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Report

Receptor-Mediated Magnetic Carriers: Basis for Targeting

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A new magnetic microsphere carrier has been formulated that may localize drugs by both biochemical and physical means. The microspheres, prepared from the polysaccharide chitosan, are designed to bind to anionic glycosaminoglycan receptors on the surface of capillary endothelial cells. The microspheres were formulated to have a controlled cationic character and had a mean diameter of 0.70 μm and a magnetite content of 16% (w/w). Formation of complexes between chitosan and heparin and between the microspheres and heparin has been demonstrated. Heparin served as a model glycosaminoglycan. The chitosan:heparin complex ratio was found to be 1:1 based on charge and was formed between ammonium ions on the chitosan and SO_3^- groups on heparin. Neutralization of the charge on the microspheres prevented their complexation with heparin. The rationale for the use of the delivery system and its potential limitation are discussed.

KEY WORDS: magnetic chitosan microspheres; heparin complex; receptor targeting.

INTRODUCTION

A targeted drug delivery system is most often a drug associated with a macromolecule or particulate carrier, designed to be localized at a specific anatomic region in the target site (1). Recently, there has been a greater focus on utilizing receptor-mediated carriers, such as monoclonal antibodies, that interact with cell surface receptors at the drug's intended site of action (2–4). An apparent limitation of this type of system is that following intravascular administration, a large fraction of the carrier may never reach an extravascularly located target site because of their systemic distribution via the blood circulation. There is nothing inherent in the design of these drug carrier systems that will enable the carrier to be localized in the capillaries of the target site.

An alternate approach is to utilize carriers that have receptors on the capillary endothelial cells. This idea has been recently expressed by Audus and Borchardt (5) with respect to targeting drugs to the brain. These authors suggest the use of carriers that could be transported across the blood–brain barrier by specialized transport processes (i.e., amino acids) or by receptor-mediated transcytosis (i.e., as suggested for insulin).

A novel approach would be to use receptor-mediated magnetic carriers. Under the influence of a suitable magnetic field, the magnetic particles will be stationary, enabling the carrier to bind to capillary endothelial cell receptors. The retained magnetic microspheres may then be taken up by endocytosis and by passage through adjacent endothelial cell gaps in fenestrated or discontinuous capillary beds (6). In continuous capillary beds, i.e., the blood–brain barrier, the receptor-mediated binding of the particle to the capillary wall may create a local concentration gradient as drug is released from the particle. The concentration gradient may lead to increased brain concentrations for drugs permeable to the blood–brain barrier.

The basis for the proposed capillary endothelial receptor-mediated delivery system is to form a complex between the anionic heparin-related glycosaminoglycans, on the luminal surface of the capillary endothelial cells (7–9), and cationic magnetic microspheres. A strong interaction between cationic microspheres and anionic glycosaminoglycan receptors should retain the microspheres in the capillary region.

In this study, cationic magnetic microspheres were prepared from approximately 83% deacetylated chitin, herein referred to as chitosan. Chitin [poly-β-(1 → 4)*N*-acetyl-D-glucosamine] and its deacetylated derivative, chitosan, are naturally occurring polysaccharides being utilized in a variety of biomedical applications (10). Both of these materials have been fabricated into controlled drug release devices (11). The purpose of this investigation was to formulate magnetic chitosan microspheres and to demonstrate their interaction with heparin.

MATERIALS AND METHODS

Chemicals

Chitosan, low-viscosity grade (MW 652,000), was purchased from Proton Laboratories, Redmond, Washington. The following were obtained from Sigma Chemical Co., St. Louis, Missouri: sorbitan sesquioleate (Arlacel 83, S3386), bovine serum albumin (A-7030), methylene blue (MB1), and heparin sodium (from porcine intestinal mucosa, H7005). Ferrofluid (EMG IIII) was purchased from Ferrofluidics Corp., Nashua, New Hampshire. Double-distilled deionized

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water was obtained from a Millipore (Bedford, Massachusetts) system. Extra heavy mineral oil was purchased from Ruger Chemical Co., Irvington, New Jersey. All other chemicals were analytical grade.

Equipment

The ultrasonic water bath was a Bransonic 220; the ultrasonic probe was a Branson sonifier. A Brinkman Rotovapor-R rotary vacuum drier was used. All absorbance data were obtained from a Varian 2200 spectrophotometer. Iron analyses were completed with a Perkin–Elmer 5000 atomic absorption spectrophotometer. A Philips 505 scanning electron microscope was used for particle size analysis.

