Synthesis of *N*-(aminoalkyl)chitosan for Microcapsules

E. J. DUNN,[1] X. ZHANG,[1] D. SUN,[2] and M. F. A. GOOSEN[1,*]

[1]Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada K7L 3N6; [2]Department of Applied Chemistry, Tianjin University, Tianjin, People's Republic of China

SYNOPSIS

Chitosan was chemically modified by alkylation with *N*-(2-bromoethyl)phthalimide, *N*-(3-bromopropyl)phthalimide, and *N*-(4-bromobutyl)phthalimide. The resulting *N*-(phthalidimidoalkyl)chitosans were treated with hydrazine to remove the phthalidimido group resulting in the final *N*-(aminoalkyl)chitosan products. For comparison purposes, poly(vinyl alcohol) (PVA) was alkylated with *N*-(3-bromopropyl)phthalimide, then treated with hydrazine to give the *N*-(3-aminopropyl)PVA product. All alkylation products were characterized by solution ^{1}H- and ^{13}C-NMR and by solid-state CP-MAS ^{13}C-NMR. The above synthetic polymer derivatives, as well as chitosan, polyallyl amine, and polyethylenimine, were used to form membrane coatings around calcium alginate beads in which blue dextran of molecular weight 7.08×10^4 or 26.6×10^4 was entrapped. These microcapsules were prepared by extrusion of a solution of blue dextran in sodium alginate into a solution containing calcium chloride and the membrane polymer. Membrane integrity and permeability were assessed by measuring the elution of the blue dextran from the capsules, spectrophotometrically. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Microcapsules can be used as devices for the controlled release of drugs, vaccines, antibiotics, and hormones. Bioactive agents may be entrapped within cores of anionic polymers such as alginate, carrageenin, carboxymethyl cellulose, and poly(acrylic acid). Similarly, membranes can be formed using various cationic polymers such as chitosan, poly-L-lysine, polyethylenimine, and poly(allyl amine).[1-7] The feasibility of employing chitosan microcapsules, or microspheres, for the oral administration of vaccines has previously been examined in our laboratory.[1] Three parameters were believed to strongly affect membrane formation. These were molecular weight of the polymer, type of reactive group on the polymer, and distance of this group from the polymer main chain.

The most promising encapsulation system yet developed appears to be the encapsulation of calcium alginate beads with poly-L-lysine. However, the use of this system on a large scale, such as for oral vaccination of animals, is not feasible due to the high cost (i.e., \$200/g) of poly-L-lysine (PLL). It would therefore be desirable to develop an economic and reliable microencapsulation system based on chitosan and alginate.

The better membrane-forming properties of PLL over chitosan were explained as follows: PLL contains a number of long-chain alkylamino groups that extend from the polyamide backbone. These chains may extend in a number of directions and interact with various different alginate molecules, through electrostatic interactions, resulting in a highly cross-linked membrane. Chitosan, on the other hand, has amino groups that are very close to the polysaccharide backbone. Interaction between the charged amino groups of chitosan and carboxylate groups of alginate may be lessened due to steric repulsions between the two molecules.

McKnight et al.[1] attempted to mimic the properties of PLL by extending the length of the cationic spacer arm on the chitosan main chain. In the chemical modification, chitosan was first reacted with α-bromoactyl bromide followed by reaction with an amine. The synthesis was adapted from a procedure by Tam et al.[8] for coupling hemoglobin

* To whom correspondence should be addressed.

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to dextran. The major problem reported in this procedure[8] was the competing hydrolysis reaction of the bromoacyl bromide. Furthermore, the lack of characterization of the modified chitosan in McKnight's study caused ambiguity on the effectiveness of the chitosan modification. No significant difference was found in membrane properties between modified and unmodified chitosan.

A two-step synthetic method for attaching long aminoalkyl side chains to chitosan is represented in Figure 1. This method is collectively called the Gabriel synthesis.[9] The approach outlined in Figure 1 is designed to attach flexible alkylamine side chains to the chitosan polysaccharide backbone, possibly simulating the behavior of PLL. The presence of two amino groups in this side chain may even enhance membrane-forming properties. Chemical modification of poly(vinyl alcohol) (PVA) by a similar procedure may also produce a polyamine with membrane-forming properties similar to that of PLL.

The objectives of our research were to examine various polymers for use as membrane coatings; to develop a synthetic approach to the chemical modification of the polymers, chitosan and PVA, to simulate the structure of PLL; and to identify analytical techniques that may be used to study the effects that chemical modification of polymers such as chitosan have on the physical structure and the membrane properties of the polymer.

EXPERIMENTAL

Materials

Low-viscosity (< 200 mPa s) chitosan (Sea Cure+ TM) was obtained from Protan Laboratories, Redmond, WA. Chitin [poly(*N*-acetylglucosamine], poly(vinyl alcohol) (PVA: Type II: low molecular weight), dextrans of average molecular weight 70.8, 155, and 266 kD, blue dextran (MW 2,000,000), Basilen Blue E-3G, and trichloroacetic acid (100%

