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Carbohydrate Research 340 (2005) 1185–1191
Carbohydrate RESEARCH

A new radiochemical method to investigate ion binding with polyelectrolytes

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Received 26 November 2004; received in revised form 9 February 2005; accepted 14 February 2005

Abstract—A new method for investigating the binding of ions with polyelectrolytes has been developed. This method, based on Donnan equilibrium and an isotope exchange between the electrolyte and polyelectrolyte, can distinguish territorial from specific binding of ions and can determine fractions of ions bound with the polyion. This method can determine ion binding with polyelectrolytes in a wide range of polyelectrolyte concentrations in multicomponent solutions. The method was tested with radioactive tracers $^{22}Na^+$, $^{36}Cl^-$ and heparin sodium salt. The influence of the ionic strength on the Na^+ binding with heparin was investigated at 310 K. In the limit of zero ionic strength, all Na^+ ions are bound to heparin, but only 45% of them are exchangeable. Thus Na^+ ions can be bound both territorially and specifically. The fraction of bound ions decreases rapidly with increasing ionic strength. The fraction of the specifically bound ions becomes negligible when the ionic strength exceeds 0.01 M, whereas the fraction of territorially bound ions can be neglected at ionic strengths higher than 0.45 M.

Keywords: Heparin; Radioactive tracers; Heparin–ion binding

1. Introduction

The purpose of the present work was to elaborate a radiochemical method to investigate the binding of the metal ions with heparin. Heparin is a linear polymer consisting of repeating units of 1→4-linked uronic acid and 2-amino-2-deoxy-D-glucopyranose (D-glucosamine) residues.[1] Typically, the uronic acid distribution in heparin consists of 90% L-idouronic acid and 10% D-glucuronic acid, both in the pyranose form. Heparin is a polyelectrolyte with the highest negative charge of any known biological macromolecule, due to a high content of negatively charged sulfate and carboxyl groups. Heparin and its derivatives are widely used in medicine as anticoagulants, antithrombotics and antilipemic agents.[2] This polysaccharide interacts with the ions present in blood, such as Na^+, Ca^{2+}, Cu^{2+}, or Mg^{2+}. The binding of counter-ions partially neutralizes the polyion charge and, consequently, the biological activity of heparin decreases.[2]

Heparin, like other polyelectrolytes, can bind ions either territorially, by electrostatic interactions, or locally, by specific interactions. Information about the binding of metal ions with heparin has been deduced from self-diffusion coefficients,[3] chiroptical properties,[4,5] NMR spectroscopy[6–12] and interactions with methylene blue.[13–15] Although these measurements can yield the fraction of ions bound with heparin, they cannot distinguish the territorial and specific ion binding.[13] Therefore the local, specific character of ion binding is usually assumed to explain large deviations[6,8] of experimental association constants from the values predicted by Manning's two-variable theory.[16,17] Such large deviations are observed for divalent ions, like Ca^{2+}, Cu^{2+} and Zn^{2+}. Therefore, these ions are assumed to be bound both by electrostatic and specific interactions, whereas the monovalent ions, like Na^+ and K^+, are believed to be bound territorially via electrostatic interactions.

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doi:10.1016/j.carres.2005.02.011

We elaborated the new radiochemical method to investigate the degree of ion binding with heparin. This method is based on a Donnan equilibrium and an isotope exchange of labeled counter-ions. This new method, which can be used for any polyelectrolyte, makes it possible to determine not only the fraction of the counter-ions bound with the polyion, but it also permits the character of the ion binding to be distinguished.

2. Experimental

2.1. Materials

Heparin sodium salt (HpNa), a commercial product (Sigma, Prod. Nos. H3393), extracted from porcine intestinal mucosa, was used as received. The material used in this study had a molecular weight within the 17–19 kDa range. The number of Na^+ ions per tetrasaccharide unit was determined as follows. An aqueous solution of HpNa was passed through a cation exchange column (Amberlit IR-124) until the effluent was neutral. A solution of this acid form of the heparin was poured into a conductimetric cell and then titrated with 0.0096 M NaOH solution. The conductance curve showed a plateau between two equivalence points.[18] The first of them is due to neutralization of $-SO_3H$ groups and the second point appears after neutralization of all carboxyl groups. The procedure described above was repeated for different concentrations of the HpNa and different flow rates. This yielded an equivalent weight of the heparin salt of 198 ± 3 and the sulfate/carboxylate ratio was 2.09 ± 0.09.

