Hydrophobic Ion-Pairing of Low Molecular Weight Heparin with Cetyltrimethylammonium Bromide

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Abstract

Purpose: Unfavorable physicochemical properties of low molecular weight heparin (LMWH) restrict membrane permeation of this therapeutic macromolecule to paracellular passive diffusion. To augment LMWH flux across biological membranes using contributions of transcellular transport mechanisms, this study explored formation of electrostatically stabilized association complexes via ion-pairing with the lipophilic cetyltrimethylammonium bromide (CTAB).

Methods: LMWH/CTAB interactions were assessed in acetate buffer, pH 5.0, using molar ratios ranging from 1:0 to1:40. Unbound LMWH was quantified spectrophotometrically at λ=630 nm using the *Azure A* assay. Dynamic laser light scattering technology was applied to estimate hydrodynamic diameter and zeta potential of electrostatically stabilized LMWH/CTAB association complexes.

Results: Unbound LMWH concentrations significantly decreased from 95.9±2.3% to 14.4±2.6% in the presence of CTAB at a molar ratio of at least 1:30. In parallel, formation of colloidal association complexes was detected exhibiting mean hydrodynamic diameters between 400-500 nm and maximum zeta potential values of +1.85±0.80 mV. Using Job's continuous variation method, a 1:1 stoichiometry of LMWH/CTAB association complexes in acetate buffer, pH 5.0, was concluded. Benesi-Hildebrand and Scatchard transformations of experimental binding data revealed an estimated formation constant for LMWH/CTAB complexes at pH 5 of 1.1×10^{-7} and 1.2×10^{-7} L×mol^{-1}, respectively.

Conclusion: The results from this study suggest weak ion-pairing between LMWH and CTAB in acetate buffer, pH 5.0. The slightly positively charged nanocomplex is predicted to access transcellular transport mechanisms more effectively than the highly negatively charged LMWH. As a consequence, electrostatically stabilized LMWH/CTAB association complexes may enhance overall permeation properties of LMWH across biological membranes.

Keywords: *association complex, stoichiometry, Job's method, Benesi-Hildebrand plot, Scatchard plot*

Running Head: Ionic Heparin Nanocomplexes

Introduction

Unfractionated heparin (MW=5,000-30,000 Da) and low molecular weight heparin (LMWH, MW<5,000 Da) are the most commonly used antithrombotic and thrombo-prophylactic agents in hospital practice. As a highly sulfated, anionic polysaccharide composed of repeating glucosamine and uronic acid residues [1], heparin and its derivatives exhibit unfavorable physicochemical properties such as size, charge, and hydrophilicity that restrict membrane permeation predominantly to paracellular diffusion. Combined with the high susceptibility of heparin for enzymatic degradation under acidic conditions in the stomach or heparinase-mediated cleavage in distal segments of the gastrointestinal tract, clinical administration is usually limited to parenteral injection [2-4].

Hydrophobic ion-pairing is a drug delivery strategy that alters unfavorable physicochemical properties of an ionizable hydrophilic molecule by simple electrostatic association with a hydrophobic counter ion. Examples of hydrophobic moieties that were successfully used for hydrophobic ion-pairing include fatty acids and ionizable surface-active agents. Since ion-pairing is driven by non-covalent electrostatic attraction between opposite ionic charges, safety and efficacy of drug molecules is usually not altered by this drug delivery approach [5]. However, ion-pairing has been demonstrated to profoundly improve solubility of ionic molecules in aprotic solvents, encapsulation efficiency of ionic compounds in lipophilic polymers, as well as membrane permeation of hydrophilic macromolecular drugs such as proteins and DNA, which resulted in significantly greater oral bioavailability of these drugs [6-8].

Earlier reports suggest that ion-pairing of LMWH with positively charged polymers such as polyethyleneimine effectively enhanced nasal absorption due to apparent reduction of the highly negative

surface charge of this drug molecule [9]. Similarly, formation of electrostatically stabilized association complexes between LMWH and deoxycholylethylamine significantly increased bioavailability and dose-dependent pharmacokinetics after oral administration [10].

