maintained at 0°C, with stirring for 2 minutes. This emulsion was then added to 175 mL of corn oil maintained at 120°C, and cross-linking was performed in a glass beaker under vigorous stirring (1000 rpm) for 40 minutes. The microspheres obtained were filtered and then washed with diethyl ether, dried, and sieved. This batch is denoted by symbol TCL.

Preparation of Microspheres by Glutaraldehyde Cross-linking

The microspheres were prepared by the method reported by Thanoo et al[19] (referred to as GCL). A 2.5% (wt/vol) chitosan solution in aqueous acetic acid was prepared. This dispersed phase was added to continuous phase (125 mL) consisting of light liquid paraffin and heavy liquid paraffin in the ratio of 1:1 containing 0.5% (wt/vol) Span 85 to form a water in oil (w/o) emulsion. Stirring was continued at 2000 rpm using a 3-blade propeller stirrer (Remi Equipments, Mumbai, India). A drop-by-drop solution of a measured quantity (2.5 mL each) of aqueous glutaraldehyde (25% vol/vol) was added at 15, 30, 45, and 60 minutes. Stirring was continued for 2.5 hours to obtain microspheres, which were separated by filtration under vacuum and washed, first with petroleum ether (60°C-80°C) and then with distilled water to remove the adhered liquid paraffin and glutaraldehyde, respectively. The volume of glutaraldehyde was varied to affect the cross-linking density. The batches are referred to as GCL10, GCL15, and GCL20 depending on the total volume of glutaraldehyde used. The microspheres were then finally dried in a vacuum desiccator.

Preparation of Microspheres by Tripolyphosphate

The microspheres were prepared by the method described by Bodmeier and Paeratakul.[25] A chitosan solution of 2.5% wt/vol concentration was prepared. Shiraishi et al[26] and Shu and Zhu[27] have also reported the interaction of chitosan with tripolyphosphate (TPP). The microspheres were formed by dropping the bubble-free dispersion of chitosan through a disposable syringe (10 mL) onto a gently agitated (magnetic stirrer) 5% or 10% wt/vol TPP solution. The batches were coded as TPP5 and TPP10, respectively. The chitosan microspheres were separated after 2 hours by filtration and rinsed with distilled water; then they were air dried.

Preparation of Microspheres by Emulsification and Ionotropic Gelation by NaOH

As reported by Singla and colleagues,[28,29] the dispersed phase consisting of 40 mL of 2% vol/vol aqueous acetic acid containing 2.5% wt/vol chitosan was added to the continuous phase consisting of hexane (250 mL) and Span 85 (0.5% wt/vol) to form a w/o emulsion. After 20 minutes of mechanical stirring, 15 mL of 1N sodium hydroxide solution was added at the rate of 5 mL per min at 15-minute intervals. Stirring speed of 2000 to 2200 rpm was continued for 2.5 hours. The microspheres were separated by filtration and subsequently washed with petroleum ether, followed by distilled water and then air dried. This batch was coded as EIG.

Preparation of Ethylcellulose Microspheres

EC microspheres were prepared according to the method reported by Ranga Rao and Devi.[30] A solution of EC in acetone was added to liquid paraffin containing emulgent (Span 85), while stirring at a speed of 1500 rpm. The emulsion was stirred for 5 to 6 hours at 25°C to 30°C. Subsequently, a suit-

able amount of petroleum ether was added to the dispersion, filtered, and dried at ambient temperature. The resultant microspheres were washed with water followed by petroleum ether to remove traces of liquid paraffin. The microspheres were desiccated under vacuum. The batch was coded as EC.

Many batches were prepared using these methods, but only those batches and conditions that led to the batches having approximately the same mean geometric diameter (50 μm) are given here.

Size Determination of Microspheres

Microspheres were sized using a Malvern Mastersizer S (Malvern Instruments Ltd., Malvern Worcestershire, UK). The distribution of particle size was measured. The data obtained were computed for goodness-of-fit Chi-square test. The distribution was found to be log normal ($P < .001$), and the geometric mean diameter (d_g) and the standard deviation (σ_g) were calculated from log probability plots of data.[31]

For carrying out the experiments (zeta potential determination and so on), a fraction of 50 ± 2 μm size was taken. The microspheres were fractionated by sieving through 2 sieves (50-52 μm, Secor Sieves, Ambala, India). With the 52-μm sieve at the top, the sieves were shaken for 15 minutes in a sieve shaker. The fraction retained on the 50-μm sieve was used for analysis.

