Interactions of Amidated Acids with Heparin

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ABSTRACT: Raman and NMR studies are performed to characterize the solution structures of complexes between heparin and a group of amidated acids, which act as delivery agents that facilitate the gastrointestinal absorption of orally administered heparin. At concentrations typically employed for the oral drug delivery of heparin, the contact points between heparin complexed with the delivery agents include points near the OH groups of heparin. The results suggest that heparin interacts rather nonspecifically with the amidated acids as monomers and with self-associated complexes of the delivery agents. It is also found that the carboxyl groups of at least one of the bioactive delivery agents easily protonates when it forms complexes with itself or heparin. This attribute may be one reason why this class of compounds is effective in the oral delivery of heparin. © 2002 John Wiley & Sons, Inc. Biopolymers (Biospectroscopy) 67: 41–48, 2002; DOI 10.1002/bip.10040

Keywords: amidated acids; heparin; oral drug delivery

INTRODUCTION

Heparin, a potent inhibitor of blood coagulation, has poor oral bioavailability. It is administered parenterally because heparin does not traverse the gastrointestinal mucosal barrier because of its size and overall negative charge.[1–3] However, intravenous delivery has substantial disadvantages and the medical implications of an orally delivered heparin solution are attractive.[2,3] A group of amidated acids (delivery agents) have been shown[4–8] to promote the gastrointestinal absorption of USP heparin in rats and primates, including humans. Although these current drug delivery agents have been shown to enhance heparin absorption without eliciting unwanted side effects,[9] the mechanism by which this is achieved has yet to be understood.[4–6]

In this study we use vibrational (Raman), Raman difference,[10] and NMR spectroscopies to characterize the solution structures and chemical states of solution mixtures of several of the amidated acids with heparin at concentrations that have been shown to be effective in oral delivery. In our previous article we reported on the solution physical properties of a series of these delivery agents at low and high concentrations using Raman and NMR spectroscopies.[11] One result of that study is that, at higher concentrations, the delivery agents form a self-associated complex that is in equilibrium with the monomer. The relative amount of the compound in the complex increases with the concentration. The complex is characterized by the formation of considerable hydrophobic interactions, ring stacking, and

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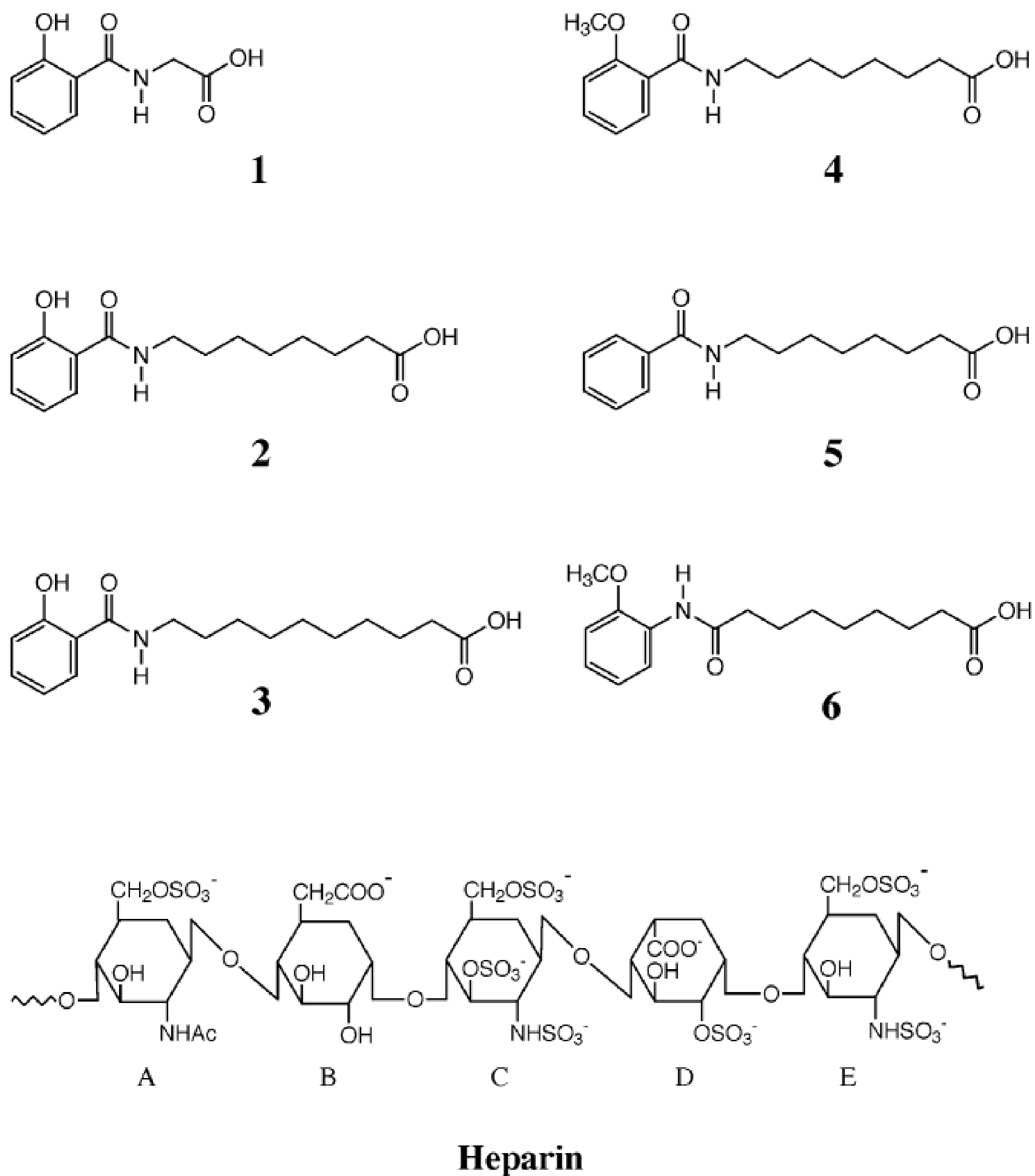