NaCl (suprapur, Merck) and radioactive tracers, $Na^{36}Cl^-$ (Polatom) and $^{22}NaCl$ (Amersham) were used as received. Water was double distilled and deionized. Solutions of HpNa and NaCl were prepared by weight. The concentration of heparin, expressed as a mol of univalent anionic charges, covered the range from 1.5 to 30 mM.

2.2. Experimental method

All experiments have been performed in a cell that consists of two vessels separated by a membrane (Fig. 1). The membrane (Spectra/Por 4) is permeable to water and small ions, but impermeable to heparin. The volumes of both compartments are equal and the solutions in both vessels were stirred. The NaCl solution, labeled with either $^{36}Cl^-$ or $^{22}Na^+$ ions, was poured into the left compartment of the cell. The right compartment of the cell was filled with the HpNa solution. Samples, of about 5 μL, were collected from both compartments in 1-h intervals. The samples were weighed and their radioactivity, A_t, was measured in 5 mL of scintillation cocktail (Ultima Gold™ XR, Packard) using a liquid scintillation counter RackBeta (LKB). In all experiments, maintained at 310.0 ± 0.01 K, equilibrium was reached after about 25 h. At this point, the final radioactivity, A_∞, of the solutions in both compartments was determined. To ensure that the equilibrium was reached, the experiments were continued up to 10 days, but the A_∞ values did not change.

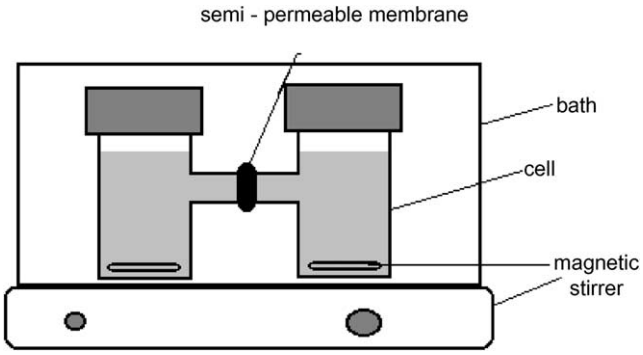

Figure 1. Scheme of the experimental apparatus.

2.3. Determination of the fraction of territorially bound ions

In solutions of an inorganic salt, MX, and a polyelectrolyte, PM, separated by a semi-permeable membrane, thermodynamic equilibrium demands an equality of electrochemical potentials of small diffusible ions, μ_M^+ and μ_X^-, in polyelectrolyte and electrolyte solutions:

$$\left[\mu^0_{M^+} + RT\ln\left(\gamma_\pm c_{M^+}\right)_\infty + F\phi\right]_P = \left[\mu^0_{M^+} + RT\ln\left(\gamma_\pm c_{M^+}\right)_\infty + F\phi\right]_{salt} \quad (1)$$

$$\left[\mu^0_{X^-} + RT\ln\left(\gamma_\pm c_{X^-}\right)_\infty - F\phi\right]_P = \left[\mu^0_{X^-} + RT\ln\left(\gamma_\pm c_{X^-}\right)_\infty - F\phi\right]_{salt} \quad (2)$$

where ϕ denotes an electric potential and $\gamma_\pm$ is the ionic mean activity coefficient.

Addition of Eqs. 1 and 2 yields the Donnan equation,[19] which interrelates equilibrium concentrations of diffusible ions, either M^+ or X^-, in polyelectrolyte $[(c_{ion})_\infty]_P$ and electrolyte $[(c_{ion})_\infty]_{salt}$ solutions, respectively.

$$[(c_{M^+})_\infty]_P \cdot [(c_{X^-})_\infty]_P = [(c_{M^+})_\infty]_{salt} \cdot [(c_{X^-})_\infty]_{salt} \quad (3)$$

The simple relation (3) is valid when ionic mean activity coefficients can be neglected, for example, either for very dilute solutions, when activity coefficients are close to unity, or for the solutions of similar ionic strengths, with almost identical activity coefficients.