Determination of Zeta Potential

The zeta potential is representative of particle charge. Zeta potentials were measured by electrophoresis, which was performed with a Malvern Zetasizer nanoZS apparatus. Phosphate buffer with pH 7.0 (0.001 M) was used as environment. The microspheres were suspended in buffer by ultrasonication for 30 minutes. The concentration of the suspension was 2% wt/vol. The cell was filled with a measured amount of sample and inserted with its integral gold electrodes close to the lid. Single-factor analysis of variance (ANOVA) using MS Excel was performed to determine the difference in zeta potential owing to different methods.

Stability of Chitosan Microspheres in 0.1N HCl

The stability of chitosan microspheres in 0.1N HCl was determined by incubating 0.5% wt/vol suspension of the microspheres in 0.1N HCl for 60 minutes and measuring the transmission of the samples at 500 nm (Spectronic 20D, Milton Roy, PA, USA) as reported by Berthold et al.[18] Chitosan is soluble in acidic pH, therefore, the purpose of carrying out this study was to determine the effect of different cross-linking methods on the solubility of chitosan, which in turn reflects the stability at acidic pH.

Mucous Glycoprotein Assay

A periodic acid/Schiff (PAS) colorimetric method reported by Mantle and Allen[32] was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the chitosan microspheres and its effect on the assessment of mucoadhesive behavior of chitosan microspheres. Two reagents were prepared. Schiff reagent contained 100 mL of 1% basic fuchsin (pararosaniline) aqueous solution and 20 mL of 1 M HCl. Sodium metabisulphite (0.1 g) was added to every 6 mL of Schiff reagent before use, and the resultant solution was incubated at 37°C until it became colorless or pale yellow. Periodic acid reagent was freshly prepared by adding 10 μL of 50% periodic acid solution to 7 mL of 7% (vol/vol) acetic acid solution.

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.25, 0.5, 0.75, and 1 mg/2 mL). After adding 0.2 mL of periodic acid reagent, the samples were incubated at 37°C for 2 hours in a water bath. Then, 0.2 mL of Schiff reagent was added at room temperature. Thirty minutes later, the absorbance of the solution was recorded at 555 nm in a UV spectrophotometer (Spectronic 20D).[33] Triplicate samples were run. All the samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve. As comparison, the mucoadhesive potential of EC microspheres was also assessed with the above procedure. Each experiment was performed 3 times and standard deviation noted.

Adsorption of Mucin on Chitosan Microspheres

Mucin aqueous solution with different concentrations (0.025, 0.05, 0.1, 0.2, and 0.5 mg/mL) were prepared. Chitosan microspheres (20 mg) prepared using different methods were dispersed in the above mucin solutions, vortexed, and shaken at room temperature.[12] Then, the dispersions were centrifuged at 4000 rpm for 2 minutes, and the supernatant was used for the measurement of the free mucin content. The data obtained were interpreted using Freundlich (1) or Langmuir (2) equations describing the adsorption isotherms:

$$C_{ads} = KC_e^n \quad (1)$$

$$C_{ads} = \frac{aC_e}{b + C_e} \quad (2)$$

Where C_{ads} is the concentration of mucin adsorbed at equilibrium and C_e is the concentration of free mucin at equilibrium. Values of different constants were obtained from the graphs of the above equations. For the Langmuir equation, $1/C_{ads}$ was plotted against $1/C_{free}$ to get the constants and for the Freundlich equation, log C_{ads} was plotted against C_{free} to get the constants.