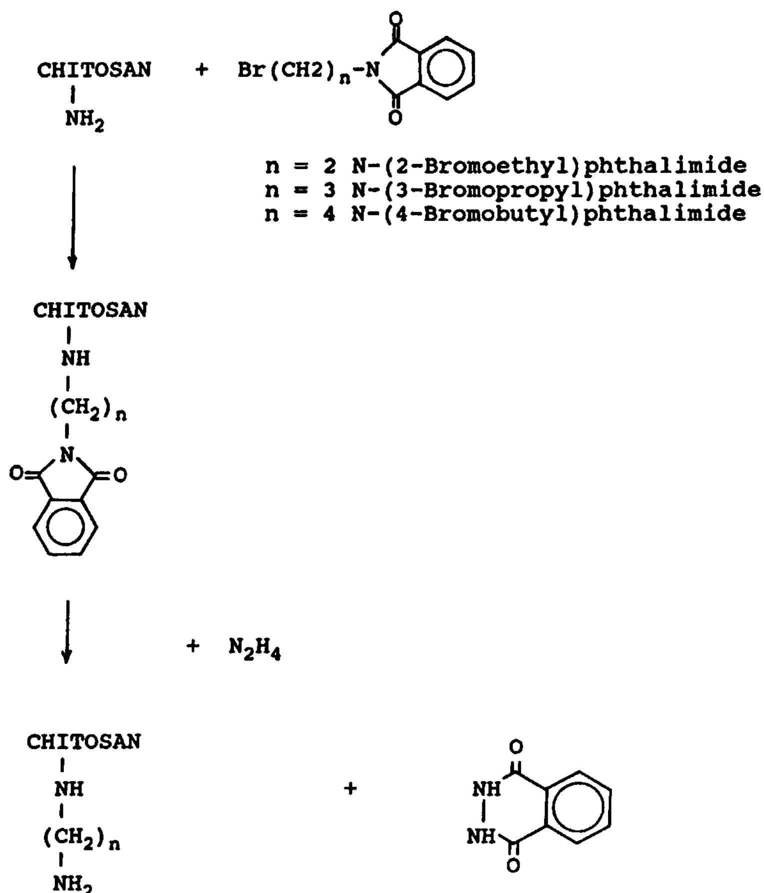

Figure 1 The Gabriel synthesis: Modification of chitosan with bromoalkylphthalimides and hydrazine.

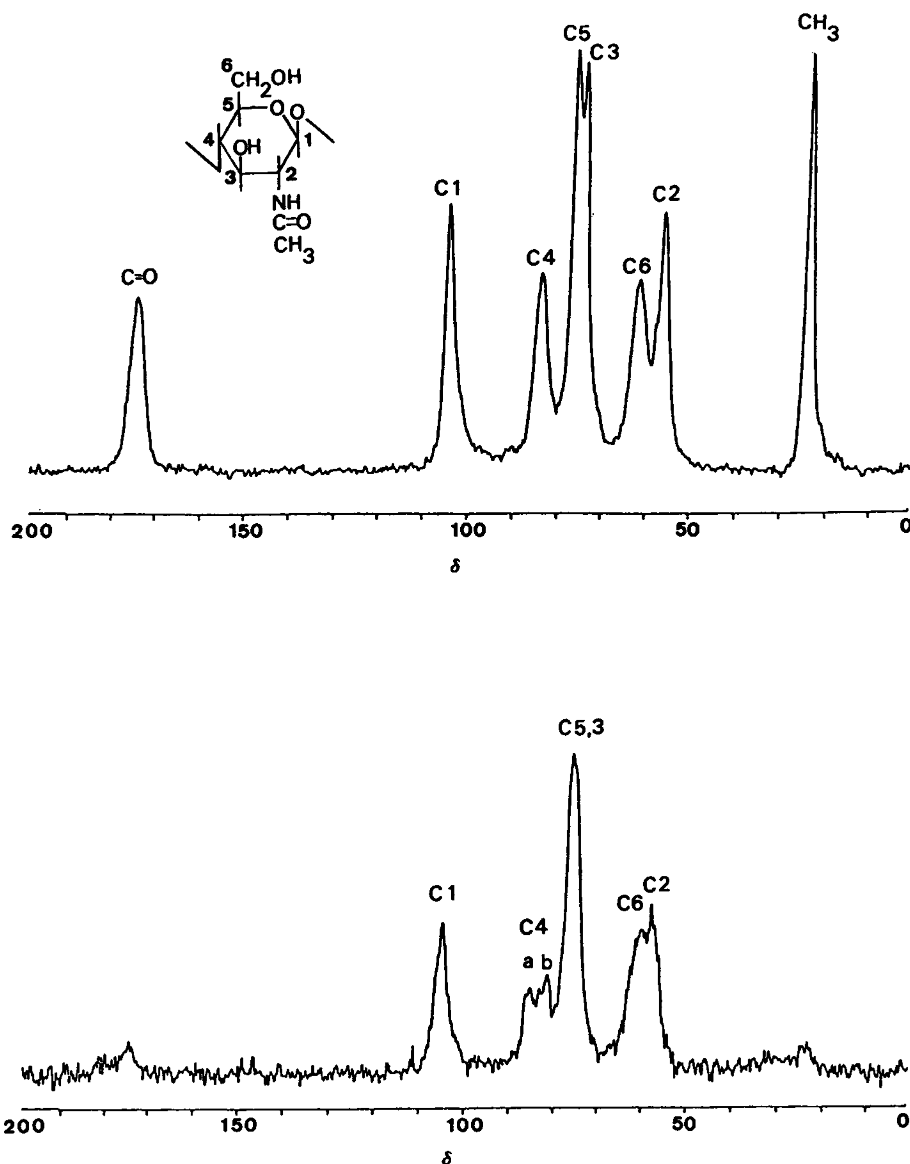

Figure 2 CP-MAS ^{13}C-NMR spectrums of (top) chitin and (bottom) chitosan.

w/v) were purchased from Sigma Chemical Co., St. Louis, MO. Poly(allyl amine) (PAAm) of high and low molecular weight, hydrazine (35 wt % in H_2O), *N*-(2-bromoethyl)phthalimide, *N*-(3-bromopropyl)phthalimide, *N*-(4-bromobutyl)phthalimide, and a 50 wt % solution of polyethylenimine (PEI) in H_2O were purchased from Aldrich Chemical Co., Milwaukee, WI. Dimethyl-d_6 sulfoxide (DMSO-d_6: 99.9 atom % D) and deuterium oxide (D_2O: 99.9 atom % D) were from MSD Isotopes, Montreal, Canada. Algin (Keltone® LV: Kelco, Chicago, IL) was also used. All other commercially available chemicals were reagent grade and used without further purification unless otherwise noted.

Synthesis of Modified Chitosans

Three different methods were investigated in an attempt to prepare the desired substituted chitosan derivatives.

