Hydrogen Bonding and Electrostatic Interaction Contributions to the Interaction of a Cationic Drug with Polyaspartic Acid

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Purpose. To determine the mechanism and identify forces of interaction between polyaspartic acid and diminazene (a model drug). Such knowledge is essential for the design of polymeric drug delivery systems that are based on molecular self-assembly into complexes or micellar type systems.

Methods. Complex formation was studied by isothermal titration microcalorimetry and the McGhee von Hippel model was applied to obtain K_{obs}, ΔH_{obs}, and n_{obs}. The calorimetry data were compared with both an optical density study and the amount of free/complexed drug.

Results. The diminazene-polyaspartic acid interaction is enthalpically driven, whereby one diminazene molecule interacts with two monomers of polyaspartic acid. The dependence of K_{obs} on salt concentration reveals a contribution of electrostatic interactions. However, applying Manning's counter ion condensation theory shows that the major driving force for the complex formation is hydrogen bonding, with interfacial water molecules remaining buried within the complex. The modelling of the pH dependence of K_{obs} and ΔH_{obs} demonstrates that the ionization of carboxylic groups of polyaspartic acid is a prerequisite for the interaction.

Conclusions. Complex formation between diminazene and polyaspartic acid is driven by both electrostatic interactions and hydrogen bonding, with the latter being the dominating force. Although electrostatic interactions are not the major driving force, ionization of the drug and polymer is essential for complex formation.

KEY WORDS: isothermal calorimetry; binding parameters; supramolecular assemblies.

INTRODUCTION

The concept of a spontaneous molecular self-assembly into complexes or micellar like structures is receiving considerable attention in the formulation of delivery systems for drug targeting and gene therapy (1–5). The interaction between a drug (or DNA) and a polymer is central for the formation and stability of the molecular assembly formed. Hence, an understanding of the molecular forces that govern the interaction and self-assembly is fundamental for the design of such delivery systems. This necessitates preliminary studies to evaluate and determine the mechanisms of interaction between a drug or DNA and a polymer.

Isothermal titration calorimetry is an ideal method for studying such systems (ligand-macromolecule or macromolecule-macromolecule interactions), and it is further the only technique which allows simultaneous determination of all binding parameters (K, ΔH, ΔS and N) in a single experiment (6). Other advantages of the system include its high sensitivity, accuracy and precision (7), non invasiveness and destructiveness (8).

A model polymer-drug system has been investigated in this study. Polyaspartic acid has been chosen as a model polyanionic polymer to study its interaction with a model cationic drug, diminazene aceturate. Polyaspartic acid was chosen because it is a water soluble, non toxic and biodegradable polymer (9,10). Also, polyaspartic acid-polyethylene oxide copolymer has been used to produce micelles loaded with the enzyme (lysozyme), and the micelle formation has been attributed to ionic interactions between the cationic enzyme and the carboxylic acid groups of polyaspartic acid (4). Moreover, some pharmaceutical applications of polyaspartic acid are based on interaction/complex formation. For example, polyaspartic acid has been shown to decrease nephrotoxicity associated with aminoglycoside drugs. This is attributed to the formation of electrostatic complexes that prevent these drugs from interacting with anionic phospholipids and from causing cell injury and necrosis (11). Hence, this study would also be beneficial for these specific pharmaceutical applications of polyaspartic acid.

This study assesses the thermodynamics of the interaction of polyaspartic acid with a cationic drug. Their mechanism of interaction has been established by investigating the influence of salt (NaCl) concentration and the effect of buffer pH using isothermal titration calorimetry. The data for heat released at various NaCl concentrations were fitted to the McGhee van Hippel model (12) to determine the thermodynamic parameters.

MATERIALS AND METHODS

Materials

Poly(alpha,beta)-DL-aspartic acid, sodium salt, average molecular weight 12 300 Da was purchased from Sigma Chemicals, UK and used as received. Diminazene aceturate, Trizma^R hydrochloride (Tris HCl) and Sodium Chloride (reagent grade) were also bought from Sigma Chemicals, UK. Water used for the preparation of buffer solutions was ultrapure Elgastat^R Option 3 water (Elga Ltd., UK).