The objective of this study was to explore electrostatic association between the negatively charged glycosaminoglycan LMWH and the hydrophobic cationic surfactant cetyltrimethylammonium bromide (CTAB) as a strategy to fabricate colloidal nanocomplexes carrying limited surface charge. The long-term goal of our research is to augment membrane permeation of LMWH by targeting transcellular transport mechanisms available for nanocolloids (e.g., vesicular transport). The results presented in this manuscript summarize physicochemical properties of electrostatically stabilized LMWH/CTAB complexes fabricated at molecular ratios ranging between 1:0 and 1:40.

Materials and Methods

Materials

Low molecular weight heparin (LMWH, average MW=4,500 Da)was obtained from MP Biomedicals (Solon, OH) whereas cetyltrimethylammonium bromide (CTAB) was purchased from Acros Organics (New Jersey, NJ). *Azure A* chloride salt was received from Amresco (Solon, OH). All other chemicals and solvents were of analytical grade and used without further purification.

Methods

Formation of LMWH/CTAB Association Complexes

Complexes between LMWH and CTAB at molar ratios ranging from 1:0 to 1:40 were prepared by mixing different volumetric aliquots of a 1.4 mM CTAB stock solution prepared in a mixture of 4.5 M acetic acid and 0.2 M acetate buffer, pH 5.0, with a 6.67 µM LMWH stock solution prepared in 0.2 M acetate buffer, pH 5.0. CTAB solution was added dropwise to the LMWH solution under periodic mixing. After a 20 min equilibration period, colloidal suspension was subjected to a 30 min centrifugation at 4000×*g* (25˚C). Subsequently, unbound LMWH in the supernatant was quantified spectrophotometrically at λ=630 nm using the colorimetric *Azure A* assay as described in detail by Cadène and colleagues [11].

Physicochemical Properties of LMWH/CTAB Association Complexes

Particle size distribution of LMWH/CTAB complexes was determined by dynamic laser light scattering (DLS) using the Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW helium/neon laser (λ=633 nm) and a thermoelectric temperature controller. Before each measurement, the colloidal suspension was vortexed for at least one minute. Particle size values reported in this study correspond to hydrodynamic diameters. The zeta potential of colloids was estimated using electrophoretic mobility data acquired in 0.2 M acetate buffer, pH 5.0. Measurements were performed in triplicate.

Stoichiometry of LMWH/CTAB Association Complexes

The stoichiometry of electrostatically stabilized LMWH/CTAB ion pairs was estimated using the Job's method of continuous variation [12]. Equimolar master solutions (6.66 µM) of LMWH prepared in 0.2 M acetate buffer, pH 5.0, and CTAB prepared in a mixture of 4.5 M acetic acid and 0.2 M acetate buffer, pH 5.0, were combined at volumetric ratios ranging from 10:0 to 0:10. One of the major assumptions of the Job's method is that the difference in absorbance measured for LMWH in the absence of CTAB and for LMWH in the presence of CTAB is proportional to the complex concentration. Consequently, the stoichiometry of the LMWH/CTAB complex is determined by the graphical peak value in the Job's plot [13].

Stabilityof LMWH/CTAB Association Complexes

To assess the strength of the interaction between LMWH and CTAB in acetate buffer, pH 5.0, the equilibrium formation constant (K_f) of this electrostatically stabilized association complex was estimated using the Benesi-Hildebrand [14] and Scatchard transformations [15]. Briefly, Benesi-Hildebrand predicts that K_f is correlated to the unbound drug concentration according to:

$$[D]_T/\Delta A = 1/([S]_T.\varepsilon.K_f) + 1/(\varepsilon) \quad \text{(Eq. 1)}$$

where $[D]_T$ and $[S]_T$ represent the total concentrations of drug and surfactant respectively, while the difference in absorbance (ΔA) = A_{free} - $A_{mixture}$, and ε is the molar extinction coefficient of complex determined by linear regression analysis (i.e., 0.44 M^{-1} cm^{-1}).