Figure 1. The molecular structures of the delivery agents and heparin.

"loose" tails of the alkane-carboxyl portion of the delivery agents. As shown below, the carboxyl moiety can exist in either the ionized or protonated form. The protonated carboxyl group seems to be favored under pH or concentration conditions that approach the limits of delivery agent solubility.

MATERIALS AND METHODS

The synthesis of the amidated acids (compounds **1-6**, Fig. 1) is described elsewhere.[6]

All samples for Raman measurement were dissolved in 15 m*M* phosphate buffer (pH 7.4) or 15 m*M* *N*-tris[hydroxymethyl]methy-3-aminopropane sulfonic acid (TAPS) buffer (pH 8.0), depending on the required experimental conditions. Once dissolved in the buffer, the pH of the solution was adjusted to the desired value by the gradual addition of either 1*N* sodium hydroxide (NaOH) or hydrochloric acid (HCl) solution. The complex between heparin (Scientific Protein Laboratories, Waunakee, WI) and the delivery agent was prepared by dissolving enough compounds to form a

solution with specified concentrations. Each side of a specially fabricated split cell cuvette (Hellma Cells, Jamaica, NY) was loaded with about 30 μL of the samples whose difference spectrum is to be determined. The cuvette, which was mounted on a translator stage-stepping motor combination device (Unidex XI with ATS302 stages, Aerotech Inc., Pittsburgh, PA), can be automatically moved side to side (or from cell to cell) without a variation in the optical alignment of the set-up. This allows for the sequential and repetitive determination of the parent spectra making up the difference spectrum.[10,12,13]

To induce Raman scattering the sample was irradiated with about 100 mW of a 530.9- or 568.2-nm line of a Coherent Innova 400-K3-krypton ion laser (Coherent Radiation Inc., Palo Alto, CA). Situated 90° from the incident beam were the detection system consisting primarily of an 1877-0.6 m Triplemate spectrometer (Spex Industries, Metuchen, NJ) and a LN/CCD-1152UV with a ST-133 controller (Princeton Instruments, Princeton, NJ). The whole optical multichannel analyzer system was interfaced with a Mac IIfx computer (Apple, Cupertino, CA), which made use of the program Igor (WaveMetrics, Lake Oswego, OR) for data collection and analyses. All spectra were corrected for the nonuniform spectral response of the detector and calibrated against the known Raman lines of toluene. The spectrometer slits were set to achieve a spectral resolution of 6 cm^{-1}. The reproducibility in the band position measurements was ± 1 cm^{-1}.