Isothermal Titration Microcalorimetry

Calorimetric experiments were performed using a Thermal Activity Monitor (TAM 2277, Thermometric AB, Sweden) operated at 298 K, as described previously (13). Samples of diminazene aceturate and polyaspartic acid were prepared in 25 mM Tris HCl buffer containing varying concentrations of NaCl, i.e., 25, 50, 150, 250, 350, 500 mM and adjusted to pH 5.3 $\pm$ 0.05. In each experiment, a 3 mL sample of a 0.4 mg/mL solution of diminazene aceturate was placed in a sample cell and the titration was performed by consecutive injections (20 μL) of a solution of polyaspartic acid (2.85 mg/mL). Heats of dilution/mixing were determined in blank titrations by injecting aliquots (20 μL) of polyaspartic acid (2.85 mg/mL) into the appropriate NaCl solution (3 mL). The experimental method was set up and data collected via the Digitam^R software. Data presented are the mean of a minimum of 2 replicate

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titrations at each NaCl concentration. A typical output of the raw microcalorimetry data for the injection of polyaspartic acid into diminazene aceturate and a blank experiment are shown in Fig. 1. The pH change of all samples after the experiment did not exceed ± 0.2.

Analysis of Binding Isotherms

The interaction of diminazene with polyaspartic acid was evaluated using the McGhee von Hippel (12) model for binding of non-interacting ligands to a lattice of ligand binding residues by applying the following equation

$$\frac{v}{[D_f]} = K(1 - nv)\left[\frac{1 - nv}{1 - (n - 1)v}\right]^{n-1} \quad (1)$$

where v denotes the ratio of bound drug (diminazene), $[D_b]$, over the total binding site concentration on the polymer (polyaspartic acid) which was set to the total number of monomers of the polymer in the solution, $[D_f]$ denotes the free drug concentration, K is the association (binding) constant, and n is the number of binding site (lattice residue) on the polymer covered by one drug molecule. The reaction heat content, Q_i after i injections is given by

$$Q_i = [D_b]\Delta H^0 V_i \quad (2)$$

where ΔH° denotes the standard enthalpy of complex formation and V_i is the volume of solution in the cell after i injection. The following relation between total drug concentration, $[D_{total}]$, $[D_f]$, and $[D_b]$ is used:

$$[D_{total}] = [D_f] + [D_b] \quad (3)$$

The equilibrium binding parameters, K, ΔH, n were determined by applying the non-linear least square routines available within the Origin 5.0 (MicroCal Software) to equations 1 to 3. It should be noted that integrated heats of bindings were corrected for the enthalpy of dilution, ΔH_d, before calculation of equilibrium binding parameters. A value of ΔH_d for each reaction was obtained by averaging the released heats for the end injections of a titration isotherm, where the complex formation was completed (14,15) (Fig. 1). However, blank titrations for each reaction were also performed to ensure that ΔH_d was small and constant in comparison to the heat of binding (Fig. 1, blank experiment). The corresponding ΔH_d was subtracted from the integrated released heat for each injection. The concentration of the reactants was such that the experimental c value was >1 (varied between 5 and 78), where $c = K_{obs}[M_{total}]$, and $[M_{total}]$ denotes the concentration of diminazene in the calorimetric cell. The validity of this condition allowed an accurate determination of the binding parameters. Since the concentration of the reactants rather than their activities was used, this is indicated by affixing the subscript "obs" to the calculated binding parameter. The free energy of complex formation (ΔG_{obs}) was determined from $\Delta G_{obs} = RT\ln(K_{obs})$, and the entropy of binding was calculated from $\Delta S_{obs} = (\Delta H_{obs} - \Delta S_{obs})/T$.

Molecular Modelling

The three dimensional structure of diminazene molecule was estimated *in vacuo* by applying the MM3 force field model