Synthesis of (3-Aminopropyl)chitosan in DMSO

A suspension was prepared of 1.5 g of chitosan (0.0094 mol) in 40 mL of dimethyl sulfoxide (DMSO). To this suspension was added a total of 8.73 g of *N*-(3-bromopropyl)phthalimide (0.0326 mol) and 5 mL of NaOH (40% w/v). These final amounts give approximately a 3.5-fold excess of phthalimide to amine residue assuming 100% de-

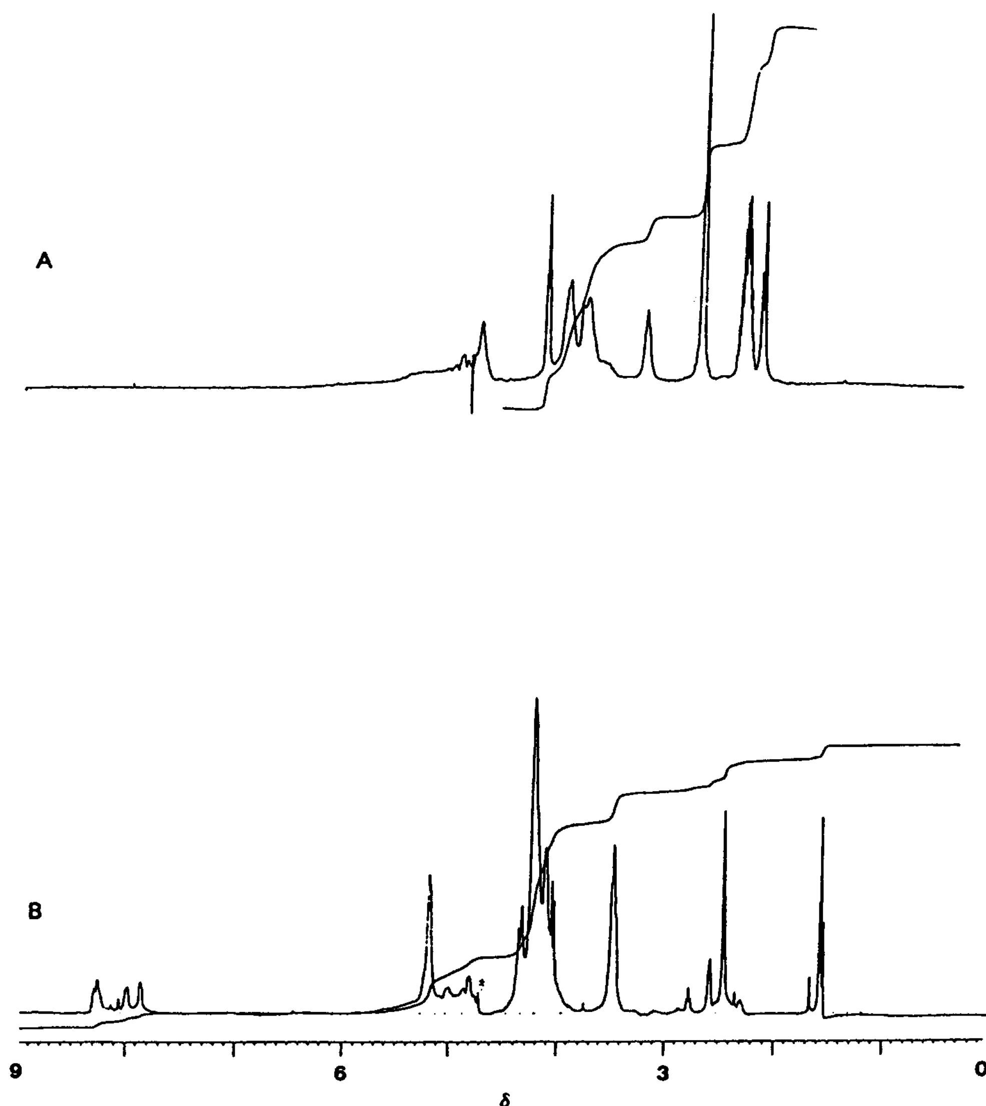

Figure 3 ^{1}H-NMR spectrums of (A) chitosan and (B) *N*-(3-phthalimidopropyl)chitosan (1% TCA in D_2O) from the DMSO method. Note: δ is in ppm.

acetylation of the chitosan, which may occur under these basic conditions. The phthalimide and NaOH were added in three approximately equal portions over a period of 24 h. After the final addition, the solution was stirred overnight. Approximately 100 mL of acetone was added to the mixture and the product was filtered and washed with acetone. A light brown solid (1.19 g) was recovered.

Synthesis of N-*(Phthalimidoalkyl)chitosans via Alkali–Chitosan*

To 40 mL of a 50% w/v solution of NaOH was added 2.0 g of chitosan. The suspension was stirred overnight and then divided equally into four flasks (10 mL each: 0.0031 mol). To each of the flasks were added 3.0 g of 2-bromoethylphthalimide (0.012 mol), 3-bromopropylphthalimide (0.011 mol), or 4-bromobutylphthalimide (0.011 mol). To each flask, ca. 100 mL of distilled water was also added and the solutions stirred for 48 h. After stirring, some of the water was removed by rotary evaporation and the products precipitated with acetone. The light brown solid products were recovered by filtration and washing with acetone to give the following yields: chitosan, 0.20 g; *N*-(2-phthalimidoethyl)chitosan, 0.20 g; *N*-(3-phthalimidopropyl)chitosan, 0.16 g; and *N*-(4-phthalimidobutyl)chitosan, 0.17 g.

Synthesis of N-(Phthalimidoalkyl)chitosans in Acetone/H_2O Mixture

The synthesis of N-(2-phthalimidoethyl)chitosan, N-(3-phthalimidopropyl)chitosan, and N-(4-phthalimidobutyl)chitosan followed the same general procedure. The details of the method are described below for the reaction of chitosan with 4-bromobutylphthalimide.

Chitosan (0.61 g, 0.0038 mol) was dissolved in 50 mL of 4% acetic acid solution. To this solution was added 2.683 g (0.0095 mol) N-(4-bromobutyl)phthalimide dissolved in 10 mL acetone. Upon addition, a white precipitate formed. Sufficient acetone (ca. 10 mL) was added to dissolve the precipitate. Sodium hydroxide (35 mL of 10% w/v) was then added slowly with stirring, resulting in a cloudy brown solution with pH 12. The solution was stirred overnight. A further 1.606 g (0.0057 mol) of phthalimide dissolved in 20 mL acetone was added and left to stir for 48 h. The product (0.466 g) was then filtered and washed with absolute ethanol and then acetone.