Table 1. Physicochemical Characteristics of Chitosan Microspheres Prepared by Different Methods*

Batch	Size Range	Mean Geometric Diameter (μm)	Zeta Potential (mV)	% Transmission
TCL	5-105	50.4	20.7	10.1
GCL10	7-110	52.1	36.2	8.9
GCL15	4-102	49.2	35.9	8.1
GCL20	5-103	51.7	32.7	8.2
TPP5	4-107	52.8	42.7	65.2
TPP10	3-99.7	48.7	40.1	62.4
EIG	5-104	52.6	50.2	64.1
EC	2-108	51.9	-15.4	

*TCL indicates thermal cross-linking; GCL, glutaraldehyde cross-linking; TPP, tripolyphosphate; EIG, emulsification and ionotropic gelation; and EC, ethylcellulose.

Rat Gut Loop Studies of Mucoadhesion

Male Wistar rats, with a mean weight ~300 g, were anesthetized and killed with an overdose of barbiturate. The small intestine was removed and washed with physiological saline, using the procedure described by Ranga Rao and Buri,[34] with a syringe (5 to 10 mL/min for 10 minutes, then 20 to 30 mL/min for ~20 minutes). At least 500 mL of saline was used for cleaning the intestine. The cleaned tissues were used immediately or kept at –15°C until use, which was within 2 days.

A required amount of chitosan microspheres or EC microspheres were suspended in physiological saline and sonicated (400 mg in 10 mL). The suspension of microspheres was filled into lengths of small intestine (~15 cm in length) and sealed. These tubes were incubated in saline at 37°C for 60 minutes. The microsphere suspension was then removed, and the number of microspheres present in the suspension before and after the adhesion study was counted using a Coulter counter (Beckman Coulter Inc., CA, USA). A control of blank saline was also taken and filled in the intestine. The reading obtained from this blank was subtracted from the observations obtained from the saline containing microsphere batches. The percentage of microspheres adhered to the tissue was calculated from the difference between the counts. The validity of the counting technique was proved by the existence of a linear relationship between the amount of chitosan microsphere suspension added to a fixed 100 mL of electrolyte, and the resultant counts. At least 6 measurements were made for each sample.

Results and Discussion

The particle sizes of the chitosan microspheres and EC microspheres are shown in Table 1. The zeta potential of EC microspheres was negative, while the zeta potential of chitosan microspheres was positive (Table 1). The method of preparation of chitosan microspheres had a marked effect on the zeta potential of microspheres. The microspheres prepared by TCL had the lowest zeta potential, while the EIG batch had the highest zeta potential ($P < .001$). With increase in glutaraldehyde volume and TPP concentration, the zeta potential decreased (Table 1). This difference was found to be statistically significant for GCL10 and GCL20 ($P < .01$) and TPP5 and TPP10 ($P < .05$). This phenomenon can be explained on the basis of preparation of microspheres. Zeta potential is a measure of net positive charge on microspheres. The ionic neutralization of positive charge was high when glutaraldehyde and thermal cross-linking were used as compared with other agents.

Stability of Chitosan Microspheres in 0.1N HCl

The stability of all the batches of microspheres was determined by measuring the transmission after the microspheres had been exposed to 0.1N HCl. In the present investigation, the acid instability of the chitosan microspheres led to the dissolution of the microspheres, and the sample became more transparent. Since the decrease in turbidity is directly dependent on the disintegration of the microspheres, transmission is a measure of the concentration of nondisintegrated microspheres. A low transmission indicates high stability, and a high transmission implies that the microspheres dissolved in HCl. As shown in Table 1, TCL and GCL did not dissolve in 0.1N HCl, and all other batches of chitosan microspheres were found to be unstable at 1.2 pH. The acidic instability of TPP and EIG microspheres can be explained by the manufacturing process of the microspheres. The addition of NaOH and TPP to the acetic acid solution of chitosan leads to poorly soluble chitosan derivatives by ionic neutralization of the positively charged amino groups. On addition of the acid (increased proton concentration), the equilibrium is shifted toward solubilization and the microspheres dissolve.