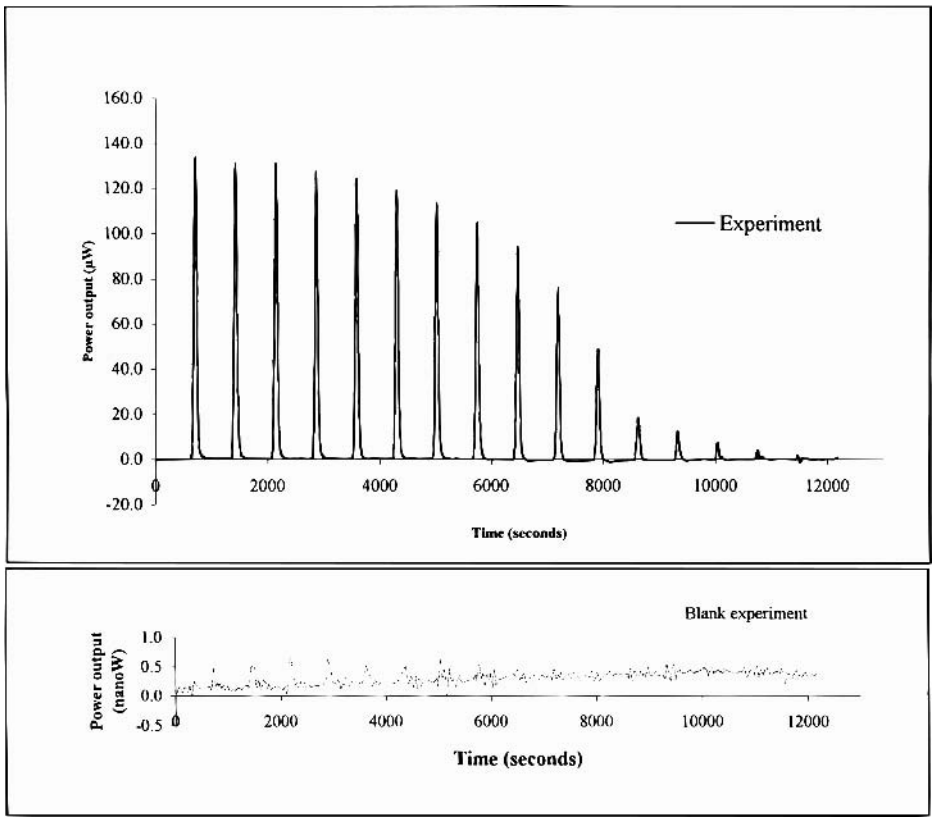

Fig. 1. Experimental calorimetric data for the isothermal titration of polyaspartic acid into diminazene. The blank titration i.e. injection of polyaspartic acid into 25 mM NaCl is also shown.

using routines available within the Chembuilder 3D programme (Interactive Simulation, Inc.).

Optical Density Studies

Aliquots (20 μL) of polyaspartic acid solution (2.85 mg/mL) were added to 3 mL diminazene aceturate (0.4 mg/mL) solution and stirred for 20 seconds. (The samples were prepared with 25 mM Tris HCl buffer and adjusted to pH 5.3.) The optical density of the preparation after each aliquot addition was determined at 490 nm (Pharmacia LKB Biochrom Ultrospec 4000 Spectrophotometer). The experimental conditions were chosen to mimic the titration calorimetry experiment.

Quantification of Free/Complexed Drug

Various aliquots of polyaspartic acid solution (2.85 mg/mL) were added into separate test tubes containing 3 mL diminazene aceturate solution (0.4 mg/mL). (The samples were prepared with 25 mM Tris HCl buffer and adjusted to pH 5.3). The contents of the test tubes were mechanically shaken for 10 minutes and centrifuged at 3600 rpm for 10 minutes. From each test tube, 1 mL of the supernatant was removed and diluted to 50 mL with 25 mM Tris HCl buffer. Diminazene aceturate in the supernatant was quantitated by UV at 369 nm (Pharmacia LKB Biochrom Ultrospec 4000 Spectrophotometer).

RESULTS AND DISCUSSION

Turbidimetry Studies and Quantification of Free/Complexed Drug

Figure 2 illustrates two corresponding experiments (i) turbidity for titration of polyaspartic acid into the diminazene solution and (ii) the amount of free drug in the buffer, expressed as a function of drug/polyaspartic acid monomer ratio. The turbidy curve shows that the optical density of the solution gradually increases as the drug/polymer monomer ratio increases. This can be attributed to the phase separation of the drug-polymer complexes (agglomerates) from the solution due to charge neutralization. The curve reaches a maximum at $x_{max} = 2$, which indicates that at this point each diminazene molecule complexes with two monomers of polyaspartic acid. At the same drug/polyaspartic acid monomer ratio there is a minimum in the curve depicting the amount of free drug in the buffer (21.74% of the initial present drug is free with 79.26% in the complex). At the ratios higher than 2:1 the amount of free drug increases and optical density decreases, both trends indicating that the complex formation is a reversible process where the complex gradually dissociates with addition of an excess of polyaspartic acid.

Salt Dependence of the Binding Constant

The integrated heats of complex formation between diminazene and polyaspartic acid (corrected for the heat of dilution at different salt concentrations) and with the best fit curves (applying the McGhee von Hippel model to the experimental data) are shown in Fig. 3. The corresponding equilibrium binding parameters are presented in Table I. The data clearly indicate that as the salt concentration increases from 25 mM to 500 mM NaCl, the observed binding constant, K_{obs}, decreases from 1.13×10^5 M^{-1} to 7.07×10^3 M^{-1}. The salt dependence of the observed binding constant is shown in Fig. 4. It can be seen that log K_{obs} varies linearly with log [NaCl]. The linear regression analysis yields a slope of -1.06 ± 0.12 and an intercept of -3.44 ± 0.12. Manning's theory (in the absence of anion release) gives the following relationship (16):