Hydrazinolysis of N-(Phthalimidoalkyl)chitosans

The N-(phthalimidoalkyl)chitosans prepared in the last section were dissolved in dilute acetic acid so-

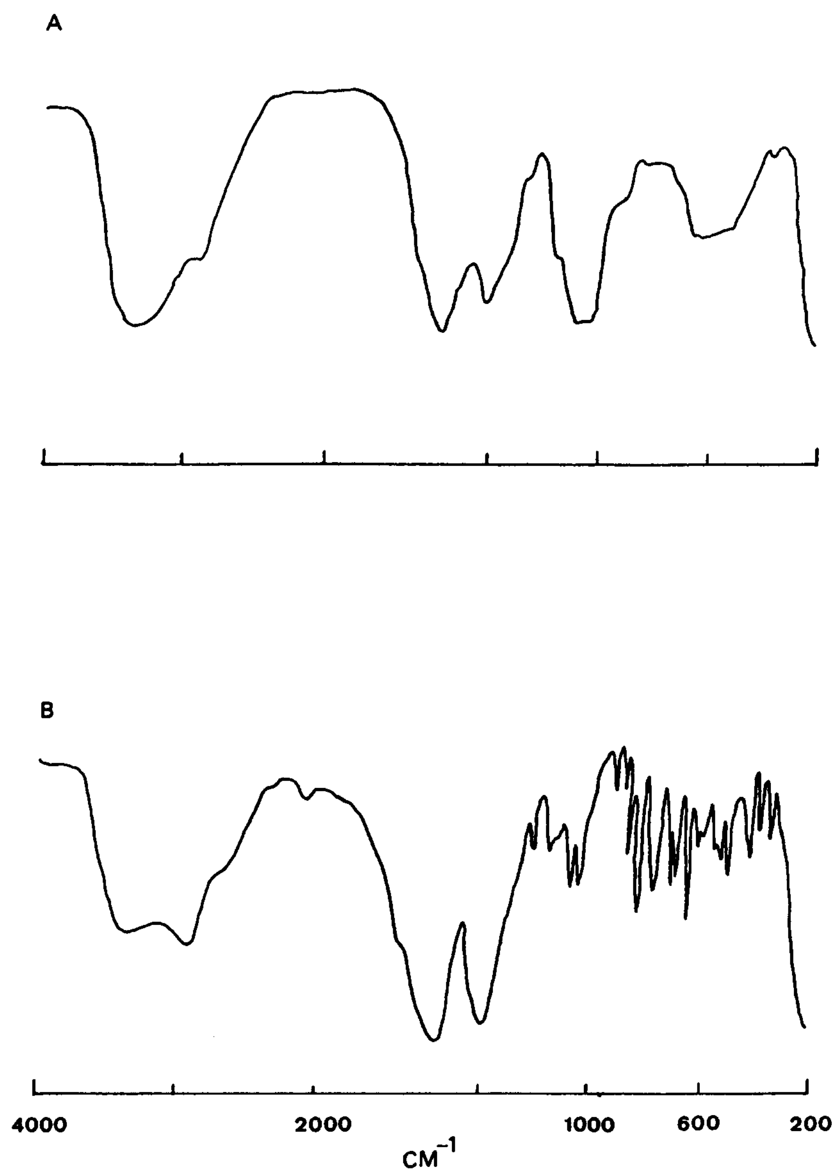

Figure 4 IR spectrums of (A) chitosan and (B) N-(3-phthalimidopropyl)chitosan (KBr disk) from the DMSO method.

lution (ca. 20 mL). Once dissolved, 5 mL of 35% (w/v) hydrazine solution in water were added. The solutions were left to stir overnight. The products were recovered by precipitation with acetone followed by filtration and washings with ethanol and acetone.

Synthesis of N-(*Phthalimidopropyl*)*PVA*

The chemical modification of low molecular weight PVA to produce a novel amine-containing polymer was also attempted. The synthetic procedure used