The NMR measurements were performed on a Bruker DRX-300 spectrometer at 27°C. The 1-D spectra of heparin and the heparin/compound **2** mixture were obtained on samples dissolved in a 90% H_2O/10% D_2O solution without buffer at pH 7.4. The solvent peak was suppressed using a double pulsed field gradients spin echo (DPFGSE) pulse sequence.[14] The proton exchange spectra were obtained using the CLEANEX pulse sequence[15,16] and the solvent peak was suppressed by the Watergate technique.[17] The selective pulse used in CLEANEX has a Gaussian shape, and the strength of the pulse was set to 10 Hz.

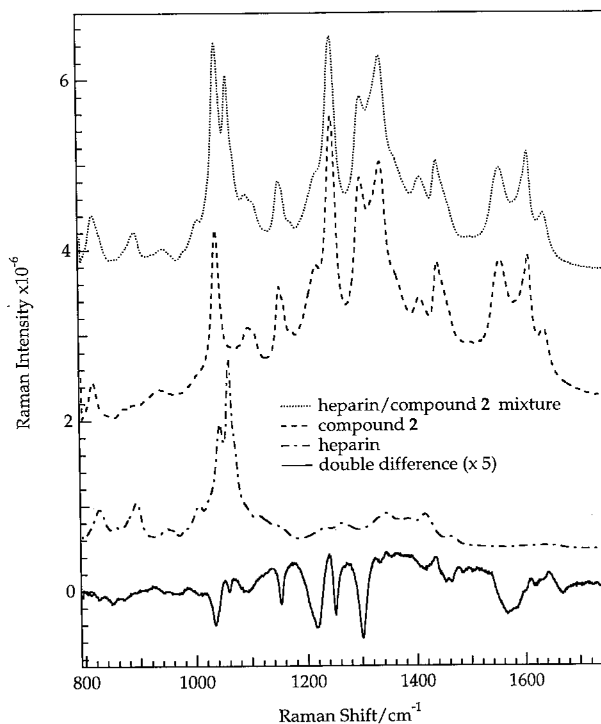

Figure 2. Raman spectra of (- - -) 50 mg/mL of compound **2**, (– · –) 33 mg/mL of heparin, and (· · ·) a mixture containing the same amounts of both. (—) A double difference Raman spectrum is obtained by subtracting the spectra of the individual compounds from that of the mixture. All solutions are buffered to pH 7.4. The double difference Raman spectrum is expanded 10 times the original.

RESULTS

Figure 1 shows the structural formulas of the compounds used in this study. Figure 2 graphs the Raman spectra of compound **2**, heparin, and a mixture containing both. The Raman spectra of the delivery agents were reported previously, and many of the bands found in their Raman spectra were assigned.[11] Most of the major bands in the vibrational spectra of the molecules arise from the ring moiety. One ring band at about 1250 cm^{-1}, assigned to the aryl-O stretch, is of special interest because it downshifts when the delivery agent molecules form a self-associated complex. The Raman spectra of those molecules whose ring moiety contains a hydroxyl moiety change drastically upon ionization of this group (pK_a of about 8.5). In particular, the relative intensity at 1300 versus 1340 cm^{-1} and at many other positions indicates the relative proportion of protonated to unprotonated hydroxyls.[11] Earlier studies on trans N-monosubstituted amides[1–3] placed the amide I (or C=O stretch) mode at 1630–1680 cm^{-1} and the amide II in the 1510–1550 cm^{-1} region. Amide II and amide III (1248–1300 cm^{-1}) bands arise from a strong coupling of the C—N—H in-plane deformation and the C—N

Table I. Changes in Chemical Properties of Drug Delivery Compounds in Presence of Heparin or Its Analog Glucosamine-2-Sulfate as Deduced from Raman and Raman Difference Spectroscopy

	Chemical Changes Induced by	
Compound	Heparin	Glucosamine-2-Sulfate
1	Increased self-association $R = 2\%$	Increased self-association $R = 1\%$
2	Increased self-association $R = 8\%$	Increased self-association $R = 6\%$
3	Increased self-association $R = 6\%$	ND
4	Increased self-association $R = 3\%$	Increased self-association $R = 3\%$
5	Increased self-association	Increased self-association
6	Increased self-association $R = 7\%$	Increased self-association $R = 7\%$