$$\frac{\partial \ln K_{obs}}{\partial \ln[NaCl]} = -\mathbf{Z}\psi \tag{4}$$

where Z represents the number of electrostatic interactions of

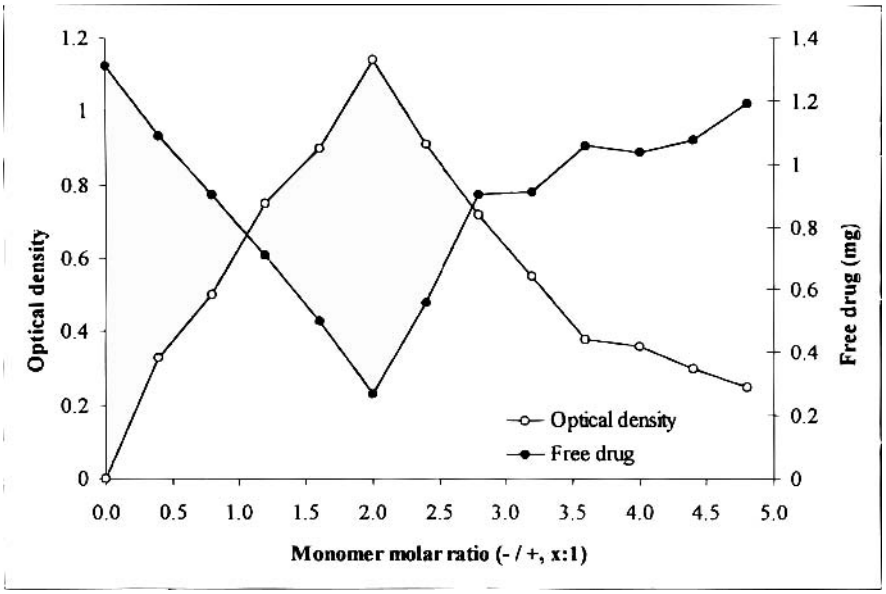

Fig. 2. Turbidimetry study for the addition of polyaspartic acid into diminazene at varying mole drug:monomer charge ratios. The free drug in the supernatant at each mole drug:monomer charge ratio is also shown.

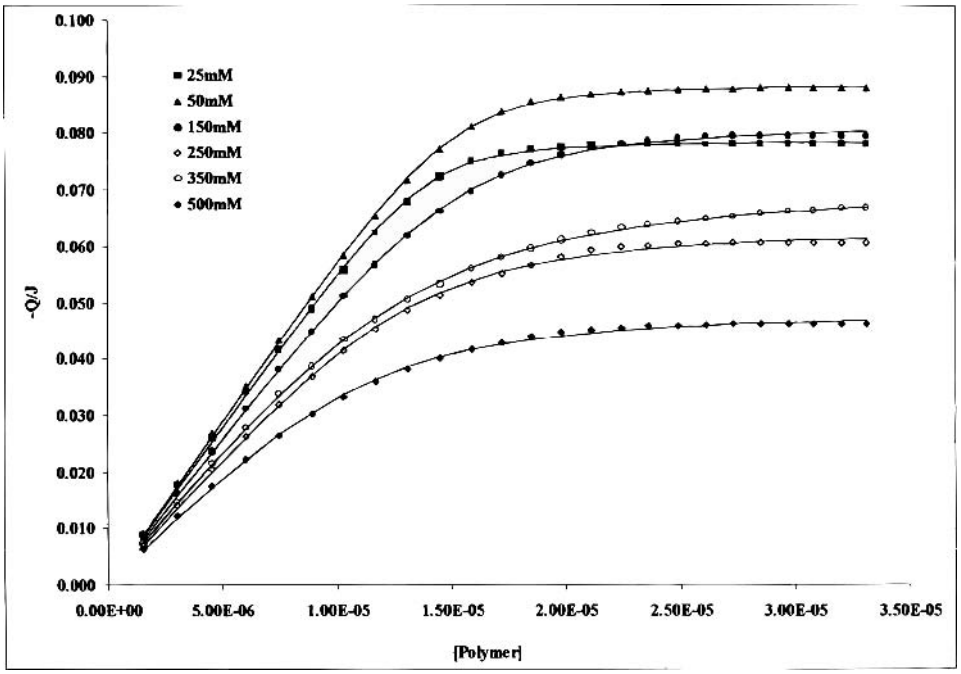

Fig. 3. Integrated heats of interaction corrected for heats of dilution from titrations of polyaspartic acid into diminazene in varying concentrations of NaCl solution. Solid lines represent the best fit binding isotherms to the experimental data.