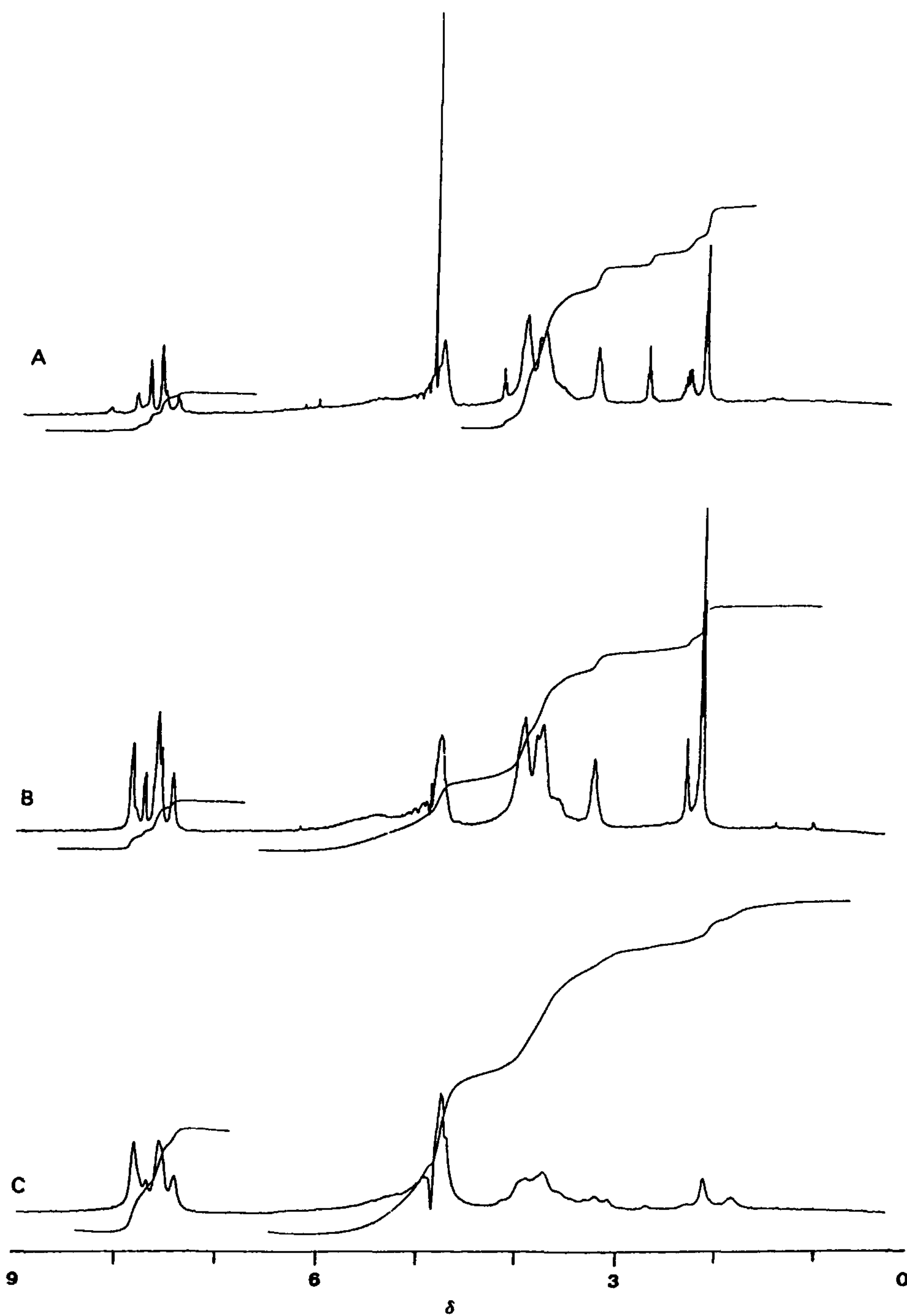

Figure 5 ^{1}H-NMR spectrums of (A) *N*-(2-phthalimidoethyl)chitosan, (B) *N*-(3-phthalimidopropyl)chitosan, and (C) *N*-(4-phthalimidobutyl)chitosan. (1% TCA in D_2O) from the acetone/H_2O method.

was similar to that described for derivatization of chitosan.

PVA (1.04 g, 0.024 mol) was dissolved in 230 mL distilled water. A total of 8.0 g (0.030 mol) of n-(3-bromopropyl)phthalimide in 250 mL acetone were added in portions over a 4 h period and the final solution stirred overnight. The volume of the mixture was reduced to ca. 50 mL rotary evaporator and an equal volume of acetone added to precipitate the product. Some of the resulting solid (5.0 g) was redissolved in a minimal amount of water and the insoluble material filtered and washed with acetone. The product (1.0 g) was then dissolved in 20 mL $DMSO/H_2O$ (50% v/v) and 10 mL N_2H_4 (35% w/v) was added and stirred 48 h. The product (0.5 g) was precipitated with 300 mL acetone, filtered, and washed.

Synthesis of Blue Dextrans

Blue dextrans of average molecular weight 70.8, 150, and 266 kD were prepared as described by Ashton and Polya.[10] The degree of substitution of each of the blue dextrans was determined by UV-vis spectrophotometry.

Polymer Characterization

Infrared spectra were recorded on a Perkin-Elmer 598 spectrometer. The solid samples were prepared as KBr disks. Nuclear magnetic resonance spectra (^1H: 400 MHz; ^{13}C: 100 MHz) were recorded either on a Bruker AM 400 MHz spectrometer (solution samples in DMSO-d_6 or D_2O containing 1–2% trichloroacetic acid) or on a Bruker CXP 200 MHz spectrometer (solid samples). UV-visible spectra were recorded on a Phillips UV 8720 scanning spectrophotometer.

Microcapsule Preparation

Solutions of sodium alginate (1.5% w/v) were prepared by dissolving 0.75 g algin in 50 mL of distilled water. Chitosan solutions (0.1% w/v) were prepared by dissolving 0.05 g chitosan in 50 mL acetic acid (1% w/v) containing 1.1 g $CaCl_2 \cdot 2H_2O$. The pH of this solution was adjusted to 6.0 by addition of 10% NaOH. Solutions of modified chitosans and polyallyl amines were prepared in the same way. Solutions of polyethylenimine were prepared by dissolving 0.1 g of a 50% w/w solution of PEI in the acetic acid–calcium chloride mixture.

Blue dextran (0.5 g) of average molecular weight 70.8 or 266 kD was added to 50 mL of 1.5% w/v algin. Capsules were prepared by extruding 3 mL of alginate solution into 15 mL of cationic polymer (chitosan, PEI, PAAm) solution through a syringe needle, jacketed by an air-jet. Conditions such as pumping rate and air flow were adjusted such that microcapsules of 0.5–1.0 mm diameter were formed. After all the alginate had been extruded, microcapsules were transferred to 50 mL centrifuge tubes and allowed to settle. The liquid was removed from the capsules by decanting and the remaining capsules rinsed twice with distilled water (40 mL), decanting between washings. After decanting the second water rinse, two acetone rinses (30 mL) were performed to dehydrate and solidify the capsules. After the final acetone rinse, the beads were transferred to a glass sample vial and allowed to air-dry in a desiccator.

Assessment of Membrane Integrity: Diffusion of Blue Dextran from Microcapsules

Approximately 20 mg of dried microcapsules containing either blue dextran or BSA were weighed into a 1 cm UV cuvette (disposable acrylic). To this, 2 mL of buffer solution (pH 6) was added and the solution shaken. Blue dextran elution was followed by measuring absorption at 620 nm as a function of time. The cuvette was shaken periodically between measurements. At the completion of sampling, an aliquot (500 μL) of sodium citrate (2M) was added to release the remaining blue dextran and a final absorbance reading taken. The final concentration was corrected taking into account the dilution.

Table I Chemical Shifts (δ) and Assignments of Solution ^{13}C-NMR Spectra Signals for N-(2-Phthalimidoethyl)chitosan (2PEC), N-(3-Phthalimidopropyl)chitosan (3PPC), and N-(4-Phthalimidobutyl)chitosan (4 PBC)

	δ (ppm)		
2PEC	3PPC	4PBC	Assignment
174.3	174.9	174.5	Acetyl carbonyl
140.0	135.0	136.0	Phthalimide group
129.3	130.8	131.0	Phthalimide group
105.4	104.8	102.1	C-1
83.5	82.9	85.0	C-4
76.0	75.3	75.5	C-5, C-3
58.8	58.8	59.2	C-6, C-2
30.0	30.0	44.4	Alkyl chain
23.5	23.8	23.7	Acetyl methyl

RESULTS AND DISCUSSION

Characterization of Chitosan

The solid-state CP-MAS ^{13}C-NMR of chitin and chitosan are shown in Figure 2. The NMR spectrum of chitosan indicates that the chitosan is not completely deacetylated. Also, the resolution between the C-5 and C-3 as well as the C-6 and C-2 in chitosan is poorer in the chitosan spectrum compared to that of chitin. An interesting feature of the chitosan spectrum is the two signals observed for the