Conversely, the Scatchard transformation facilitates quantitative estimation of the complex dissociation constant (K_{dis}) according to:

$$V_n/S_f = \left(n/K_{dis}\right) - \left(V_n/K_{dis}\right) \quad \text{(Eq. 2)}$$

where V_n is the fractional degree of saturation of the available macromolecule binding sites at increasing ligand concentrations, which can be expressed as the concentration of bound surfactant (S_b) to the total LMWH concentration (D_{tot}). S_f denotes the concentration of free surfactant in solution, whereas *n* represents the number

of binding sites. If all binding sites are equivalent and independent, a plot of $V/[S]_{free}$ vs. V results in a straight line. The slope of the line represents $-1/K_{dis}$, which numerically equals K_f.

Statistical Analysis

Experiments were performed at least in triplicate, and results are reported as mean ± standard deviation (S.D.). Statistical difference among various treatment groups was assessed using one-way ANOVA or two-sided Student's *t*-test for pairwise comparison. A probability of $p<0.05$ was considered statistically significant (GraphPad Prism 6.0, GraphPad, San Diego, CA).

Results and Discussion

LMWH/CTAB Interactions

In acetate buffer, pH 5.0, the predicted degrees of ionization of LMWH and CTAB are expected to facilitate ionic interactions between the positively charged ammonium ion of CTAB and the negatively charged sulfate groups of LMWH. A schematic of the proposed arrangement of LMWH and CTAB in these electrostatically stabilized association complexes is shown in Figure 1.

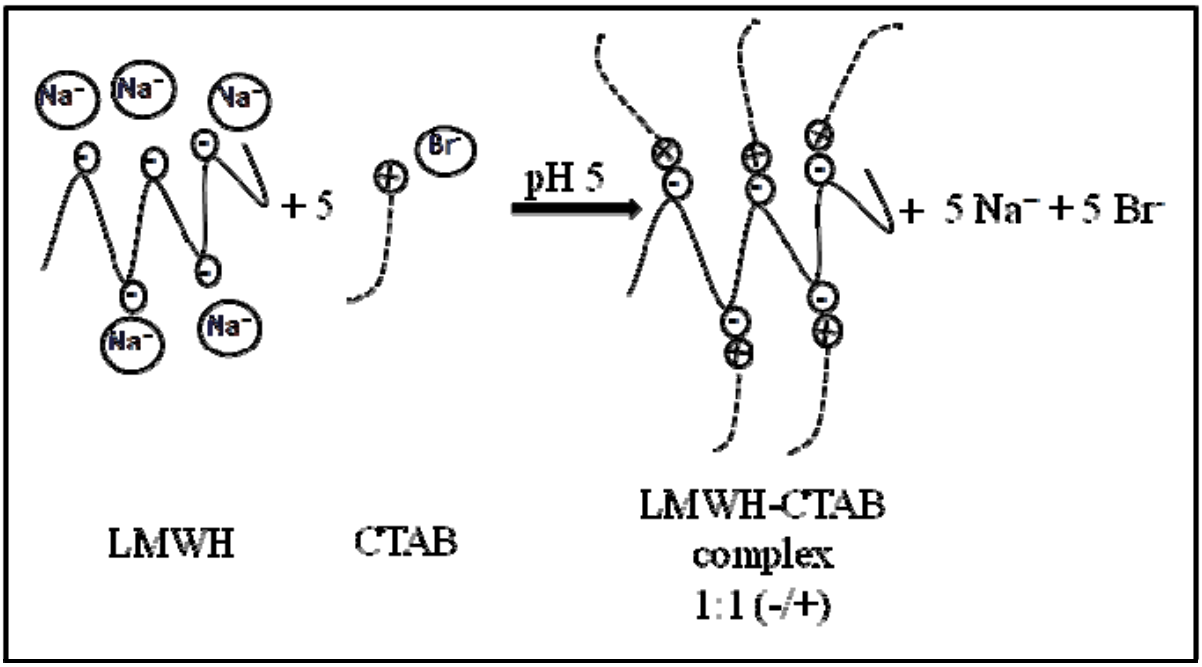


Figure 1: Schematic illustration of the predicted interaction of negatively charged LMWH with positively charged CTAB in acetate buffer, pH 5.0.