Electric neutrality of both solutions is required, therefore equilibrium concentrations of small diffusible ions M^+, $[(c^+_M)_\infty]_{salt}$ and X^- $[(c^-_X)_\infty]_{salt}$ in the electrolyte solution are equal, whereas in the polyelectrolyte solution

the equilibrium concentrations of counter-ions M^+, $[(c_M^+)_\infty]_P$ exceeds the concentration of X^- anion $[(c_X^-)_\infty]_P$. These concentrations can be expressed as follows:

$$[(c_{M^+})_\infty]_{salt} = [(c_{X^-})_\infty]_{salt} \equiv (c_{MX})_\infty \quad (4a)$$

$$[(c_{M^+})_\infty]_P = \Delta c_{MX} + [\alpha \cdot (c_{PM})_0] \quad (4b)$$

$$[(c_{X^-})_\infty]_P = \Delta c_{MX} \quad (4c)$$

$(c_{MX})_\infty$ denotes the equilibrium concentration of electrolyte and Δc_{MX} represents a change of the electrolyte concentration due to diffusion of small ions. $(c_{PM})_0$ is the initial concentration of polyions, expressed as moles of univalent charges, thus $\alpha(c_{PM})_0$ represents the initial concentration of free counter-ions in polyelectrolyte solution. Substitution of Eqs. 4a–c into Eq. 3 yields the dissociation degree, α, that is, a fraction of free counter-ions that are non-associated with the polyion:

$$\alpha = \frac{1}{(c_{PM})_0} \cdot \frac{[(c_{MX})_\infty]^2 - [\Delta c_{MX}]^2}{\Delta c_{MX}} \quad (5)$$

The equilibrium concentration of electrolyte MX in both compartments of the cell, that is, in solutions of the electrolyte and polyelectrolyte, can be easily determined using an anion labeled with a radioisotope. Diffusion of small ions does not change the molar radioactivity of the tracer $*X^-$. Therefore, the molar radioactivity of a labeled anion, a_X, can be expressed as follows:

$$a_X = \frac{[A_0(^*X^-)]_{salt}}{(c_{MX})_0 \cdot V_{salt}} \equiv \frac{[A_\infty(^*X^-)]_{salt} + [A_\infty(^*X^-)]_P}{[(c_{MX})_\infty]_{salt} \cdot V_{salt} + [(c_{MX})_\infty]_P \cdot V_P} \quad (6)$$

$[A_0(^*X^-)]_{salt}$ is the total radioactivity of the tracer in electrolyte solution, determined at the beginning of the experiment. $[A_\infty(^*X^-)]_{salt}$ and $[A_\infty(^*X^-)]_P$ represent the total equilibrium radioactivities of the tracer in solutions of the electrolyte and polyelectrolyte, respectively, and are measured at the end of the experiment, that is, after 30 h. All radioactivities are determined experimentally and they can be used to calculate the equilibrium concentration of MX in the solution of electrolyte, $[(c_{MX})_\infty]_{salt}$, and polyelectrolyte, Δc_{MX}. Under experimental conditions, the volumes of both solutions are equal, $V_{salt} = V_P = V$, thus substitution of Eq. 6 into Eqs. 4a–c gives the equilibrium concentrations of ions in both solutions, separated by the membrane:

$$[(c_{M^+})_\infty]_{salt} \equiv [(c_{X^-})_\infty]_{salt} = \frac{[A_\infty(^*X^-)]_{salt}}{[A_0(^*X^-)]_{salt}}(c_{MX})_0 \quad (7a)$$

$$[(c_{M^+})_\infty]_P \equiv \Delta c_{MX} + [\alpha \cdot (c_{PM})_0] = \frac{[A_\infty(^*X^-)]_P}{[A_0(^*X^-)]_{salt}}(c_{MX})_0 + [\alpha \cdot (c_{PM})_0] \quad (7b)$$

$$[(c_{X^-})_\infty]_P \equiv \Delta c_{MX} = \frac{[A_\infty(^*X^-)]_P}{[A_0(^*X^-)]_{salt}}(c_{MX})_0 \quad (7c)$$

Substitution of Eqs. 7a–c into the Donnan equation (3) yields the fraction of ions not associated with the polyion:

$$\alpha = \frac{(c_{MX})_0}{(c_{PM})_0} \cdot \frac{\{[A_\infty(^*X^-)]_{salt}\}^2 - \{[A_\infty(^*X^-)]_P\}^2}{[A_0(^*X^-)]_{salt} \cdot [A_\infty(^*X^-)]_P} \quad (8)$$