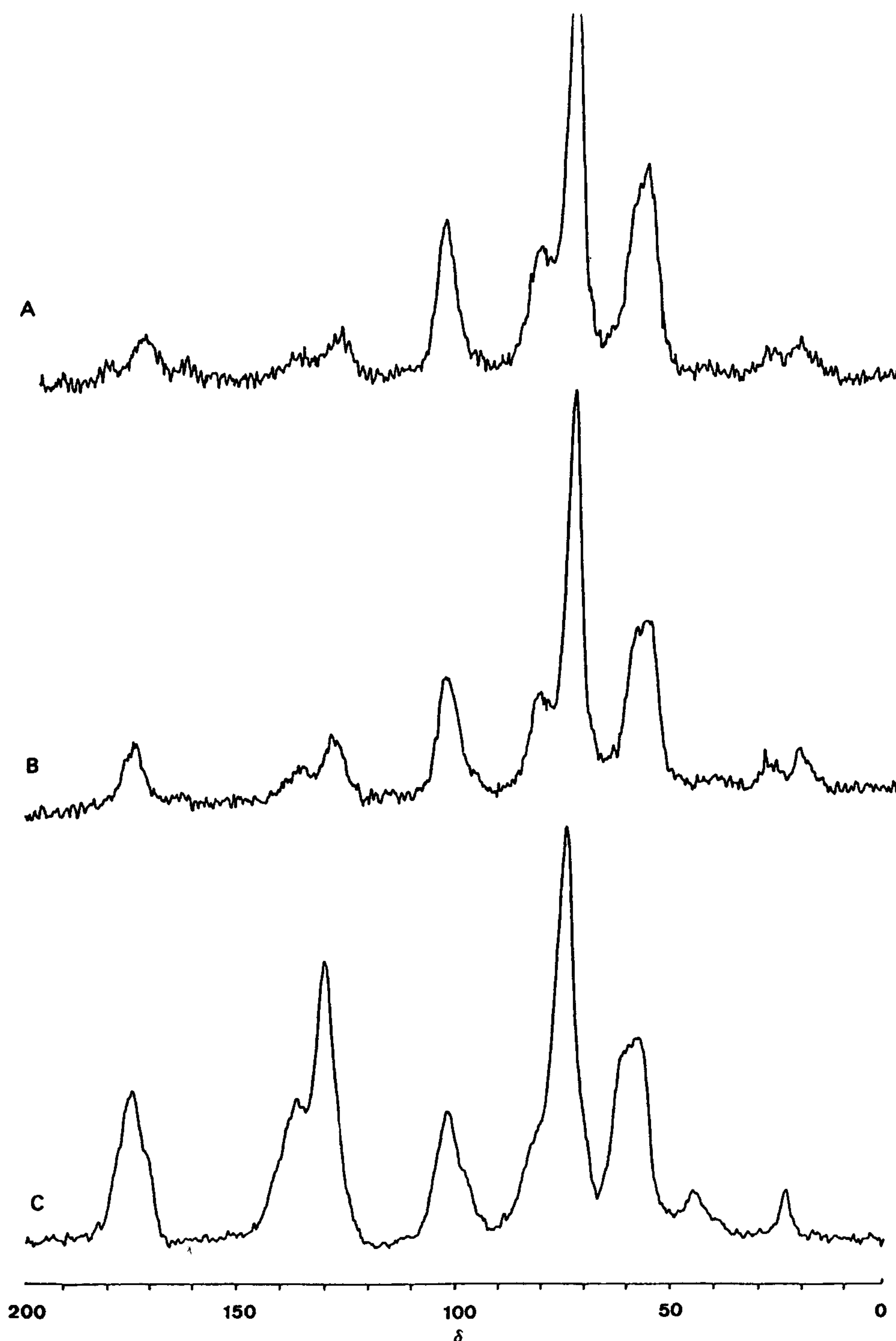

Figure 6 CP-MAS ^{13}C-NMR spectrums of (A) *N*-(2-phthalimidoethyl)chitosan, (B) *N*-(3-phthalimidopropyl)chitosan, and (C) *N*-(4-phthalimidobutyl)chitosan from the acetone/H_2O method.

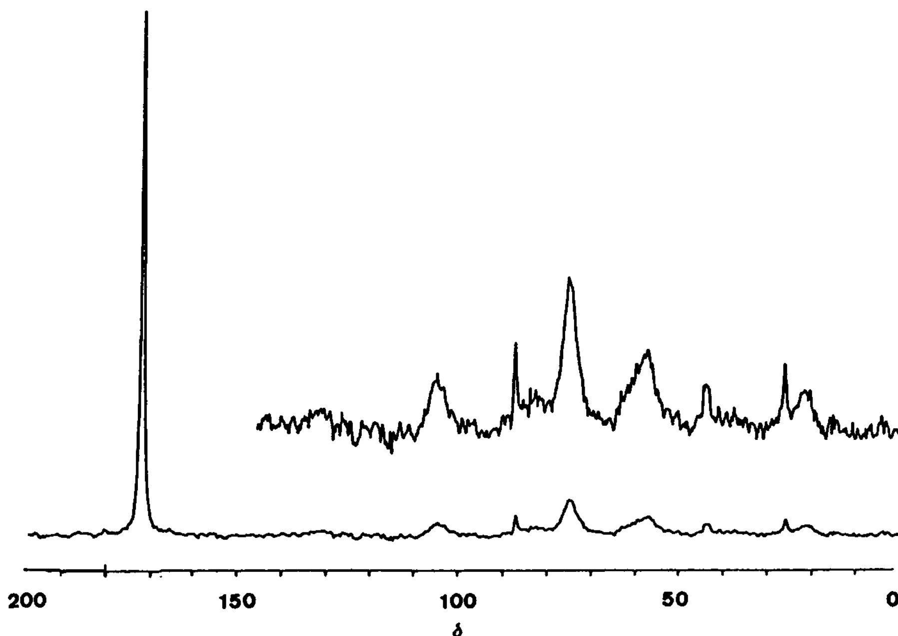

Figure 7 CP-MAS ^{13}C-NMR spectrum of *N*-(2-aminoethyl)chitosan.

C-4 carbon. This has been ascribed to the presence of two polymorphic forms of chitosan.[11] When chitosan is heated at 220°C in the presence of water (annealed), the two signals coalesce to give one signal. The annealed form is believed to be similar to that of chitin and native cellulose, both of which have extended helical conformations.