Table I. Binding Parameters of the Interaction of Diminazine with Polyaspartic Acid at Different Salt Concentrations and Contribution of Electrostatic and Non-Electrostatic Free Energy to the Total Free Energy Change of Interaction

[NaCl] (mM)	n	K_{obs} (M^{-1})	ΔH_{obs}° (kJ/mol)	$T\Delta S_{obs}^{\circ}$ (kJ/mol)	ΔG_{obs}° (kJ/mol	ΔG_{es}° (kJ/mol)	ΔG_{ns}° (kJ/mol)	$\Delta G_{ns}^{\circ}/\Delta G_{es}^{\circ}$
25	1.76	1.13×10^5	−37.53	−8.70	−28.83	−9.14	−19.69	2.15
50	1.88	8.46×10^4	−42.37	−14.26	−28.11	−7.42	−20.69	2.78
150	1.83	2.71×10^4	−39.16	−13.87	−25.29	−4.71	−20.59	4.38
250	1.56	1.19×10^4	−30.39	−7.13	−23.25	−3.43	−19.18	5.77
350	1.52	5.44×10^3	−34.14	−12.83	−21.31	−2.60	−18.71	7.19
500	1.35	7.07×10^3	−23.46	−1.50	−21.96	−1.72	−20.24	11.79

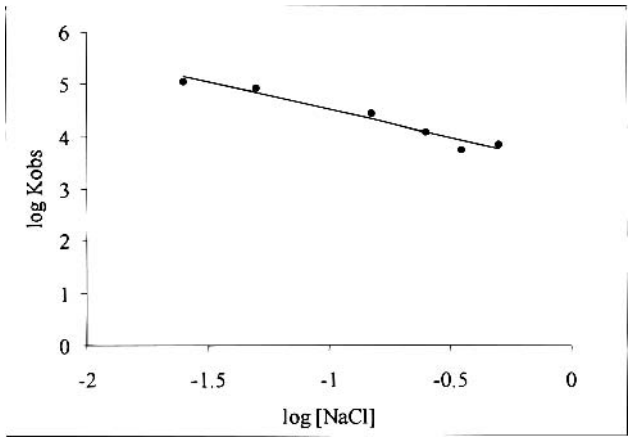

Fig. 4. Variation of the association constant (K_{obs}) for the interaction of polyaspartic acid with diminazene as a function of salt concentration.

a drug molecule (usually the electrical charge of the drug molecule) with a polymer, and ψ denotes the fraction of a counterion, M^+, that is thermodynamically bound to the polymer (polyion). Since diminazene molecule has two amine groups ionized at pH 5.3 (the experimental condition) Z can be assumed to be 2. The experimental results shown in Fig. 2 are in agreement with this assumption and they suggests that diminazene molecule neutralizes the carboxyl groups in the ratio of one diminazene molecule to two polyaspartic acid monomer units. Also, it has been shown that $Z = 2$ for the interaction of 4′,6-diaminodino-2-phenylindole (molecular structure of which is similar to diminazene) with DNA and that this molecule can be accounted as a divalent cation (17). By setting $Z = 2$ and using Equation 4, ψ was calculated to be 0.5 ± 0.05. According to Manning's theory, ψ is related to the average distance of electrical charge points on the polymer (polyion), b, with:

$$\psi = 1 - \frac{1}{2\zeta} \tag{5}$$

and

$$\xi = \frac{e^2}{\varepsilon kTb} \tag{6}$$

where ε denotes the dielectric constant of the solvent, k is the Boltzmann constant, and T denotes the absolute temperature. By introducing the calculated ψ into equations 5 and 6, b was obtained to be 7.2 ± 0.7 Å (at 25°C). This value shows that the distance between the electrical charges on the polyaspartic acid molecule is considerable. NMR studies on the hydrated polyaspartic acid in the literature support this finding (18).

From the calorimetry results at different salt concentrations (Table I and Fig. 4) it can be concluded that electrostatic interactions between diminazene and polyaspartic acid contributes to the interaction. However, the considerable intercept of the best-fit curve in Fig. 4 suggests the existence of an appreciable non-electrostatic interaction between the polymer and the drug. This is supported strongly by the entropy changes of the complex formation (Table I) which have negative values.

It should be noted that by increasing the salt concentration ΔH_{obs}° also decreased (Table I). The possible explanation may be that an increased salt concentration affects the polymer conformation in such way that it hinders its optimal interaction with the drug (which is reflected in n_{obs}) and also that the salt concentration affects the interaction by affecting the polymer ionic atmosphere (which is not predicted by Manning's theory).