Assessment of the Mucoadhesive Behavior of Chitosan Microspheres by Mucus Glycoprotein Assay

Since a strong interaction exists between mucin and chitosan, mucin should be spontaneously adsorbed to the surface of the chitosan microspheres. For this reason, the mucoadhesive behavior of chitosan microspheres was assessed by the suspension of chitosan microspheres in different amounts of mucin (Type III) in aqueous solutions at room temperature. As comparison, the mucoadhesive potential of EC microspheres was also assessed following the same procedure. The amount of mucin adsorbed increased with the increasing mucin concentration (Figure 1). In contrast, little mucin was adsorbed on negatively charged EC microspheres (Figure 1). These results confirm that chitosan microspheres have the ability to adsorb mucin. The amount of mucin adsorbed was dependent upon the method of preparation of chitosan microspheres. For GCL

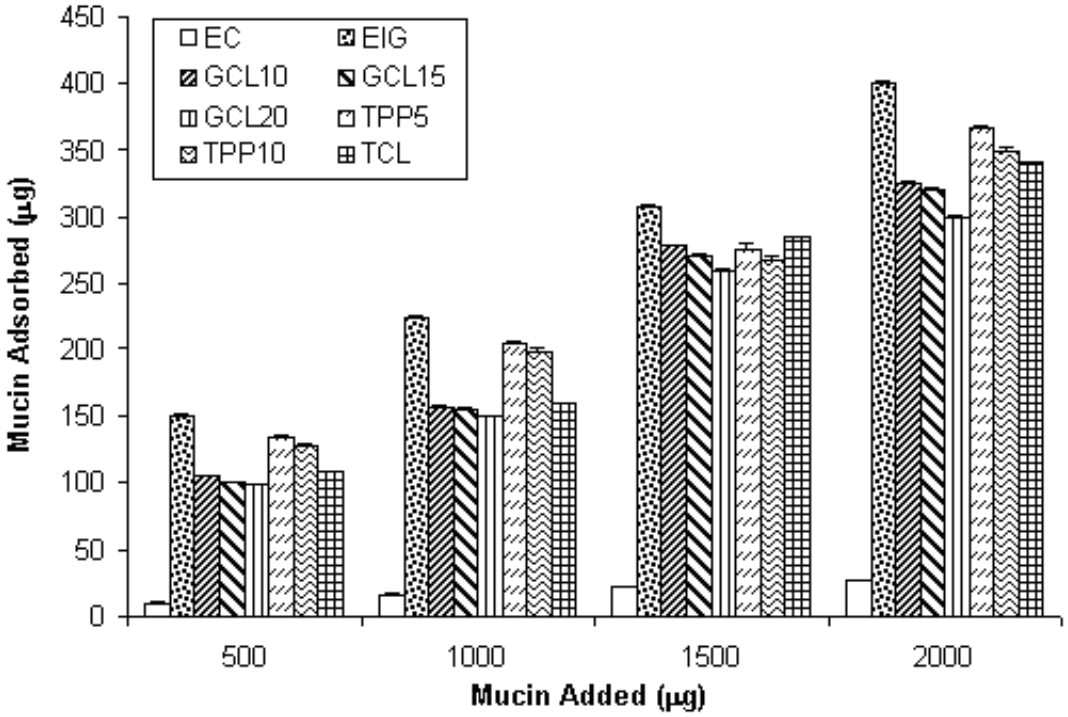

Figure 1. Adsorption of mucin on different microspheres with respect to the amount of mucin added.

Table 2. Constants Obtained by Fitting the Data to Langmuir and Freundlich Equations*

	Langmuir Isotherm†			Freundlich Isotherm‡		
Batch	**a**	**b**	r^2	**K**	**n**	r^2
TCL	1.3×10^{-1}	2.9×10^{-1}	0.97	1.47	1.41	0.90
GCL10	5.10^{-1}	2.9×10^{-1}	0.99	1.31	1.41	0.93
GCL15	8.5×10^{-1}	2.9×10^{-1}	0.99	1.19	1.42	0.95
GCL20	0.000	5.7×10^{-1}	0.95	1.43	1.13	0.92
TPP5	9.8×10^{-1}	4.2×10^{-2}	0.94	1.18	3.72	0.90
TPP10	9.8×10^{-1}	6×10^{-2}	0.92	1.17	3.10	0.78
EIG	1.05	8.8×10^{-3}	0.95	1.04	10.63	0.93

*Abbreviations are explained in the footnote to Table 1.