R is the ratio of the intensity of the 1240 cm^{-1} band found in the double difference spectrum to that of the intensity of the parent peak in the compound spectrum located at circa 1245 cm^{-1}; it is a measure of the amount of enhanced self-association. ND, Not determined.

stretching modes. The Raman spectra of heparin were reported previously.[18] The prominent bands in its Raman spectrum are assigned to the N-sulfate symmetric stretch (—SO_3 vibrations) at 1039 cm^{-1} while the 6-O-sulfate and 2-O-sulfate symmetric stretches are assigned to 1055 and 1065 (shoulder) cm^{-1}, respectively.

The interactions between heparin and compound **2** can be deduced from the shifts (either positions or intensity) in the Raman modes of either or both molecules. In order to reveal the generally small shifts in the Raman spectrum in the heparin/compound **2** mixture (Fig. 2, top spectrum) relative to the spectra of heparin or compound **2** alone, a double difference spectrum is generated by subtracting the spectra of each molecule from the spectrum of the mixture. This double difference spectrum is shown in the lower trace of Figure 2. There are some very small changes that show up near the sulfate stretch band positions that may indicate a small shift in their frequencies. However, the major spectral features in the double difference spectrum arise essentially from bands that have shifted and/or undergo intensity changes that can be assigned to motions located on compound **2**. A very careful examination of the double difference spectrum shows that it is essentially the same as that found by the formation of the self-associated complex of compound **2** (as is detailed in our previous study[11]). In this complex, compound **2** forms a stacking arrangement with its ring moiety and the apparent pK_a of the ring —OH increases. Stacking downshifts the 1250 cm^{-1} band (aryl-O stretch) slightly, which gives rise to a positive intensity at 1240 cm^{-1} in the double difference spectrum, and a change in the pK_a of the ring —OH changes the relative intensity of numerous bands in the spectrum of compound **2**, which gives rise to most of the difference bands observed in the double difference spectrum. Hence, we conclude that the addition of heparin to a solution of compound **2** promotes the amount of self-associated compound **2**. The same result was observed when compound **2** was mixed with glucosamine-2-sulfate, a monosaccharide found in the polysaccharide chain of heparin (data not shown; Table I).

All the delivery agents or compounds tested behaved similarly in the presence of heparin or glucosamine-2-sulfate. These results are summarized in Table I. The same bands, which we previously showed in the difference spectrum between high and low concentrations of the delivery agent (a spectrum indicative of the self-associated complex), appear in the presence of heparin. Table I indicates this by noting that “ring stacking” is promoted as evidence by the band at 1240 cm^{-1} in the double difference spectrum. Table I also indicates the degree of stacking by tabulating the size of the 1240 cm^{-1} band in the double difference spectrum relative to the size of the parent peak (the higher the number the higher relative proportion of self-associated complex).

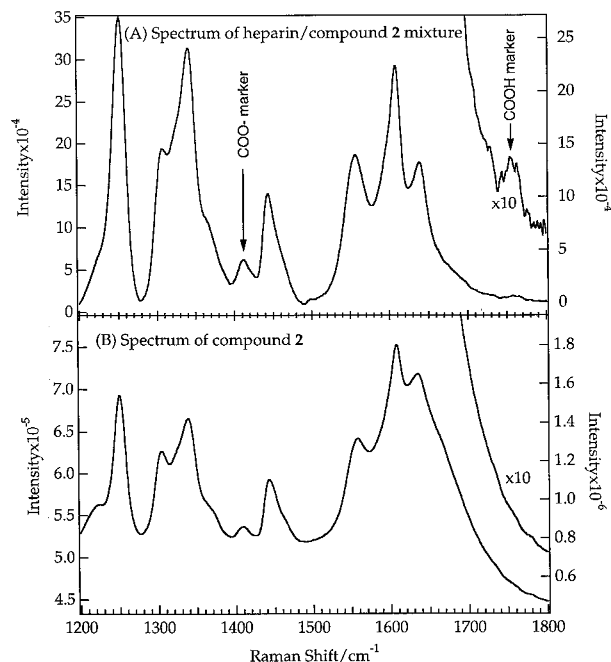

Figure 3. (a) The Raman spectrum of compound **2** complexed with heparin. The concentrations are 100 mg/mL for each species. (b) The Raman spectrum of compound **2** at a concentration of 10 mg/mL. The pH in both cases is 7.5.