Synthesis and Characterization of *N*-(Phthalimidopropyl)chitosan from the DMSO Method

The ^{1}H-NMR spectrum of the isolated product is given in Figure 3 and the IR spectrum is given in Figure 4. It can be seen, in the former figure, that there are additional resonance peaks in the region from 1 to 6 ppm, as compared to that of chitosan. Detailed assignment of all peaks was not attempted since this would involve complicated NMR decoupling experiments, which are costly and time-consuming and would not add a great deal to this discussion. However, one feature of this spectrum that is of great importance is the appearance of new resonance signals in the region of 8.0 ppm. This region is typical for aromatic proton resonances and would indicate the presence of the phthalimide moiety in the compound. For example, the chemical shift of the phthalimido group of *N*-(2-benzamidooxyethyl)-, *N*-(3-benzamidooxypropyl)-, and *N*-(4-benzamidooxybutyl)phthalimides is reported at 7.4 ppm in $CDCl_3$ solvent.[12] Thus, the presence of signals in this region would indicate that the alkyphthalimide group has been successfully attached to the chitosan polymer backbone.

The relatively low intensity of these signals would suggest that either hydrolysis of the phthalimide group has occurred under the reaction conditions and/or the degree of substitution of the modified chitosan is low. The ^{13}C-NMR spectrum of this solution was not observed due to the extremely low concentration of dissolved chitosan and the low natural abundance of the ^{13}C nuclei.

Although this synthetic method would appear to have limited success, there are still a number of problems associated with it. The major problem is the insolubility of chitosan in DMSO. The reaction mixture is heterogeneous and will be limited by the availability of reactive groups on the chitosan and the permeability of the polymer to the alkylating agent.

Synthesis and Characterization of *N*-(Phthalimidoalkyl)chitosan via Alkali–Chitosan

In this procedure, it was thought that pretreating the chitosan in concentrated sodium hydroxide solution would swell the polymer and facilitate diffusion and reaction of the bromoalkylphthalimides. However, the ^{1}H-NMR spectra for the *N*-(phthalimidoalkyl)chitosan showed very weak or no signals around 8.0 ppm. This would indicate that the alkylation did not occur to any great extent. Similarly, it was observed from the ^{13}C-NMR spectra of *N*-(3-phthalimidopropyl- and *N*-(4-phthalimidobu-

tyl)chitosan (data not shown) that there were no additional signals, above the background noise, that would indicate that a reaction had occurred.

This method proved to be less successful than the DMSO method, again primarily due to the insolubility of the reagents. Chitosan is not soluble in very basic aqueous solutions. As well, the bromoalkylphthalimides are not very soluble in water. It is likely, therefore, that hydrolysis of the bromoalkylphthalimides occurs more readily than does the alkylation reaction of the chitosan.

Synthesis and Characterization of *N*-(Phthalimidoalkyl)chitosan from Acetone/H_2O

The major problem cited in the previous two methods was poor solubility. In an attempt to overcome this problem, chitosan was first dissolved in an acetic acid solution and the bromoalkylphthalimides added as solutions in acetone. The acetone/water mixture and the pH of the solution was adjusted to maximize the amount of both reagents in solution at one time.

The ^{1}H-NMR spectra of the substituted chitosan are given in Figure 5(A)-(C). The presence of signals around 8.0 ppm indicates the presence of the phthalimide group in all the samples. Also, from the relative intensities of the peaks, it would appear that a high degree of substitution was achieved.

Table I contains the chemical shift data and the assignments for the CP-MAS ^{13}C-NMR spectra of the (phthalimidoalkyl)chitosans [Fig. 6(A)-(C)]. Again, the presence of new signals in the CP-MAS ^{13}C-NMR at 30–45 and 130–140 ppm indicate that alkylation of the chitosan has occurred.

Characterization of *N*-(2-Aminoethyl)chitosan

The *N*-(phthalimidoalkyl)chitosans described above were treated with hydrazine (35% w/v in H_2O) to remove the phthalimide group and generate the free aminoalkyl chitosans. This step proved to be more difficult than anticipated due to the apparently decreased solubility of the (phthalimidoalkyl) chitosans compared with the original chitosan. This may be due to the attachment of the hydrophobic side chain.

The solid-state ^{13}C-NMR spectrum of *N*-(2-aminoethyl)chitosan isolated from the hydrazinolysis of *N*-(2-phthalimidoethyl)chitosan is given in Figure 7. The NMR chemical shifts of the resonances and their assignments are given in Table II.

Table II Chemical Shifts (δ) and Assignments of Solution ^{13}C-NMR Spectra Signals for *N*-(2-Aminoethyl)chitosan

δ (ppm)	Assignment
172.2	Acetyl carbonyl
105.0	C-1
87.5	C-4
75.7	C-5, C-3
57.4	C-2, C-6
44.1, 26.4	$—NHCH_2CH_2NH_2$
21.7	Acetyl methyl

The interesting feature of this spectrum is the absence of signals at 140 and 129.3 ppm as seen in Figure 6 for *N*-(2-phthalimidoethyl)chitosan. The disappearance of these signals for the hydrazine-treated product would indicate cleavage of the phthalimido group from the alkyl side chain. The presence of the aminoalkyl side chain attached to the chitosan backbone is indicated by the signal at 44.1 ppm. This corresponds to the reported chemical shift of the carbons in diaminoethane ($H_2N—CH_2—CH_2—NH_2$) of 44.3 ppm.[13]

Synthesis and Characterization of *N*-(3-phthalimidopropyl)PVA

The ^{1}H-NMR spectrums of PVA and its reacted product are shown in Figure 8(A) and (B), respectively. The spectrum shows the presence of signals around 8.0 ppm, which would indicate the presence of the phthalimide group. Therefore, it would appear that alkylation of PVA with 3-bromopropylphthalimide is possible. The ^{13}C-NMR was not obtained.

Assessment of Capsule Integrity: Diffusion of Blue Dextran out of the Capsule

The diffusion of blue dextran out of alginate beads with and without membrane coatings was studied spectrophotometrically by observing the increase in absorbance at 620 nm due to the appearance of blue dextran in the extracapsular fluid as a function of time (Table III).