To test this hypothesis, increasing amounts of CTAB were added to a constant LMWH concentration, and the free LMWH concentration was quantified spectrophotometrically using the selective *Azure A* method. As summarized in Figure 2, the free LMWH concentration gradually decreased upon addition of CTAB suggesting molecular association of the oppositely charged ion species. At molar ratios ≥1:30, however, the free LMWH concentration remained unchanged, which implies that charge neutrality is reached at 1:30 [mol/mol] where all solvent-accessible sulfate groups within LMWH are engaged in ionic interactions with positively charged CTAB molecules. These conclusions are consistent with data from previous studies that demonstrated the formation of electrostatic interactions between the negatively charged heparin and the positively charged amino groups of polyethyleneimine in aqueous solution [9].

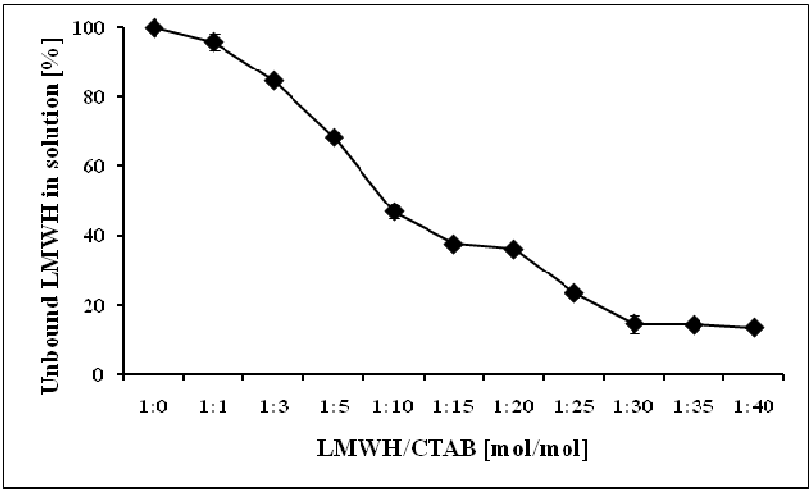
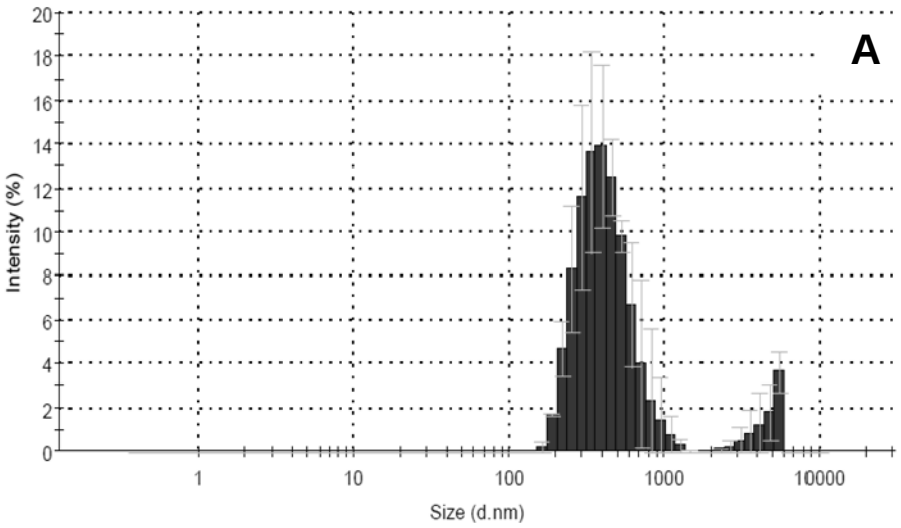


Figure 2: Complex formation of LMWH with CTAB in acetate buffer, pH 5.0. Data represent mean ± S.D. (n=3).