2.4. Determination of a fraction of specifically bound ions

Application of the counter-ion labeled with radioisotope $*M^+$ allows the determination of the fraction of counter-ions bound to polyion by specific interactions. When a semi-permeable membrane separates solutions of polyelectrolyte PM and of electrolyte MX, which contains tracer $*M^+$ two processes occur simultaneously—diffusion of the tracer and an isotope exchange between solution and polyion. The isotope exchange of the tracer $*M^+$ can occur only between the solution and counter-ions bound with polyion by nonspecific, electrostatic forces according to the following scheme:

$$(^*M^+)_{solution} + P^-M^+ \rightarrow (M^+)_{solution} + P^-(^*M^+)$$

An equilibrium of the isotope exchange is reached when the $*M^+$ tracer is distributed uniformly between electrolyte and all exchangeable counter-ions bound nonspecifically to the polyion. The isotope exchange causes a decrease of the molar radioactivity of the tracer, but at equilibrium the molar radioactivities of the electrolyte and the polyelctrolyte, expressed as mol of univalent charges, are equal, $[(a_M)_\infty]_{MX} = [(a_M)_\infty]_{PM} = (a_M)_\infty$. The equilibrium molar radioactivity depends on the total concentration of the exchangeable counter-ions. When the volumes of both solutions, electrolyte and polyelectrolyte, are equal ($V_{salt} = V_P = V$), the equilibrium molar radioactivity of the tracer $(a_M)_\infty$ fits the following relation:

$$(a_M)_\infty = \frac{[A_0(^*M^+)]_{salt}}{V \cdot [(c_{MX})_0 + \beta \cdot (c_{PM})_0]} = \frac{[A_\infty(^*M^+)]_{salt} + [A_\infty(^*M^+)]_{salt}}{V \cdot [(c_{MX})_0 + \beta \cdot (c_{PM})_0]} \quad (9)$$

$[A_0(^*M^+)]_{salt}$ is the total radioactivity of the tracer in the electrolyte solution at the beginning of the experiment. $[A_\infty(^*M^+)]_{salt}$ and $[A_\infty(^*M^+)]_P$ denote the total equilibrium radioactivity of tracer in the electrolyte and

polyelectrolyte solutions, respectively. $\beta(c_{PM})_0$ represents a concentration of the 'exchangeable' counter-ions.

The total equilibrium radioactivities of tracer $*M^+$, $[A_\infty(*M^+)]_P$ and $[A_\infty(^*M^+)]_{salt}$, respectively, are proportional to the concentrations of M^+ ions in polyelectrolyte and electrolyte solutions:

$$[A_\infty(^*M^+)]_P = (a_M)_\infty \cdot V_P \cdot [(c_{M^+})_\infty]_P \quad (10a)$$

$$[A_\infty(^*M^+)]_{salt} = (a_M) \cdot V_{salt} \cdot [(c_{M^+})_\infty]_{salt} \quad (10b)$$

Substitution of Eqs. 4a–c and 9 into Eqs. 10a and 10b interrelates the experimentally measured equilibrium radioactivities of the tracer, $[A_\infty(^*M^+)]_{salt}$ and $[A_\infty(^*M^+)]_P$, with the change of the electrolyte concentration, Δc_{MX}, initial concentrations of electrolyte $(c_{MX})_0$ and polyelectrolyte $(c_{PM})_0$ and the fraction of the 'exchangeable' ions β:

$$[A_\infty(^*M^+)]_P = \frac{[A_0(^*M^+)]_{salt}}{(c_0)_{MX} + \beta(c_{PM})_0}[\beta(c_{PM})_0 + \Delta c_{MX}] \quad (11a)$$

$$[A_\infty(^*M^+)]_{salt} = \frac{[A_0(^*M^+)]_{salt}}{(c_0)_{MX} + \beta(c_{PM})_0}[(c_{MX})_0 - \Delta c_{MX}] \quad (11b)$$

Division of the Eq. 11a by Eq. 11b and substitution of Eqs. 7a and 7b yields the fraction β:

$$\beta = \frac{(c_{MX})_0}{(c_{PM})_0} \cdot \frac{[A_\infty(^*M^+)]_P}{[A_\infty(^*M^+)]_{salt}} \cdot \left(\frac{[A_\infty(^*X^-)]_{salt} - [A_\infty(^*X^-)]_P}{A_0(^*X^-)_{salt}}\right) \quad (12)$$

This equation represents the fraction of 'exchangeable' M^+ ions, that is, those ions bound with the polyion by electrostatic interactions. The rest of the ions, represented by the $(1-\beta)$ fraction, must be bound to the polyion by specific interactions and therefore remain unexchanged. Eq. 12, which represents the fraction of nonspecifically bound ions, and Eq. 8, which yields fraction of ions nonassociated with polyion, were derived without any assumptions about properties of the polyion and the character of the ion binding. Thus, the method described above can be applied to investigate the binding character of any ion with any polyion. We have used this method to study the binding of Na^+ ion with heparin.