Preparation of Magnetic Chitosan Microspheres

One hundred fifty milligrams of chitosan was dissolved in 6 ml of 10% (v/v) acetic acid in deionized water. To this solution, 4 ml of acetone was added and the contents were vortexed until a clear solution was obtained. In a 75-ml double-walled beaker, 1 ml of chitosan solution was sonicated with 100 μl of a 10% (w/v) ferrofluid suspension in a water bath for 10 min. To this mixture, 50 ml of mineral oil was added, and an emulsion was formed by sonification for 3 min at 200 W with an ultrasonic probe. The temperature of the emulsion was controlled with a circulating water system maintained at 4°C. One hundred microliters of Arlacel 83 was then added to the emulsion as a stabilizer. The emulsion was then placed in a rotary vacuum drier under nitrogen atmosphere at 70°C for 2 hr to evaporate the acetone and water in the internal phase. Following evaporation, the microsphere suspension was diluted with 60 ml of hexane containing 1% (v/v) Arlacel 83 and centrifuged at 4500 rpm for 15 min. The supernatant was decanted and the microsphere pellet was washed twice in 80 ml of hexanes containing the Arlacel 83. The solid microsphere pellet was then suspended in 15 ml of anhydrous ether and transferred with two 300-G bar magnets in place to a 20-ml centrifuge tube to retain any free magnetite. After centrifugation at 2000 rpm for 5 min, the ether was removed and the microspheres were stored at room temperature.

Particle size analyses were done with a scanning electron microscope. The magnetite content of the microspheres was obtained from atomic absorption spectroscopy after digestion of the microspheres. Approximately 600 μg (accurately weighed) of the microspheres was digested in 1 ml of concentrated hydrochloric acid for 12 hr. The samples were diluted 20 times with deionized water and measured for iron at a wavelength of 248.3 nm.

Chitosan–Heparin Complexation Studies

The chitosan:heparin complex was studied on the basis of the competitive binding displacement method (12), using methylene blue as the competing marker (13).

The following solutions were prepared: 570 μg/ml of methylene blue in deionized water (MB1), 5.7 μg/ml of methylene blue in 1% (v/v) acetic acid in deionized water (MB2), 2.25 mg/ml of heparin in deionized water (H), 2.25 mg/ml of chitosan in 1% (v/v) acetic acid in deionized water (C), and 2.25 mg/ml of bovine serum albumin in deionized water (BSA). The pH of 1% (v/v) acetic acid in deionized water was 3.5.

In stoppered vials, the following solutions were mixed: 1 ml of H and 9 ml of MB2; 0.1 ml of MB1, 7.9 ml of 1% (v/v) acetic acid, 1 ml of H, and 1 ml of C; and 0.1 ml of MB1, 7.9 ml of 1% (v/v) acetic acid, 1 ml of H, and 1 ml of BSA. The visible absorbance spectrum was obtained for each mixture between 520 and 700 nm with 1% (v/v) acetic acid in the reference cell. Spectra of MB1 and 10-fold dilutions of H, C, and BSA in 1% (v/v) acetic acid were also obtained.

The interaction between heparin and the magnetic chitosan microspheres was also studied by the competitive binding displacement method. The following mixture was