Physicochemical Properties of LMWH/CTAB Association Complexes

The interaction of oppositely charged species in aqueous systems is thermodynamically driven by both electrostatic and hydrophobic forces. In addition to macromolecule and ligand concentrations, the phase behavior of these mixtures is influenced by many molecular features. For the macromolecular species, this includes molecular weight, rotational freedom, degree of branching, charge density, hydrophobicity, and backbone rigidity. For the ligand molecule, polar head group and aliphatic chain length are known to most dramatically influence association behavior [16,17]. Particle size distribution measurements performed by DLS for LMWH/CTAB complexes at different molar ratios revealed a generally smaller particle size at low surfactant concentrations. In contrast, association complexes prepared at higher surfactant concentration appear to be larger in diameter (Table 1). Representative intensity-weighted size distribution profiles are shown in Figure 3. For LMWH/CTAB complexes prepared at a molar ratio of 1:1 (Figure 3A), two major particle populations with a mean hydrodynamic diameter of ~481nm and >1µm were identified. Considering the presence of excess negative charge due to LMWH at this equimolar ratio, it is predicted that the electrostatically stabilized LMWH/CTAB complex coexists with distinct LMWH and CTAB aggregates represented by the size range >1µm. When CTAB concentration increased, the larger particle size population disappeared suggesting greater efficiency in ion-pairing of individual LMWH molecules (Figure 3B). It is also conceivable that stronger macromolecule–surfactant associations at LMWH/CTAB = 1:30 [mol/mol] induced significant reduction in molecular extension of individual polysaccharide chains, hence resulting in a pronounced decrease in mean hydrodynamic radius. Alternatively, the reduction in overall negative charge of LMWH due to increased



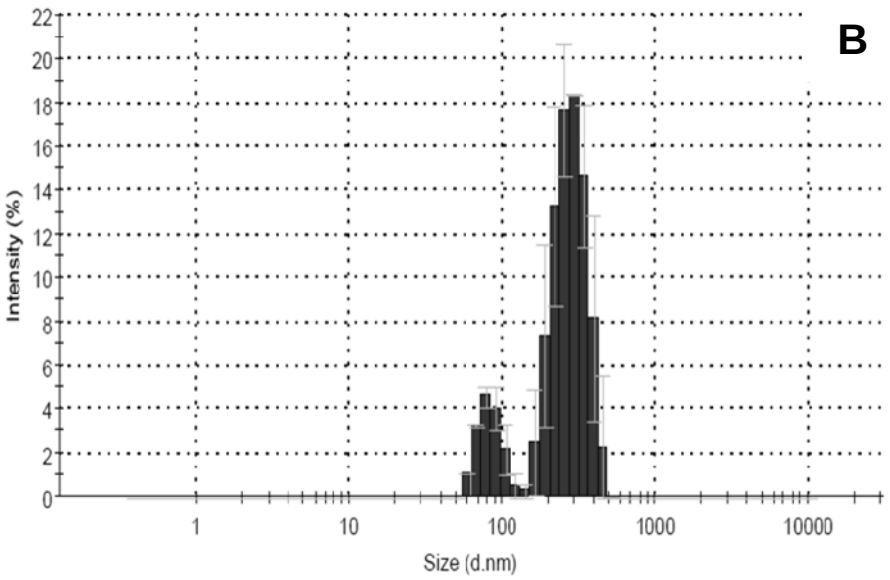


Figure 3: Representative intensity-weighted particle size distribution profiles of LMWH/CTAB association complexes measured in acetate buffer, pH 5.0, at a molar LMWH:CTAB ratio of 1:1 (Panel A) and 1:30 (Panel B), respectively.

CTAB concentrations may have been the results of limited polysaccharide chain extension, which translated into a reduced hydrodynamic particle diameter [18,19]. Trabelsi and colleagues studied the effect of chain length of cationic surfactants, incl. CTAB, on the internal organization of association complexes formed with carboxymethyl cellulose [20]. Although it is acknowledged that the chemical structure of LMWH significantly differs from that of carboxymethyl cellulose, thermodynamic processes such as rigidity of hydrophilic polymer chains as a function of accessible hydration layers may also impact the size distribution of electrostatically stabilized LMWH/CTAB association complexes. Consequently, future thermodynamic evaluation of these interactions using isothermal titration calorimetry may be suitable to understand molecular events underlying LMWH/CTAB ion pairing in greater detail.