Non-Electrostatic Interaction between Drug and Polymer

The existence of non-electrostatic interactions between a ligand and polyion can be assessed from the study on salt dependence of the binding constant. Applying this approach, the total free energy change of the interaction, ΔG_{obs}, is dissected to the free energy change of electrostatic ΔG_{es} and free energy change of non-electrostatic interactions. ΔG_{ns}, ΔG_{obs}, ΔG_{es}, and ΔG_{ns} are calculated from the following equations (19).

$$\Delta G_{obs} = -RT \ln K_{obs} \tag{7}$$

$$\Delta G_{es} = RTZ\psi \ln [M^+] \tag{8}$$

$$\Delta G_{ns} = \Delta G_{obs} - \Delta G_{es} \tag{9}$$

The corresponding values of ΔG_{obs}, ΔG_{es}, and ΔG_{ns} at different salt concentrations are shown in Table I. The values obtained clearly show that by increasing the salt concentration, the effect of the electrostatic contribution decreases (the absolute value of ΔG_{es} decreases) whereas ΔG_{ns} remains nearly unchanged at about −20 kJ/mol. Consequently, the ratio of $\Delta G_{ns}/\Delta G_{es}$ increases from 2.15 at 0.025mM NaCl to 11.79 at 500 mM NaCl concentration. This indicates that the non-electrostatic interaction plays the major part in the binding. ΔG_{ns} has a negative sign (Table I), which indicates that the non-electrostatic interaction is a favourable interaction. The nature of the non-electrostatic interaction can be categorised from the ΔH_{obs}° and ΔS_{obs}° values (Table I). It can be seen that both ΔH_{obs}° and ΔS_{obs}° have negative signs, which is characteristic of hydrogen bond formation (20). The negative entropy change indicates that the mobility of the interacting sites is decreased as the result of hydrogen bonding and that the interaction is further probably not accompanied by a release of water molecule(s) from the polymer drug interface (although the release of condensed counterions upon complex formation of the drug and polymer is entropically favourable). The existence of the water molecules within a complex has been demonstrated previously (21). Moreover, we found that K_{obs} (at 25 mM NaCl) in the presence of 500 mM sucrose was not significantly different from K_{obs} in the absence of sucrose, indicating that complex formation was not accompanied by the significant release of water molecules (data not shown).

Applying the force field MM3 molecular modelling, the lowest molecular energy of diminazene *in vacuo* was obtained. It was found that the distance between amine groups fall in the range of 10–12 Å. It was calculated in the above section that for polyaspartic acid the mean distance, b, between two successive carboxylic groups equals to 7.4 Å. It can therefore be suggested that in the complex form, an amine group of diminazene and a carboxylic group of polyaspartic acid are in close proximity. Also, since our previous study for the interaction of procaine with polyacrylic acid revealed that the interaction of amine groups with carboxylic acid provides a very weak hydrogen bond formation (13), it is more likely that the hydrogen bonds are formed between the amidine groups of diminazene and carboxylic groups of polyaspartic acid.

Dependence of Binding Constant on pH

Our study shows that by increasing the pH of the buffer K_{obs} for the diminazene-polyaspartic acid interaction also increases (Table II). The plausible explanation is that the NH_2 groups in polyaspartic acid chain become deprotonated at a higher pH. This results in a decreased repulsion between the carboxyl groups and consequently, an increase in the electrical charge density of polyaspartic acid. A similar finding has been shown for the α-NH_2 group of polylysine (pK_a value 7.2) which becomes deprotonated by increasing the pH (22).

Another aspect to be considered in the pH dependence of K_{obs} is the pH dependence of the ionization of the carboxylic group of polyaspartic acid. Assuming that the pK_a for carboxyl group of polyaspartic acid is about 4, the ionisation degree for polyaspartic acid in the calorimetry studies performed at different pH values (Table II) will change from approximately 0.5 at pH 4 to 0.95 at pH 5.3 with almost complete ionisation at pH values 7 and 9. Therefore, the pH determines the fraction of ionised carboxyl groups available for interaction with diminazene.

Hence, from the above discussion the pH dependence of K_{obs} can be divided into two contributions. The first one, increased charge density of polyaspartic acid at higher pH due to deprotonation of NH_2 groups, may be described by (23):

$$\frac{1}{b} = \frac{[1 + (1 + K_{\alpha-NH_2}\, a_H)^{-1}]}{b_0} \tag{10}$$

Table II. pH Dependence of K_{obs} and ΔH_{obs}^{o}

pH	K_{obs} (M^{-1})	n_{obs}	ΔH_{obs}^{o} (kJ/mol)
4.0	5.68×10^3	1.37	−14.47
5.3	1.44×10^4	1.58	−30.45
7.0	2.13×10^4	1.71	−32.06
9.0	2.85×10^4	1.81	−34.09

where b_0 is the axial charge spacing when all NH_2 groups are protonated, a_H is the proton activity, and $K_{\alpha-NH2}$ is the protonation constant of NH_2 groups. The second contribution, the effect of degree of ionization of carboxylic groups on the number of electrostatic interactions, Z, can be given by

$$Z = 2(1 + K_{COOH}\, a_H)^{-1} \quad (11)$$

where K_{COOH} denotes the protonation constant of carboxylic groups of polyaspartic acid. Assuming that each carboxyl group in polyaspartic acid represents one binding site and that one amine group of diminazene interacts electrostatically with the carboxyl group, then $Z = n$. This assumption is supported by the studies shown in Fig. 2. Equation 11 is valid as long as the amine groups of diminazene are completely protonated. Coefficient 2 in Eq. 11 implies the maximum number of electrostatic interactions when all the carboxylic groups are completely ionized.