3. Results and discussion

Although the HpNa is commonly used in medicine, data concerning Na^+ binding with heparin are scarce. A lack of such data can be understood because NaCl is commonly used to keep a constant ionic strength and in many experiments the NaCl concentration significantly exceeds the concentration of other electrolytes.

The method described above is based on the transport of radioactive tracers and thus the membrane must be completely permeable and neutral to them. Therefore, we have investigated transport of tracers through such a membrane by measurements of the solution radioactivity as a function of time. Typical dependencies of the solution radioactivity on time during experiments are shown in Figure 2.

An exponential decay of tracer radioactivity, either $^{36}Cl^-$ or $^{22}Na^+$, in NaCl solution has been observed in all experiments. This decrease has been accompanied by the exponential increase of radioactivity in heparin solution. The final radioactivities of $^{36}Cl^-$ in the solutions of heparin and NaCl were equal, whereas the equilibrium radioactivity of $^{22}Na^+$ in the heparin solution significantly exceeded the $^{22}Na^+$ radioactivity in the NaCl solution. In all experiments the sum of radioactivities in both solutions was constant and equal to the initial radioactivity of the tracer. This confirms that the membrane was completely permeable for small ions.

Tracer transport coefficients have been calculated from the dependence of $\ln|A_t - A_\infty|$ versus diffusion time.[20] These dependencies, representing both decay and increase of the radioactivity, are linear. Examples of these rate coefficients for ion transfer are listed in Table 1. As can be seen, the rate coefficients calculated from the increase, k_I, of radioactivity in HpNa and from the decay, k_D, of radioactivity in NaCl solution, are equal within the experimental error. This equality is additional evidence that the membrane is completely permeable and neutral to small ions.

The fractions of the free Na^+ ions, α, were calculated from Eq. 8 using the equilibrium radioactivity of $^{36}Cl^-$. Numerical results of the association degree $(1-\alpha)$, that is, the fraction of Na^+ ions bound with heparin by electrostatic interactions are summarized in Table 2. An association constant, K_{as}, has been computed from Eq. 13 and these results are also listed in Table 2.

$$K_{as} = \frac{c_{Hp^-Na^+}}{(c_{Na^+}) \cdot (c_{Hp^-})} \equiv \frac{[(1-\alpha) \cdot (c_{HpNa})_0]}{[\alpha \cdot (c_{HpNa})_0 + \Delta c_{NaCl}] \cdot [\alpha \cdot c(_{HpNa})_0]} \quad (13)$$

In Figure 3, the influence of the ionic strength on the association degree $(1-\alpha)$ is presented. The dependence of $(1-\alpha)$ on I is linear and has been fitted to Eq. 14 by the least-squares method:

$$(1-\alpha) = (1.006 \pm 0.005) - (1.49 \pm 0.07) \cdot \sqrt{I} \quad (14)$$

This equation shows that in the limit of zero ionic strength, all Na^+ ions are associated with heparin. These ions can be considered as territorially bound and thus