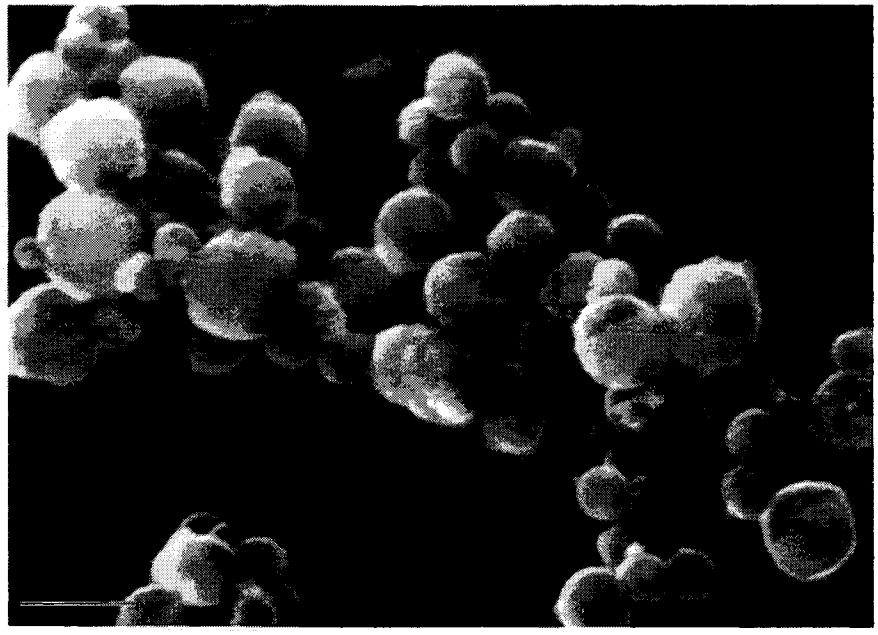

Fig. 1. Photomicrograph of magnetic chitosan microspheres. Bar indicates 1 μm. 25,000 ×; reduced 35% for reproduction.

prepared: 0.1 ml of H, 0.1 ml of MB1, 9.8 ml of deionized water (pH 7), and 4 mg of magnetic chitosan microspheres. Then the absorbance scan was recorded. An exactly analogous mixture was prepared with alkali-treated microspheres obtained after mixing the magnetic chitosan microspheres with 2% (v/v) ammonium hydroxide in acetone for 10 min. This basic treatment was used to neutralize the ammonium ions on the chitosan molecules.

Characterization of the complex ratio was completed as follows. To 0.1 ml of MB1, volume increments of a 0.225 mg/ml heparin solution were added. After each volume addition of heparin, 1% (v/v) acetic acid in deionized water was added to obtain a final volume of 15 ml. The absorbance of the resulting solution was recorded at 660 nm. In a second experiment, a 0.225 mg/ml chitosan solution was added in volume increments to the methylene blue:heparin complex (0.1 ml of MB1 and 1.5 ml of 0.225 mg/ml heparin). The resulting mixture was diluted to 15 ml with 1% (v/v) acetic acid, and the absorbance measured at 660 nm.

Because of the heterogeneity of the heparin polymer, the chitosan: heparin complex ratio calculation was based on a heparin tetrasaccharide with a molecular weight of 1026 (14). The corresponding molecular weight of the chitosan tetramer was 632.

RESULTS

Preparation of Magnetic Chitosan Microspheres

Numerous batches of microspheres were prepared by the above method. It was found that the mean particle diameter was 0.70 ± 0.20 μm (N = 350). The magnetite (Fe_3O_4) content of the microspheres was 16 ± 2% (w/w) (N = 4). Figure 1 is a photomicrograph of the magnetic chitosan microspheres.

Heparin–Chitosan Complexation Studies

Figures 2 through 5 give the absorbance spectra from the complex studies. In Fig. 2, the methylene blue:heparin spectrum was similar to previous studies and indicates the formation of a complex (15). It can be observed that the addition of chitosan to the heparin:methylene blue complex produced the methylene blue spectrum, indicating the formation of a chitosan:heparin complex. Bovine serum albumin (Fig. 3) did not affect the absorbance spectrum of the methylene blue:heparin complex. Similar to the results in Fig. 2, the magnetic chitosan microspheres (see Fig. 4) displaced methylene blue from heparin binding sites, causing the methylene blue spectrum to be regenerated. The methylene blue:heparin spectrum remained the same after the addition of the alkali-treated microspheres and indicated that a complex was not formed between alkali-treated microspheres and heparin.

In Fig. 6, curve 1, the addition of heparin causes the absorbance to decrease, a hypochromic effect, indicating that methylene blue is being bound to heparin. The slower decline in absorbance (second phase of curve 1) most likely represents rearrangement of methylene blue molecules on heparin binding sites. The curve reaches a plateau when all methylene blue is bound to heparin. The addition of chitosan to the methylene blue:heparin complex causes an increase in absorbance (curve 2) consistent with methylene blue's displacement from heparin binding sites. The sharp increase in absorbance represents the complete displacement of methylene blue from heparin binding sites by chitosan. The small increase in absorbance prior to the plateau region of the curve is most likely due to polymer–dye interactions. The chitosan:heparin complex ratio was determined to be 1:1 based on charge.