† $C_{ads} = \frac{aC_e}{b+C_e}$

‡ $C_{ads} = KC_e^n$

and TCL batches, the amount of mucin adsorbed was less compared with the EIG and TPP batches ($P < .001$). The amount of mucin adsorbed decreased with increase in the amount of glutaraldehyde ($P < .01$) and TPP ($P < .01$). Thus, these studies indicate that there may be an increase in the residence time of the formulation in the gastrointestinal tract, depending upon the method of preparation of chitosan microspheres. Although the zeta potential of TCL batch is the lowest, the amount of mucin adsorbed is comparable to GCL batches. This may be explained on the basis that besides neutralization of the positive charge, thermal cross-linking might have distorted the polymer chains leading to different results. For all other batches we can say that the amount of mucin adsorbed decreased with decrease in zeta potential.

Adsorption Isotherms

The adsorption of mucin on chitosan microspheres was measured by the determination of the equilibrium free concentration of mucin in solution. The data thus obtained were fitted with Freundlich and Langmuir equations. Straight lines were obtained, and the constants from these lines are listed in Table 2. It was observed that the values of r^2 were statistically significantly higher ($P < .01$) for the Langmuir equation as compared with the Freundlich equation. This is indicative of a more specific adsorption process where electrostatic interaction is involved. The adsorption of mucin to chitosan is expected to be dominated by the electrostatic attraction between the positively charged chitosan and negatively charged mucin (the

Table 3. Mucoadhesive Measurements of Chitosan Microspheres on Rat Small Intestine by Particle Counting Technique*

Batch	Counts of Chitosan Suspension Before Incubation	After Incubation	Adhered %
EC	6768 ± 543	6629 ± 578	2.05
TCL	5987 ± 458	3789 ± 547	36.71
GCL10	5568 ± 543	3987 ± 345	28.39
GCL15	6238 ± 589	4563 ± 489	26.85
GCL20	6138 ± 612	4678 ± 345	23.78
TPP5	5987 ± 568	2768 ± 245	53.76
TPP10	6215 ± 612	2965 ± 236	52.29
EIG	5899 ± 546	1876 ± 167	68.19

*Abbreviations are explained in the footnote to Table 1. Values are ± SD; n = 3.

negative charge of mucin is due to the ionization of sialic acid). Therefore, the surface charges of chitosan microspheres represented by zeta potential would influence the amount absorbed. The amount of mucin adsorbed increased with increasing mucin concentration. Also, in conformity with the electrostatic attraction theory, the amount of the adsorption decreased with decreasing zeta potential. Microspheres with highest zeta potential (EIG) had the largest amount of adsorbed mucin. TCL is an exception among these as it has lower zeta potential than GCL batches but higher mucin adsorption than GCL batches. The reason for this has already been explained.

Mucoadhesion of Chitosan Microspheres in Rat Small Intestine

The adsorption of chitosan microspheres on rat small intestine was tested by counting the number of the particles adsorbed to the tissue. The results are shown in Table 3. EC microspheres were assessed by the same procedure, as a comparison. Chitosan microspheres were adsorbed on the tissue, whereas only a few of the EC microspheres were adsorbed to the tissue. This is the further evidence for the strong interaction between chitosan microspheres and mucus glycoprotein and/or mucosal surfaces. The interaction was found to be dependent upon the method of preparation of microspheres.

It was observed that the percentage of microspheres that adhered to the intestine was maximal for EIG batch followed by TPP, TCL, and GCL batches. The amount of chitosan adsorbed on the tissue increased with the decreasing cross-linking level ($P < .01$ for GCL batches and $P < .05$ for TPP batches).

CONCLUSION

Chitosan microspheres prepared by different methods were evaluated for their mucoadhesive properties. Mucous glycoprotein assay and rat gut loop studies were performed to determine the amount of mucin adsorbed on chitosan microspheres. Positively charged chitosan microspheres (as determined by zeta potential) had the ability to adsorb mucus glycoprotein. The extent of adsorption of mucin was dependent upon zeta potential of chitosan microspheres. Any factor leading to reduced absolute values (different cross-linking level and method of preparation) reduced the adsorption amount. Chitosan microspheres prepared by emulsification ionotropic gelation were found be more mucoadhesive as compared with other methods.

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