Table 1: Hydrodynamic diameter of LMWH/CTAB association complexes prepared in 0.2 M acetate buffer, pH 5.0

LMWH/CTAB [mol/mol]	Size [nm] [a]	PDI [b]
1:1	481.5 ± 43.8	0.389
1:3	507.7 ± 30.2	0.711
1:5	280.7 ± 20.5	0.421
1:10	607.3 ± 38.1	0.814
1:15	92.6 ± 2.8	0.436
1:20	80.6 ± 0.9	0.349
1:25	301.2 ± 7.9	0.419
1:30	416 ± 3.4	0.465
1:35	517.7 ± 11.7	0.517
1:40	367.2 ± 24.6	0.435

[a] Data represent mean ± SD (n=3-4)
[b] PDI = polydispersity index.

As the association of oppositely charged species is expected to affect electrophoretic mobility of colloidal complexes within an electric field, the zeta potential of LMWH/CTAB complexes formed at different molar ratios was determined experimentally. In the absence of the cationic CTAB, electrostatic repulsion of negatively

charged LMWH was predicted to limit formation of colloidal association structures exhibiting a distinct zeta potential. Experimental data supported this hypothesis as only insignificant deviations from 0 mV were measured (-0.04±0.03 mV). However, inclusion of increasing CTAB concentrations resulted in formation of measurable particles with a maximum zeta potential of+1.85±0.8 mV at LMWH/CTAB = 1:30 [mol/mol]. These data imply that formation of LMWH/CTAB colloids is predominantly driven by ionic interactions and reaches saturation at charge neutrality. Similar results were reported by Langevin and co-workers, who assessed the formation of carboxymethyl cellulose/CTAB association complexes [21].

Stoichiometry and Stability of LMWH/CTAB Association Complexes

To estimate the molecular arrangement or binding stoichiometry of LMWH/CTAB association complexes and predict the stability of these ion pairs, conventional mathematical conversion strategies using experimentally determined binding data were applied. The results of Job's continuous variation method [22,23] suggest that LMWH/CTAB complex formation follows a 1:1 binding stoichiometry (Figure 4). This implies that at a molar ration of 1:30 every accessible anionic site of LMWH was interacting with a CTAB cation. The results were in good agreement with the reported values for the interaction of heparin with the *Azure B* dye [24]. It is important to note that the curvature of the line shown in the Job's plot (Figure 4) indicates formation of an association complex exhibiting relatively low stability. In this case, the predicted stoichiometry is obtained from the intersection of the extensions of these extrapolated lines. Reliability of this interpolation critically depends on the number of experimental data points coinciding with experimental data points [25].

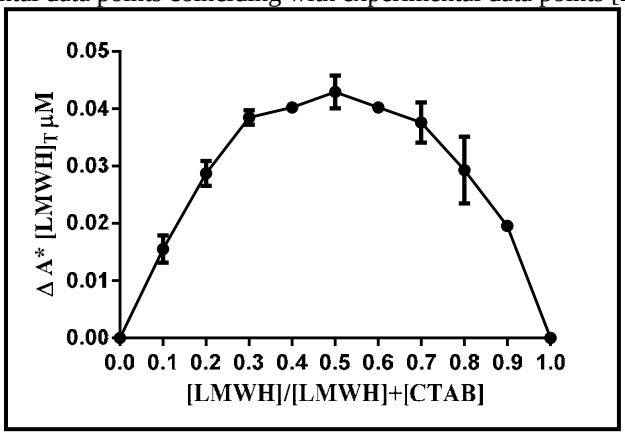
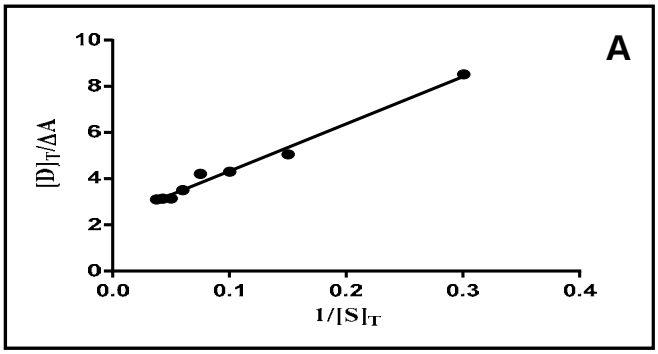


Figure 4: Continuous variation plot (i.e., Job's plot) of the change in absorbance as a function of the free LMWH mole fraction in the presence of CTAB. Experiments were performed in triplicate. Data are shown as mean ± S.D.