Figure 3(a) shows the Raman spectra of the heparin/compound **2** mixture at high concentration and Figure 3(b) shows that of compound **2** at low concentration. A clear set of marker bands showing the ionization state of the end group carboxyl of compound **2** are at 1410 cm^{-1} for ionized and 1710–1770 cm^{-1} for protonated.[19] The values in the frequency of a protonated carboxyl are variable because various environments interact more or less strongly with the polar C=O bond of the —COOH moiety; the protonated marker band essentially consists of the C=O stretch, and interactions that polarize this bond (e.g., hydrogen bonding) lower the frequency of the stretch. It is clear that at low concentrations (here 10 mg/mL) the carboxylate moiety of compound **2** is completely ionized, as we reported previously. However, in its mixture with heparin, a portion of the carboxyls protonate as shown by the appearance of the weak band at 1760 cm^{-1} in Figure 3(b). It should be noted that this is not from the precipitated form of the delivery agent because the frequency of the protonated carboxyls in the precipitates was found to lie at 1715 cm^{-1} in these aggregates.[11] The interaction of the drug delivery agent/heparin complex is such that the C=O bond of the portion of those carboxyls that are protonated is nonpolarizing; in fact, the observed frequency at 1760 cm^{-1} is typical of protonated carboxyls in hydrophobic environments. The relative concentration of protonated to ionized carboxyls is easily determined from the relative intensities of the protonated and ionized marker bands. For a one to one mixture of protonated to ionized species, the ratio of the intensities of the respective marker bands (I_{1750}/I_{1420}) is approximately 0.35.[19] A comparison of the intensities at 1420 and 1760 cm^{-1} shows that about 25% of compound **2** is in the protonated form in the heparin/compound **2** mixture. Other studies (data not shown) show that compound **2** forms similar amounts of protonated carboxyl at similar concentrations and pH, concomitant with the formation of self-associated complex, although the exact percentage varies somewhat from run to run.

Figure 4 shows the 1-dimensional (1-D) proton NMR spectra of heparin [Fig. 4(a)] and its mixture with compound **2** [Fig. 4(b)] in 90% H_2O/10%

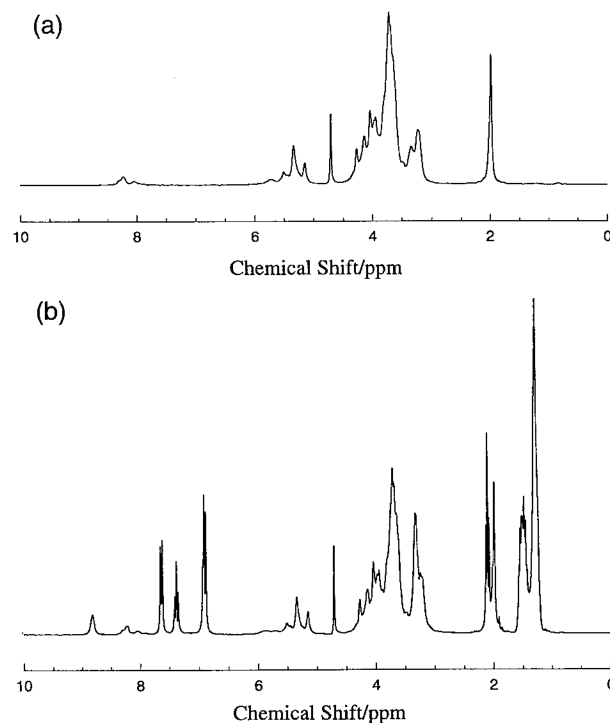

Figure 4. The NMR spectra of (a) heparin at a 50 mg/mL concentration in 90% H_2O/10% D_2O at pH 7.5 without buffer and (b) the heparin/compound **2** mixture at a 30 mg:10 mg/mL ratio in 90% H_2O/10% D_2O at pH 7.5 without buffer. The water peak at about 4.7 ppm was suppressed by the DPFGSE method.