Considering both discussed effects, the dependence of K_{obs} on pH and $[M^+]$ can be given by:

$$\log K_{obs} = \log K(1M) - 2(1 + K_{COOH}\, a_H)^{-1} \times \left[1 - \left[14.28\, \frac{[1 + (1 + K_{\alpha-NH_2} a_H)^{-1}]}{b_0}\right]^{-1}\right] \log[M^+] \quad (12)$$

Equation 12, with the following parameters log K (1 M) = 3.49 M^{-1}, pK_{COOH} = 4.25, p$K_{\alpha NH2}$ = 7.33, b_0 = 5.81 A and with log $[M^+]$ = −0.6, fits the experimental data (Table II) with good agreement (Fig. 5). It should be noted that a good fit to the experimental data was obtained when the values used for the equation parameters are in agreement with the experimental or reported values for these parameters. For example, the calculated p$K_{\alpha-NH2}$ and pK_{COOH} values are very close to the reported values, log$K$ (1 M) is in line with the values calculated from the salt studies (Table I), and b_0 value indicates the extended structure of polyaspartic acid. Therefore, Eq. 12 can be applied to predict the dependence of K_{obs} on pH. Also, it is interesting to note that Eq. 11 predicts that Z (alternatively n) should approach 2 when pH increases, which is indeed exactly what the values for n_{obs} in Table II are depicting.

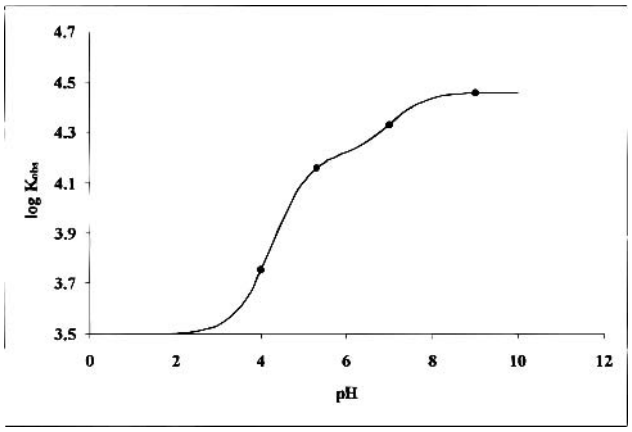

Fig. 5. Variation of the association constant (K_{obs}) for the interaction of polyaspartic acid with diminazene as a function of pH. Solid lines represent the best fit binding isotherms to the experimental data.

pH Dependence of ΔH_{obs}°

The results in Table II show that ΔH_{obs}° decreases (the absolute value increases) by increasing the pH. The pH dependence of H_{obs}° can be expressed by taking the derivative of Equation 12 with respect to 1/T, given by the following equation (23),

$$\Delta H_{obs}^{\circ} = \Delta H^{\circ}(1M) + 2\,(1 + K_{COOH}\, a_H)^{-1} \log[M^+]\, \frac{b_0}{14.28} \times \frac{K_{\alpha-NH_2}\, a_H}{(1 + K_{\alpha-NH_2}\, a_H)^2\,(1 + (1 + K_{\alpha-NH_2} a_H)^{-1})^2} \Delta H_{\alpha-NH_2} + 2 \log [M^+] \left[1 - \left[14.28\, \frac{[1 + (1 + K_{\alpha-NH_2} a_H)^{-1}]}{b_0}\right]^{-1}\right] \frac{K_{COOH} a_H}{(1 + K_{COOH} a_H)^2}\, \Delta H_{COOH} \quad (13)$$

where $\Delta H_{\alpha-NH2}$ and ΔH_{COOH} denote the enthalpy of protonation of α-NH_2 and COOH groups respectively, and ΔH° (1 M) represent the value of ΔH_{obs}° at 1 M $[M^+]$. Figure 6 illustrates the experimental data for ΔH_{obs}° at different pH values (Table II) with the generated fitted curve obtained from Eq. 13 with the following parameters: $\Delta H_{\alpha-NH2}$ = −14.4 kJ/mol, ΔH_{COOH} = −47.85 kJ/mol, ΔH° (1M) = −34.17 kJ/mol, log$[M^+]$ = −1.386, pK_{COOH} = 4, p$K_{\alpha-NH2}$ = 7.33, and b_0 = 5.8 Å (the same values that were used for the simulation in Fig. 5). It can be seen that the generated curve fits the experimental data in good agreement.