To estimate K_f of the electrostatically stabilized LMWH/CTAB association complex, binding data measured in the presence of different molar LMWH and CTAB ratios were transformed as described by Benesi-Hildebrand [14] and Scatchard [15].



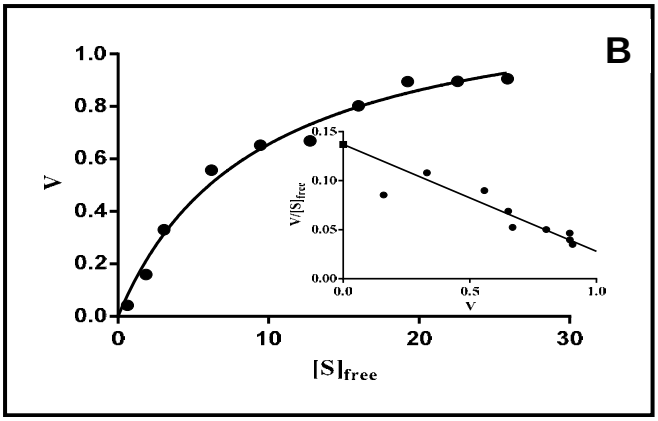


Figure 5: Estimation of the formation constant (K_f) for electrostatically stabilized LMWH/CTAB association complexes using the Benesi-Hildebrand (Panel A) and Scatchard (Panel B) transformations as outlined in Methods.

Figure 5A shows the result from the Benesi-Hildebrand transformation where the linear relationship between $[D]_T/\Delta A$ and $1/[S]_T$ predicted a K_f of 1.1×10^{-7} L×mol^{-1}. Conversely, Scatchard transformation (Figure 5B) predicts a K_f of 1.2×10^{-7} L×mol^{-1}. Both values obtained using different transformation approaches underline formation of thermodynamically weak LMWH/CTAB association complexes under given conditions. Table 2 also includes the complex formation constant estimated by the Job's method ($K_f = 2.6\times10^{-6}$ L×mol^{-1}). This value predicts a slightly more stable complex than the results from the Benesi-Hildebrand and Scatchard transformations. However, the numerical value of K_f estimated by the Job's methods may lack accuracy as only one LMWH master solution concentration was used.

Table 2: Estimated formation constants (K_f) for LMWH/CTAB association complexes

Model	Equation	K_f [L×mol^{-1}]
Benesi-Hildebrand plot	$\frac{[D]_T}{\Delta A} = \frac{1}{([S]_T \cdot \varepsilon \cdot K_f)} + \frac{1}{(\varepsilon)}$	1.1×10^{-7}
Scatchard plot	$\frac{V}{[S]_{free}} = n \cdot k_f - K_f \cdot V$	1.2×10^{-7}
Job's plot	$K_f = \frac{A/A_m}{[(1 - A/A_m)^{n+1}]C^n n^n}$	2.6×10^{-6}

Conclusion

The results from this study demonstrate that combination of CTAB and LMWH in acetate buffer, pH 5.0, facilitates formation of nanosized association complexes stabilized by ionic interactions. LMWH/CTAB ion pairs fabricated at a molar ratio of 1:30 exhibit significantly reduced surface charge, which is predicted to augment encapsulation of the highly hydrophilic LMWH into hydrophobic polymers. Future studies will focus on utilizing this hydrophobic ion pairing strategy to improve oral bioavailability of LMWH with the objective to augment absorption via transcellular transport mechanisms. Once released from hydrophobic carriers after absorption, the thermodynamically weak ion complex will assure complete dissociation of LMWH from CTAB, thus, regenerating the pharmacologically active moiety for therapeutic intervention.

List of Abbreviations

LMWH= low molecular weight heparin; CTAB= cetyltrimethylammonium bromide; DLS = dynamic laser light scattering

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