D_2O at pH 7.5 without buffer. The water resonances at about 4.7 ppm in these spectra were suppressed by the DPFGSE method.[14] In Figure 4(a) most of the resonances from heparin's CH protons were assigned in previous NMR studies of this molecule.[20] Three resonances near 8 ppm can be assigned to heparin's NH protons and the resonance at 5.7 ppm is assigned to its OH protons on the basis of their disappearance in D_2O. A comparison of Figure 4(a) and 4(b) indicates that none of the CH resonances from heparin are significantly affected in the presence of compound **2** under our experimental conditions, suggesting that the environments of the CH groups of heparin are not changed in heparin/compound **2** mixtures. The NH resonances of heparin are not noticeably affected by the addition of compound **2** either. However, the change of the OH resonance of heparin at 5.7 ppm [Fig. 4(a)] upon the addition of compound **2** is apparent. It is either shifted or its intensity is greatly reduced in the spectrum of the heparin/compound **2** mixture [Fig. 4(b)], suggesting that there may be significant interactions between the OH groups of heparin and compound **2**. Thus, several pulse sequences specifically developed to study the NH/OH hydrogen exchange were applied to heparin and heparin/compound **2** mixtures to characterize such interactions.

Figure 5(a,b) shows the spectra of heparin and its mixture with **2** obtained by the CLEANEX method[16] with a series of different mixing times. In these two spectral series the positive peaks are the exchange peaks from NH/OH protons, and the negative peaks that appear at longer mixing times are mostly nuclear Overhause effect (NOE) peaks between water and compound **2**, except the peak at 2 ppm, which is the NOE between water and a very flexible CH proton in heparin. Because CLEANEX was designed to eliminate NOEs between water and CHs of large molecules, most of the CH resonances of heparin in these spectra are effectively suppressed. At the short mixing times (<100 ms) the intensity of the NH/OH peaks in the CLEANEX spectra are mainly governed by the proton exchange rate of the specified NH/OH protons. The faster rising time of the peak intensity with the longer mixing time is thus correlated with the faster proton exchange rate.

In Figure 5(a) three relatively sharp heparin OH resonances at 5.3, 5.7, and 5.9 ppm show almost full intensity after 10 ms of mixing time while the NH resonances near 8 ppm show their full intensity at about 70 ms. This suggests the proton exchange of the OH group of heparin with