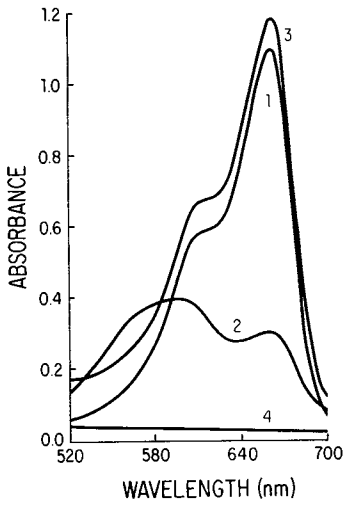

Fig. 2. Absorbance spectra of chitosan–heparin interactions. (1) Methylene blue (5.7 μg/ml); (2) methylene blue (5.1 μg/ml), heparin (0.225 mg/ml); (3) methylene blue (5.7 μg/ml), heparin (0.225 mg/ml), chitosan (0.225 mg/ml); (4) chitosan (0.225 mg/ml).

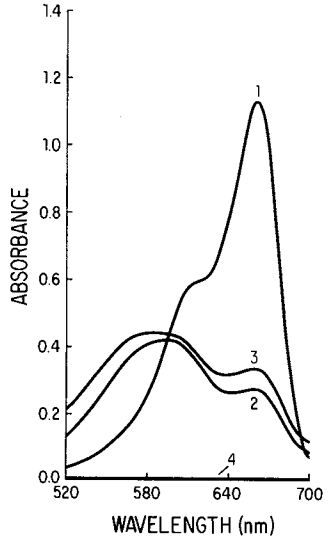

Fig. 3. Absorbance spectra of albumin–heparin interactions. Curves 1 and 2 are the same as in Fig. 2. (3) Methylene blue (5.7 μg/ml), heparin (0.225 mg/ml), bovine serum albumin (0.225 mg/ml); (4) bovine serum albumin (0.225 mg/ml).

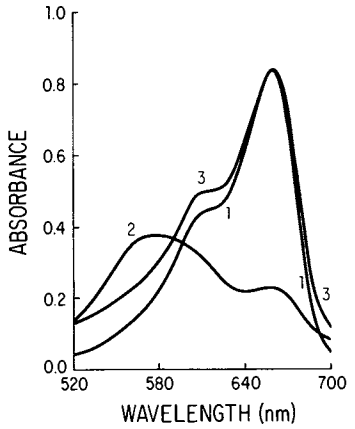

Fig. 4. Absorbance spectra of magnetic chitosan microsphere–heparin interactions. Curves 1 and 2 are the same as in Fig. 2. (3) Methylene blue (5.7 μg/ml), heparin (0.225 mg/ml), magnetic chitosan microspheres (4 mg).

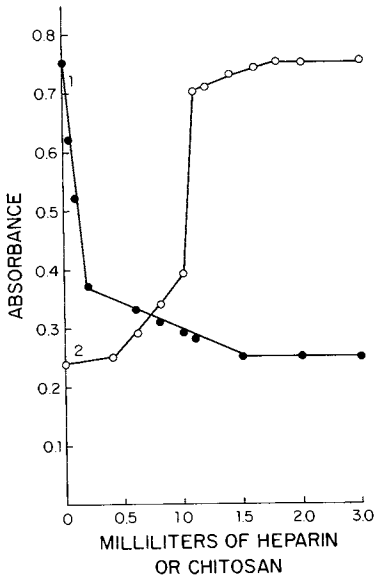

Fig. 6. Chitosan–heparin complex formation. (1) Absorbance following heparin additions to methylene blue. (2) Absorbance following chitosan additions to the saturated methylene blue:heparin complex.

DISCUSSION

The magnetic chitosan microspheres produced were less than 1 μm in diameter, which will enable them to distribute to capillary endothelial cells following intravascular administration. The magnetite content of 16% (w/w) should be sufficient to retain the particles at capillary blood flow rates under the influence of a suitable magnetic field (16).

The method of magnetic chitosan microsphere preparation is similar to techniques used for magnetic albumin microspheres (6). The primary difference is that solid chitosan microspheres are obtained by solvent evaporation rather than by protein denaturation of the emulsion. The nitrogen atmosphere increased both the rate of solvent evaporation and the stability of the microspheres. Arlacel 83 was added to prevent breaking of the emulsion at temperatures above 50°C. The surfactant also increased the recovery of the microspheres by preventing their adhesion to glass surfaces. Approximately 10 mg of microspheres was recovered per batch.