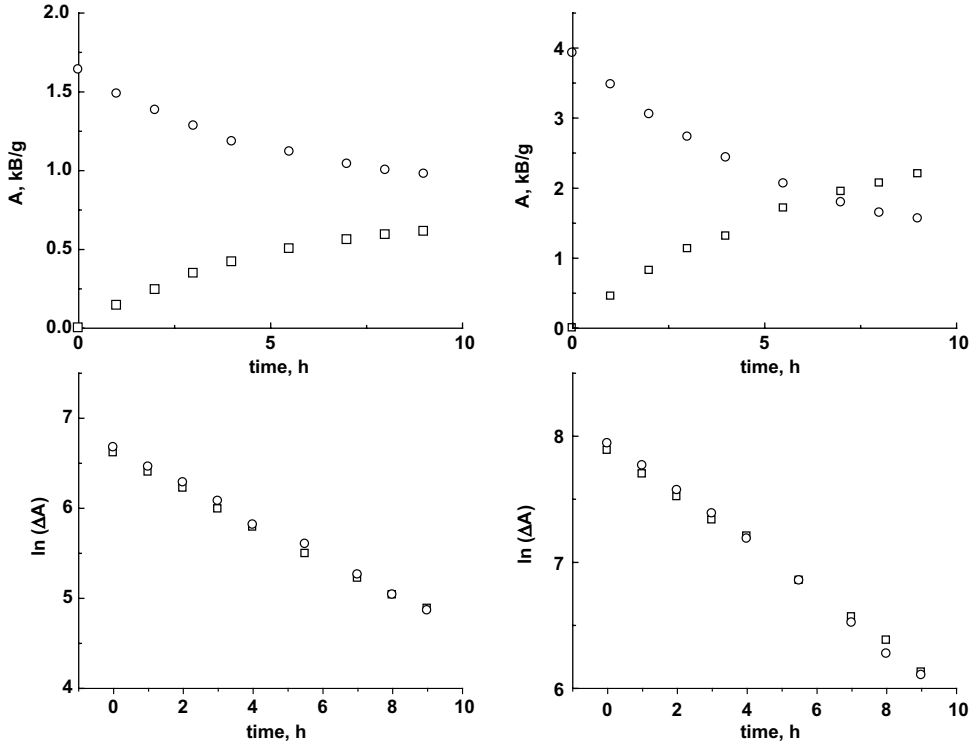

Figure 2. Time dependencies of total radioactivity A_t (upper) and $\ln|(A_t - A_\infty)|$ (bottom) in NaCl (○) and heparin (□) solution for $^{36}Cl^-$ (left) and $^{22}Na^+$ (right) at 310 K.

Table 1. Rate coefficients of $^{36}Cl^-$ and $^{22}Na^+$ transfer from NaCl solution into heparin sodium salt solution at 310 K

Tracer	$c_{NaCl} \times 10^3$ M	$c_{HpNa} \times 10^3$ M	Transfer rate coefficient, h^{-1}	
			k_D	k_I
$^{36}Cl^-$	0.01	0.01	0.192 ± 0.006	0.195 ± 0.002
$^{22}Na^+$	0.01	0.01	0.209 ± 0.005	0.195 ± 0.003
$^{36}Cl^-$	0.0015	0.0015	0.165 ± 0.009	0.158 ± 0.008
$^{22}Na^+$	0.0015	0.0015	0.144 ± 0.008	0.137 ± 0.009

k_D and k_I coefficients are calculated from the decay and increase of tracer radioactivity in solutions of NaCl and HpNa, respectively.

Table 2. Influence of the ionic strength I on Na^+ binding with heparin

$(c_{HpNa}) \times 10^3$ M	$(c_{NaCl}) \times 10^3$ M	$I \times 10^3$ M	$1 - \alpha$	K_{as}	$1 - \beta$	K_b
1.5	0.74	0.74	0.98	4.6×10^4	0.47	574
5	2.42	2.75	0.93	5.1×10^3	0.32	81
6	2.89	3.44	0.91	2.9×10^3	0.31	64
10	4.74	5.84	0.89	1.4×10^3	0.28	34
15	7.04	9.01	0.87	7.4×10^2	0.25	18
30	13.60	19.77	0.79	2.0×10^2	0.21	7

The association degree $(1 - \alpha)$, the fraction of specifically bound ions $(1 - \beta)$, the association (K_a), and binding (K_b) constants at 310 K.

they can move along the polyion surface, but they cannot leave the bound region. When the ionic strength increases, the electrostatic interactions of heparin are screened and the fraction of unbound Na^+ ions increases. Eq. 14 shows that all Na^+ ions can move freely in the solution when the ionic strength exceeds 0.45 M. Eq. 14 is in very good agreement with the recently published data of Winzor et al., who investigated the association of Na^+ ions with heparin, dextran sulfate, and polygalacturonate in solutions of the constant ionic strength, $I = 0.08$ M.[21] The association degree determined for heparin sodium salt is 0.68, whereas Eq. 14 gives the value 0.60 ± 0.02.