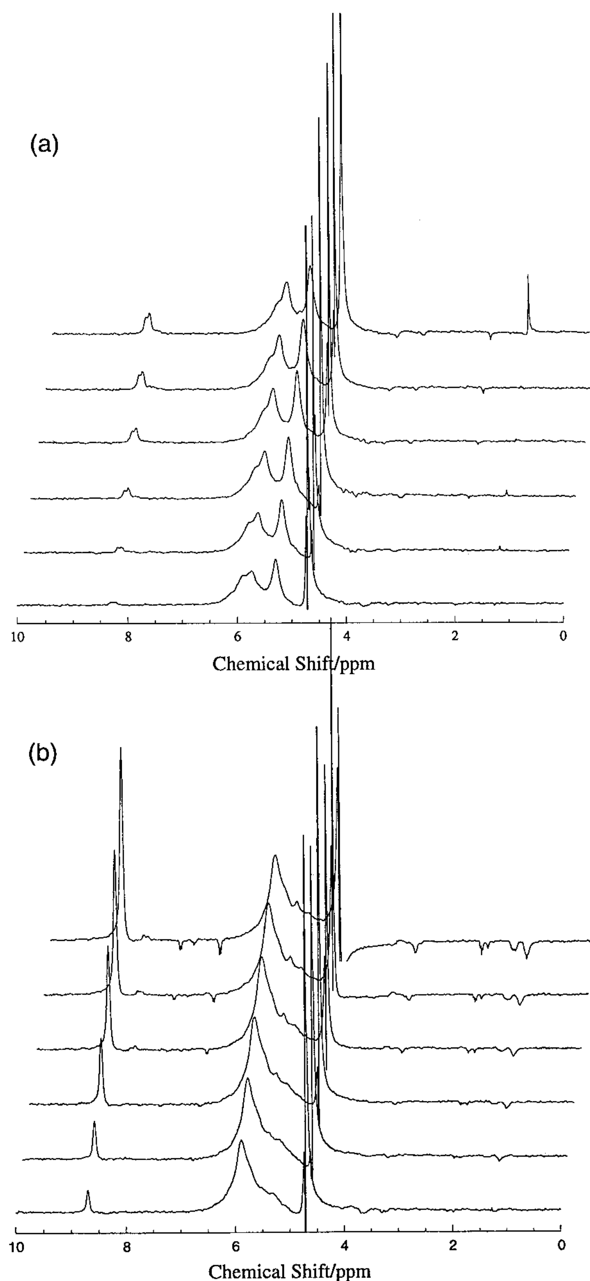

Figure 5. The results of the CLEANEX experiments on (a) heparin at a 50 mg/mL concentration in 90% H_2O/10% D_2O at pH 7.5 without buffer and (b) the heparin/compound **2** mixture at a 30 mg:10 mg/mL ratio in 90% H_2O/10% D_2O at pH 7.5 without buffer. The mixing times were set to 10, 20, 40, 70, 100, and 230 ms in the series. A low strength gradient (~0.1 G/cm) was applied during the mixing time to prevent radiation damping. The solvent water was on resonance with the selective pulse. The water peak at about 4.7 ppm was suppressed by the Watergate technique.

water is faster than that of the NH group, and the exchange half-time is <10 ms. On the other hand, as the OH proton exchange rate approaches ~300/s, which is the frequency difference between the water resonance and the OH resonance, the OH resonance will become broad and eventually become too broad to be observed for exchange rates above 300/s.

Comparing Figure 5(b) with 5(a), we can see that there are drastic changes in the OH spectral region. The relatively sharp resonance at 5.3 ppm in Figure 5(a) either becomes much broader or shifted than in Figure 5(b). A new OH resonance at 5.9 ppm is also observed. This resonance can be due to either compound **2** or heparin. Because there is no such peak observed in the CLEANEX spectra of compound **2**, the most likely explanation for the appearance of this resonance is that the interaction between heparin and compound **2** reduces the proton exchange rate of OH on compound **2**. However, further studies are required to assign this resonance and determine if there are other possibilities.

DISCUSSION

We would assume that the interactions that exist between the delivery agents and heparin include electrostatic interactions (hydrogen bonds, dipole–dipole interactions, etc.), as well as hydrophobic interactions. The heparin and delivery agents include charged and polar moieties that favor electrostatic interactions, as well as alkane parts, that would favor hydrophobic interactions.

The NMR results suggest that the interaction between heparin and compound **2** speeds up the proton exchange rate of several OH groups in heparin while likely slowing down the proton exchange rate of the OH group on compound **2**. Thus, it is reasonable to suggest that the contact points between heparin and compound **2** include points near these OH groups. These data then clearly show that heparin and the delivery agents are interacting in solution, although the results do not show the specific nature of the interaction.

The Raman results do not show any indication of band shifts in either the delivery agent or heparin, which would indicate specific contact points between the two molecular systems. It seems clear from the NMR and Raman results that the two molecules, while interacting, do so rather nonspecifically, except for the contact points near the OH groups of heparin. The Raman results do show that the presence of heparin at high concentrations in solution mixtures with the delivery agents alters the monomer versus the self-associated product(s) toward self-association of the delivery agent. This may be due to the direct association between heparin and the delivery agent. It may also be due to the change in the ionic character of the aqueous environment when heparin is present. For example, it was also previously shown that compound **2** behaved similarly: it formed self-associated complexes when mixed with simple salts (e.g., NaCl or sodium sulfate).[11] It may be that the sodium ions, which are brought into solution by the addition of the heparin sodium salt, shield the ionized carboxylate group of the delivery agents, permitting a greater amount of self-associated complex to form (i.e., one that does not become so negative so as to not be able to stay together).