Comparison of the initial blue dextran in capsule values indicates that capsules with PAAm (low and high MW), PEI, and, to a lesser extent, chitosan have higher initial concentrations of blue dextran than do capsules with no membrane. The lower initial concentration of blue dextran means more blue dextran was lost during microcapsule preparation. Since different amounts of blue dextran were entrapped in each capsule during the encapsulation

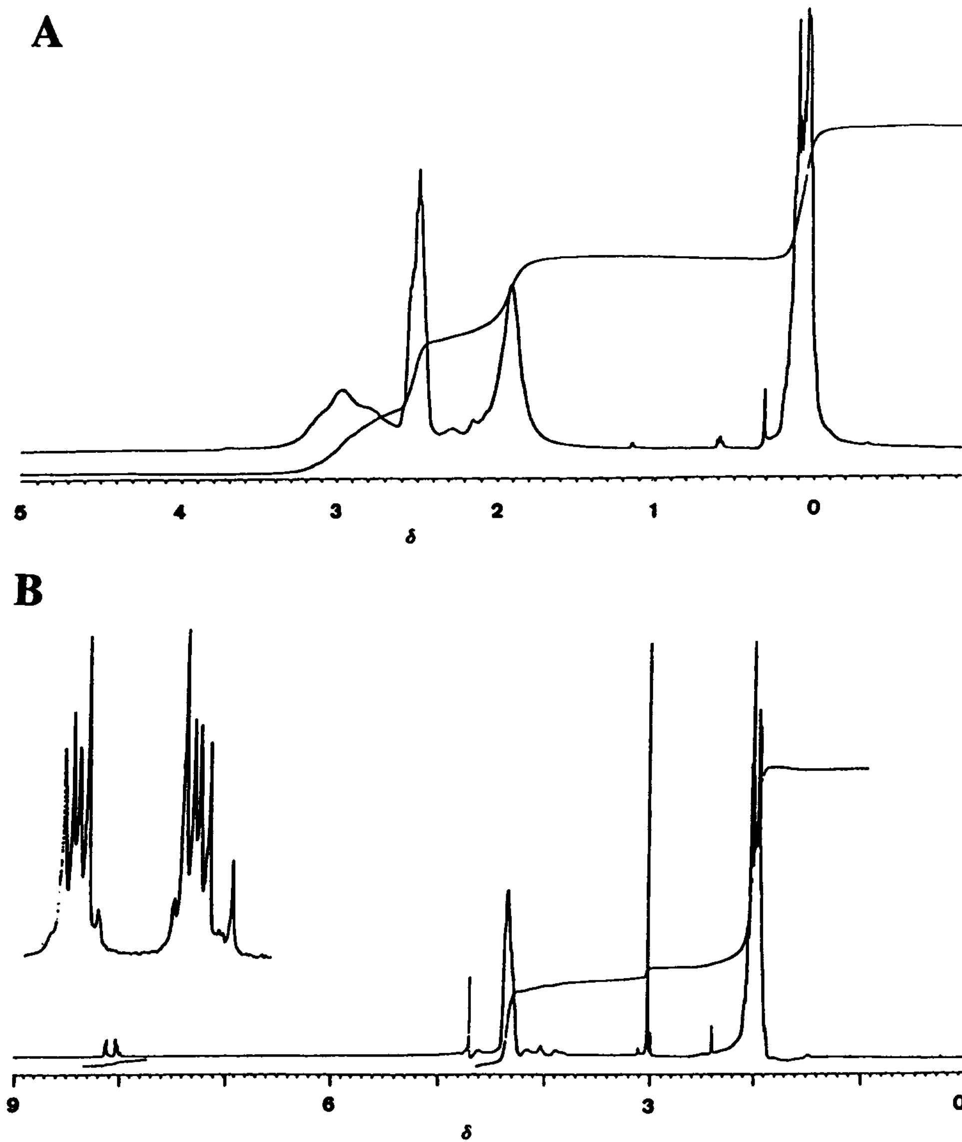

Figure 8 ^{1}H-NMR spectrums of (A) PVA and (B) N-(3-phthalimidopropyl)PVA.

procedure, the initial concentration of dextran within the capsules, and, hence, the diffusion driving force, was not the same in each case. Thus, direct measurement of diffusion rates and comparison between each system was not possible. However, based on the approximate time corresponding to 50% of the final measured absorbance before citration, the 266 kD dextran appeared to diffuse much more slowly out of the capsules than did the 70.8 kD dextran. This is reasonable due to the larger size of the 266 kD dextran. Also, release of both 70.8 and 266 kD dextrans appears to occur fastest in alginate capsules that have no membrane coating. Thus, alginate capsules with membrane coatings seem to delay the release of both molecular weight dextrans from the alginate core. Capsules with a PEI membrane coating had a significant fraction of 70.8 kD blue dextran that was unable to diffuse from the capsule. Similarly, capsules with chitosan, PAAm (high and low MW), and PEI membranes contained a considerable amount of 266 kD dextran that was only released after citration.

Finally, the comparable time scale for 50% of the blue dextran being released from alginate capsules with chitosan, modified chitosans, and modified PVA membranes suggests that there was no beneficial effect of the chemical modifications of chitosan on the permeability of the capsule membrane.

Table III The Release Properties of Microcapsules

	Mol. Wt. of Blue Dextran												
	266 kD								70.8 kD				
Membrane Material	None	PAAm Low MW	PAAm High MW	PEI	Chitosan	2AMET CH[a]	3AMPR CH[b]	3AMPR PVA[c]	None	PAAm Low MW	PAAm High MW	PEI	Chitosan
Release Properties													
Original blue dextran content of capsules, (% wt)	5.2	39.9	30.3	32.4	24.1	11.5	15.7	6.9	7.0	31.1	18.8	19.3	13.2
Releasable fraction of blue dextran[d]	0.86	0.70	0.61	0.236	0.344	0.789	0.690	0.690	1.0	1.0	1.0	0.5	1.0
Time required for one-half of the releasable fraction to come out of the capsules (min)	68	63	109	135	147	171	205	146	35	70	100	132	65

[a] *N*-(2-aminoethyl)chitosan.
[b] *N*-(3-aminopropyl)chitosan.
[c] *N*-(3-aminopropyl)PVA.
[d] Capsules citrated to allow all of blue dextran to elute from capsules.

CONCLUSIONS

The aminoalkylation of chitosan and PVA was accomplished in a simple two-step process, the first step being the alkylation of the polymer with *N*-bromoalkylphthalimides under alkaline conditions followed, subsequently, by the removal of the protective phthalimido group by treatment with hydrazine to generate the free (aminoalkyl) chitosans and (aminoalkyl) PVA. These modified polymers were able to form membrane coatings on alginate beads. In permeability studies with blue dextran, however, the capsules prepared with the modified polymers did not behave differently from capsules prepared with unmodified polymers.

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