The association constant K_{as}, calculated from Eq. 13 decreases with increasing ionic strength. Such tendency is predicted by the Manning's theory.[16,17] Figure 3 shows a comparison of the theoretical and experimental values of K_{as}. As can be seen, the theoretical values of

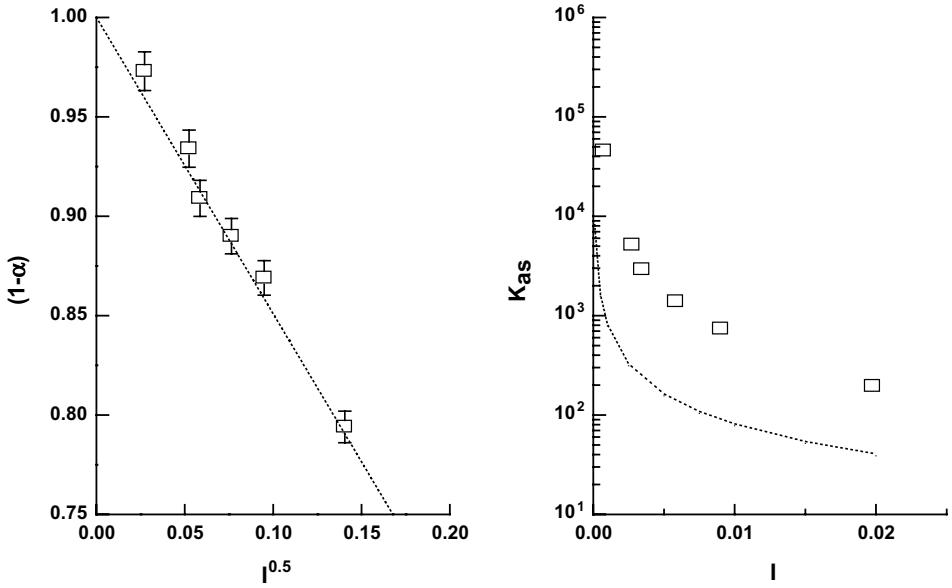

Figure 3. Influence of the ionic strength on the association degree $(1-\alpha)$ (left) and the association constant K_{as} (right) calculated from Eq. 13 (points) and estimated from Manning's theory (dotted line) in aqueous solution of heparin sodium salt at 310 K.

K_{as} are noticeably smaller than the experimental ones. Such discrepancy can be understood, because the values estimated from Manning's theory strongly depend on the assumed charge density of polyion.[16]

The association constant K_{as} decays exponentially with the increasing ionic strength, similar to what has been observed previously for binding of divalent ions[13] and methylene blue.[13–15] The K_{as} value, estimated in the limit of zero ionic strength, is about 5×10^5 and coincides with the extrapolated binding constant, about 1.15×10^6, for methylene blue[14] and the dissociation constant of carboxylic group of heparin, about 7×10^{-6}, determined in 0.009 M NaCl solution.[10]

The fraction of the exchangeable Na^+ ions, β, has been calculated from Eq. 12. $(1-\beta)$ represents the fraction of nonexchangeable Na^+ ions, which are bound with heparin by specific interactions. These results are listed in Table 2. The corresponding binding constant, K_b, has been computed from the following relation:

$$K_b = \frac{(c_{Na^+})_{loc}}{(c_{Na^+})_{non\text{-}loc}\cdot(c_{Hp^-})_{non\text{-}oc}} \equiv \frac{(1-\beta)\cdot(c_{HpNa})_0}{[\beta\cdot(c_{HpNa})_0+\Delta c_{NaCl}]\cdot[\beta\cdot c_{(HpNa)_0}]} \quad (15)$$

$(c_{Na})_{loc}$ denotes the concentrations of Na^+ ions bound locally with heparin. These ions interact with heparin by specific interactions. $(c_{Hp})_{non\text{-}oc}$ and $(c_{Na})_{non\text{-}loc}$ are concentrations of non-occupied negative charges of heparin and cations, which are non-localized on heparin surface, respectively.

There seems to be a common agreement[10] that Na^+ binding with heparin has a nonspecific, electrostatic character and thus the incomplete isotope exchange of $^{22}Na^+$ between solutions of NaCl and heparin sodium salt was astonishing. To be quite sure that not all Na^+ ions are exchangeable, the experiment was allowed to proceed for several days. However, this extension of time does not increase the β ratio. This incomplete isotope exchange suggests, therefore, that some of Na^+ ions must be bound with heparin by specific interactions.