The data clearly show (Table II and Fig. 6) that ΔH_{obs}° decreases by increasing the pH. This can be explained by an increased number of ionized carboxylic groups at a higher pH, which enables the diminazene molecules to interact with both its amine and amidine groups with polyaspartic acid, therefore resulting in the formation of more hydrogen bonds. This explanation can be supported by the n_{obs} values presented in Table II that approaches 2 as the pH increases to 9. Also it is seen from Fig. 6 that ΔH_{obs}° becomes almost independent of pH changes above pH 6. This can be explained by the relationship between ΔH_{obs}° and n_{obs}. As n_{obs} becomes pH independent at a pH above 6, so does the ΔH_{obs}°.

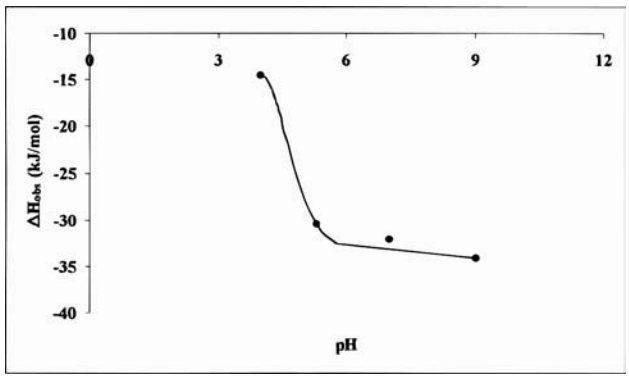

Fig. 6. Variation of the enthalpy change (ΔH_{obs}) for the interaction of polyaspartic acid with diminazene as a function of pH. Solid lines represent the best fit binding isotherms to the experimental data.

Taking into account that the enthalpy of electrostatic interaction is very small and close to zero (20), it is likely that the obtained ΔH_{obs}° originates mostly from a formation of hydrogen bonds. ΔH of hydrogen bond formation in bulk water (at 298 K) was calculated to be −9.8 kJ/mol (24). However in the vicinity of solutes the hydrogen bond is stronger, due to perturbation from the solute (25). For example, ΔH for the hydrogen bond in the hydration shell of human recombinant FKBP-12 is −17.6 kJ/mol (25). Therefore the observed enthalpy of binding for the interaction of diminazene with polyaspartic acid of −37.51 kJ/mol (at 25mM NaCl and pH 5.3) would represent a formation of 2 to 3 hydrogen bonds. It is possible that ΔH_{obs}° from the hydrogen bonds arises mostly from formation of hydrogen bonds with the amidine groups of diminazene and polyaspartic acid. Namely, we have shown in our previous work (13) that an amine group can form a hydrogen bond with a carboxyl group, however this does not have an appreciable enthalpy of interaction.

It is questionable whether the ΔH_{obs}° value also includes the enthalpy of the ionization of buffer and the intrinsic enthalpy of complex formation (26) and also if these significantly contribute to ΔH_{obs}°. This is unlikely for the present study since the ΔH_{obs}° and K_{obs} both become pH independent at a higher pH, where the interacting sites are completely ionized, and do not need to consume a proton from the buffer for the complex formation (27).

CONCLUSIONS

Although complex formation between diminazene and poly(aspartic) acid is driven by electrostatic interactions, however, it is hydrogen bond formation which is the dominant force in the complex. The enthalpy of complex formation, ΔH_{obs}°, suggests formation of two to three hydrogen bonds between the drug molecule and polymer. The entropy of complex formation, ΔS_{obs}° is negative, indicating that the mobility of the interacting sites is decreased significantly on complexation. Water molecules remain in the drug-polymer complex interface and they mediate the hydrogen bonds between the drug and the polymer. Therefore moderate variations in water activity in different environments (*in vitro vs in vivo*) do not affect on the affinity of the drug-polymer interaction.

Application of Manning's theory allowed modelling of the salt and pH dependence of K_{obs}. The modelling shows that the binding parameters (K_{obs} and ΔH_{obs}°) are dependent on the pH in the region between 4 and 6 and that at higher pH values this dependence declines. This indicates that ionization of both species, carboxyl groups of polyaspartic acid and amine groups of diminazene, are required for the optimal interaction of the drug and polymer.

Modelling of pH and salt dependence of binding parameters and also the determination of detailed thermodynamic characteristic of the drug-polymer complex allow a prediction of the state of the complex in different biological environments It also aids to determine optimum conditions for the production of drug-polymer complexes as a drug delivery system.

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