A very important result from the Raman studies is that compound **2** forms a significant amount of protonated carboxyl at pH 7.5 in the presence of heparin (and at high concentration). This is much higher than the normal pK_a of an alkylated carboxyl (ca. 4.2). This implies that intermolecular complexes of compound **2** with itself or with heparin are made up of a portion of compound **2** that has a protonated carboxyl moiety. It is not hard to understand how the apparent pK_a of the carboxyl group of the delivery agent is raised several units. Presumably the intermolecular forces that favor complex formation would ultimately be overcome by charge–charge interactions between negative carboxyl units. Hence, a protonated carboxyl moiety in the complex (of a certain size) would be lower in energy and therefore compound **2** could easily adopt a neutral form when it forms complexes. Because the delivery agent/drug complex must be stable in both aqueous solutions, which is favored by a charged (ionized) carboxyl, as well as the hydrophobic environment of the interior of a membrane, favored by a neutral (protonated) carboxyl, this observation that the ease with which the ionization state of the carboxyl can switch, depending on the environment, is likely one reason why the amidated acids of a certain alkane chain length are effective delivery agents.

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REFERENCES

1. Colthup, N. B.; Daly, L. H.; Wiberley, S. E. Introduction to Infrared and Raman Spectroscopy; Academic: San Diego, CA, 1990.
2. Lin-Vien, D.; Colthup, N. B.; Fateley, W. G.; Grasselli, J. G. The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules; Academic: San Diego, CA, 1991.
3. Deng, H.; Schindler, J. F.; Berst, K. B.; Plapp, B. V.; Callender, R. Biochemistry 1998, 37, 14267–14278.
4. Leone-Bay, A.; Leipold, H.; Agarwal, R.; Rivera, T.; Baughman, R. A. Drugs Future 1997, 22, 885–891.
5. Leone-Bay, A.; Paton, D. R.; Variano, B.; Leipold, H.; Rivera, T.; Miura-Fraboni, J.; Baughman, R. A.; Santiago, N. J Controlled Release 1998, 50, 41–49.
6. Leone-Bay, A.; Paton, D. R.; Freeman, J.; Lercara, C.; O'Toole, D.; Gschneider, D.; Wang, E.; Harris, E.; Rosado, C.; Rivera, T.; DeVincent, A.; Tai, M.; Mercogliano, F.; Agarwal, R.; Leipold, H.; Baughman, R. A. J Med Chem 1998, 41, 1163–1171.
7. Brayden, D.; Creed, E.; O'Connell, A.; Leipold, H.; Agarwal, R.; Leone-Bay, A. Pharm Res 1997, 14, 1772–1779.
8. Baughman, R. A.; Kapoor, S. C.; Agarwal, R. K.; Kisicki, J.; Catella-Lawson, F.; Fitzgerald, G. A. Circulation 1998, 98, 1610–1615.
9. Rivera, T.; Leone-Bay, A.; Paton, D. R.; Leipold, H. R.; Baughman, R. A. Pharm Res 1997, 14, 1830–1834.
10. Callender, R.; Deng, H. Annu Rev Biophys Biomol Struct 1994, 23, 215–245.
11. Desamero, R.; Cheng, H.; Cahill, S.; Girvin, M.; Callender, R.; Rath, P.; Variano, B.; Smart, J. Biopolym Biospectrosc, submitted.
12. Deng, H.; Callender, R. Comments Mol Cell Biophys 1993, 8, 137–154.
13. Callender, R.; Deng, H.; Gilmanshin, R. J Raman Spectrosc 1998, 29, 15–21.
14. Stott, K.; Stonehouse, J.; Keeler, J.; Hwang, T.-L.; Shaka, A. J. J Am Chem Soc 1995, 117, 4199–4200.
15. Hwang, T.-L.; Shaka, A. J. J Magn Reson Ser A 1995, 112, 275–279.
16. Hwang, T.-L.; Susumu, M.; Shaka, A. J.; van Zijl, P. C. M. J Am Chem Soc 1997, 119, 6203–6204.
17. Piotto, M.; Saudek, M.; Sklenar, V. J Biomol NMR 1992, 2, 661–665.
18. Atha, D. H.; Gaigalas, A. K.; Reipa, V. J Pharm Sci 1996, 85, 52–56.
19. Chen, Y.-Q.; Kraut, J.; Callender, R. Biophys J 1997, 72, 936–941.
20. Rabenstein, D. L.; Bratt, P.; Schierling, T. D.; Robert, J. M.; Guo, W. J Am Chem Soc 1992, 114, 3278–3285.