The influence of the ionic strength on $(1-\beta)$ and the binding constant K_b is presented in Figure 4. As can be seen, in the limit of zero ionic strength, the fraction of unexchangeable Na^+ ions does not exceed 0.5. With increasing ionic strength the $(1-\beta)$ value decays exponentially and when the ionic strength exceeds 0.01 M the fraction of nonexchangeable sodium ions is less than 20%. It is important to stress that in most of studies of ion binding with heparin, the constant ionic strength is kept using NaCl solution and its concentration exceeds 0.01 M. Under such conditions, only a small percentage of Na^+ ions can be bound specifically with heparin and this can explain why Na^+ ions are commonly considered as territorially bound ions.

In the limit of zero ionic strength, the K_b value is less than 1×10^3 and decays exponentially with the ionic strength. When the ionic strength is higher than 0.01, the K_b value is less than 10 and thus is significantly smaller than the binding constant determined for divalent ions.[10]

4. Conclusions

The radiochemical method described above, which is based on Donnan equilibrium and radioisotope

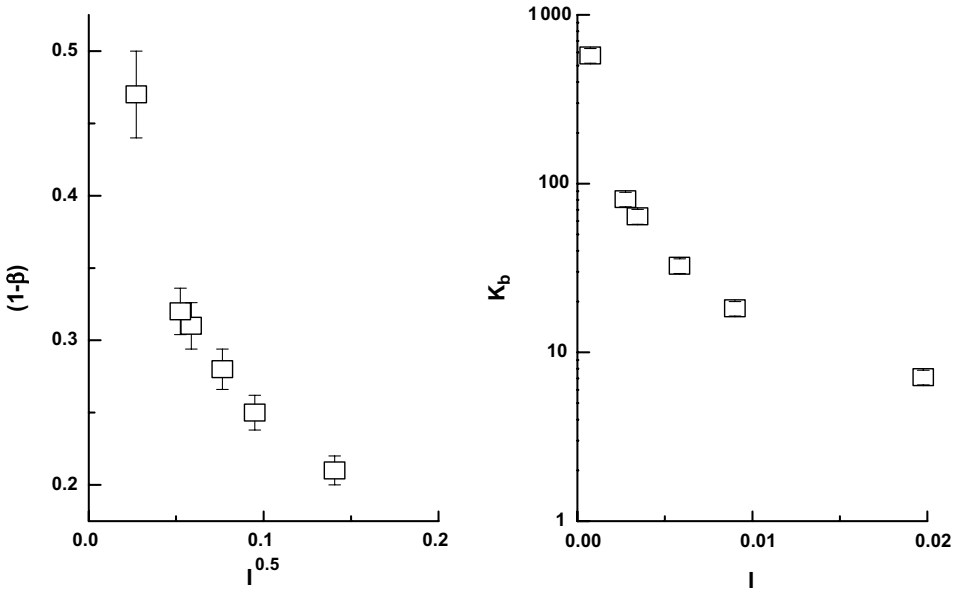

Figure 4. Influence of the ionic strength on the fraction of specifically bound Na^+ ions $(1-\beta)$ (left) and the binding constant K_b (right) in aqueous solution of heparin sodium salt at 310 K.

exchange, allows territorially and specifically bound counter-ions to be distinguished. Calculations of the fractions of the associated and nonexchangeable counter-ions have been derived without any ambiguous assumptions and therefore this method can be applied to the investigation of the binding of any ion with any polyelectrolyte. The only requirement of the method is to have ions labeled with radioisotopes. Measurements of the radioactivity are very precise and independent of system composition, therefore this method can be applied in a wide range of concentrations and for multicomponent systems.

In an aqueous solution of HpNa, heparin binds all Na^+ ions and about 45% of these ions are not exchangeable. The association degree decays exponentially with increasing ionic strength. When the ionic strength exceeds 0.45 M the screening of the electrostatic interactions of heparin is efficient therefore the association of Na^+ ions becomes negligible.

The fraction of Na^+ ions specifically bound with heparin decays exponentially with the increasing ionic strength and becomes negligible when the ionic strength is higher than 0.01. Although the extrapolated value of the binding constant is about 10^3, the K_b value decreases rapidly. When the ionic strength exceeds 0.01 the binding constant is less that 10. This explains why the specific binding of Na^+ ions has not been noticed previously.

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