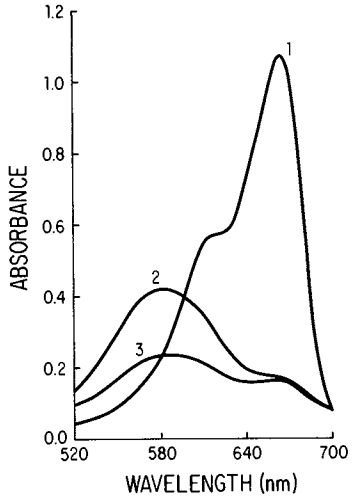

Fig. 5. Absorbance spectra of alkali-treated magnetic chitosan microsphere–heparin interactions. Curves 1 and 2 are the same as in Fig. 2. (3) Methylene blue (5.7 μg/ml), heparin (0.225 mg/ml), alkali-treated magnetic chitosan microspheres (4 mg).

Heparin was used as a structural analogue for the glycosaminoglycans and has been used for this purpose in cell binding studies (17). Since the cationic moiety of methylene blue attacks anionic heparin binding sites (15), the competitive binding displacement method was valid to study the chitosan:heparin complex. A polylysine:heparin complex has been extensively studied (18) and a chitosan:heparin complex has been observed (19); both complexes are indicative of macromolecular ion complexes. The reaction between chitosan and heparin was studied at an acid pH in which chitosan is soluble. The similar spectra obtained from the methylene blue:heparin complex and following the addition of albumin (see Fig. 3) indicate that albumin does not specifically bind to anionic sites on heparin. Thus, albumin microspheres should not interact with glycosaminoglycan receptors at ionic binding sites.

The complex formed between the magnetic chitosan microspheres and heparin (see Fig. 4) was formed in deionized water at pH 7. At this pH, SO_3^- and COO^- groups are present on heparin and will complex with ammonium ions on the microspheres. A physiological pH of 7.4 could not be used since methylene blue is not ionized at pH values above 7, thus preventing the formation of a methylene blue:heparin complex. Chitosan is insoluble at pH 7 but ammonium ions are apparently present due to the association of the acetic acid with the microspheres. Acetic acid is used in the formulation of the microspheres to dissolve the polymer prior

to emulsification and may also serve to control the ionization of the amino group on the chitosan molecule. The association of the acetic acid with the microspheres will provide a local acid environment *in vivo* to facilitate microsphere binding to the glycosaminoglycan receptors. The cationic:anionic nature of the microsphere:heparin complex is supported by the fact that neutralized microspheres did not displace methylene blue from heparin (see Fig. 5). The neutralization procedure will produce ammonium acetate and water and make available free amino groups on chitosan.

It is thought that the ionic and hydrogen bonding forces between the chitosan microspheres and the glycosaminoglycans on the capillary endothelial cells will be sufficient to retain the microspheres in the capillaries. Heparan sulfate proteoglycans are considered to form microdomains on capillary endothelial cell surfaces and are involved in endocytoctic and transcytotic events (20). Heparan sulfate proteoglycans are involved in the receptor-mediated uptake of low-density lipoproteins (21). Thus, binding of chitosan microspheres to glycosaminoglycans should result in endocytosis in peripheral capillary beds. Further, the carrier:receptor complex may trigger the receptor-mediated endocytosis of the glycosaminoglycans. The later process is a normal turnover mechanism for damaged or aged glycosaminoglycans on the cell surface (22,23). The complex of polylysine:heparin is taken up at a greater rate than the unassociated species into hamster ovarian cells (24).

Potential limitations of magnetic chitosan microspheres *in vivo* include blood cell agglutination and carrier opsonization. Since all blood cells carry net negative charges, and agglutination of red blood cells by polycations has been demonstrated (25), interactions of these cells with chitosan microspheres may be expected. Opsonization may interfere with the microsphere–endothelial cell surface interaction. However, Wilkins and Myers (26) have suggested that opsonization of charged particles may not change the particle's surface potential. The potential deleterious effects of these processes have not yet been investigated for this carrier system.

A strategy for targeting receptor-mediated magnetic carriers has been presented. Through both biochemical and physical means of particle retention, advantageous target tissue drug concentrations are anticipated. Several studies are required to quantitate the microsphere–cell surface interactions and to substantiate the rationale presented.

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