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Effect of buffer at nanoscale molecular recognition interfaces – electrostatic binding of biological polyanions

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We investigate the impact of an over-looked component on molecular recognition in water – buffer. The binding of a cationic dye to biological polyanion heparin is shown by isothermal calorimetry to depend on buffer (Tris-HCl > HEPES > PBS). The heparin binding of self-assembled multivalent (SAMul) cationic micelles is even more buffer dependent. Multivalent electrostatic molecular recognition is buffer dependent as a result of competitive interactions between the cationic binding interface and anions present in the buffer.

In biomolecular recognition, it is desirable to work in competitive aqueous media to mimic biological environments.[1] Binding must withstand electrolyte and buffers. Electrolyte can affect binding through charge-screening[2] or Hofmeister effects.[3] The impact of ions on host-guest binding,[4] self-assembly[5] and multivalent recognition[6] have been reported, and discussed with regard to binding constant determination.[7] However, the impact of buffer is less often explored.

In early biological studies it was recognized some buffers, particularly phosphate, have disruptive effects. A poorly chosen buffer can induce protein folding/unfolding,[8] interact with cell membrane components[9] or even affect cell growth.[10] As long ago as 1966, Good and co-workers outlined criteria for bio-relevant buffers.[11] In a key recent review, Soares and co-workers considered the (un)suitability of buffers, noting that even amongst Good's buffers differences could occur, especially as a result of metals interacting with buffer components.[12]

In supramolecular chemistry, although there have been reports in which buffer modifies metal selectivity of sensors,[13] buffer effects are rarely considered. Influential reviews on supramolecular chemistry in water[14] indicate many buffers are used, but with little discussion of the potential impact. A rare example of a buffer effect was reported in 2000 – increasing phosphate buffer concentration changed the binding of cationic porphyrins to anions,[15] primarily a result of ionic strength. Very rarely, specific buffer effects have been reported. Seto and co-workers reported the buffer effects on the electrostatic binding component between cationic cyclodextrins and phosphates;[16] Rebek and co-workers reported hydrophobic hosts with different binding affinities in pure water, tris and phosphate buffers, but said it was 'not readily understood'.[17] Specific buffer effects are more recognized in biochemical studies,[18] and given the emerging importance of supramolecular chemistry in biological settings,[1] buffers clearly deserve greater attention.

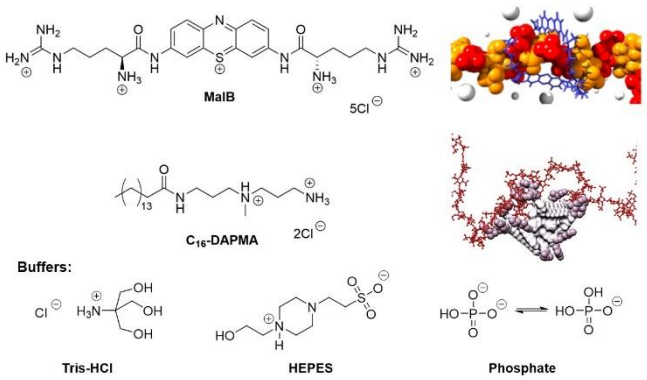

Figure 1. Structures of MalB, C_{16}-DAPMA and buffers, and computer modelling of the complexes formed between MalB and heparin (top right)[23a] and SAMul C_{16}-DAPMA and heparin (centre right).[25d]

The ionic nature of buffers means electrostatic binding is a prime candidate to be influenced by specific buffer effects. Electrostatic binding is a key biological mechanism, providing adhesion in competitive aqueous media.[19] Polyanions are vital in biology,[20] and we have been interested in binding polyanionic heparin as a result of its role in blood coagulation.[21] We developed Mallard Blue (MalB), a heparin-sensing dye (Fig. 1),[22] and in very preliminary work using UV-Vis spectroscopy, noted heparin binding varied in different buffers.[23] We have also developed self-assembled multivalent (SAMul) systems, in

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which self-assembly generates a nanoscale cationic ligand display that binds polyanionic heparin (Fig. 1).[24] In this paper, we use isothermal titration calorimetry (ITC) to determine the effect of buffer, and hence understand how nanoscale electrostatic binding interfaces are affected by buffer. Heparin was ideal for this study as it is a typical highly charged polyanion, which is bound using a large electrostatic component.

We performed detailed characterization of MalB-heparin binding using ITC (Figs. 2 and S5).[23] We titrated MalB into heparin (20 μM) in each buffer (10 mM, pH 7.4, Fig. 1), also containing background electrolyte (ca. 150 mM) – i.e., [heparin] << [buffer] << [Cl^-]. The free energies of binding (ΔG) confirmed qualitative observations from our previous study with stronger binding in Tris-HCl than HEPES, than PBS (ΔG = -8.51, -7.87 and -7.31 kcalmol^{-1} respectively, Table 1). The binding has a small favourable enthalpy in each case, indicative of electrostatic binding between oppositely charged species, and a larger favourable entropy, related to desolvation of charged surfaces (i.e., release of water molecules and counterions into bulk solvent). The importance of entropy in guanidinium-anion interactions has been highlighted previously.[25] The end of titration (EOT) values, corresponding to the binding saturation of heparin with MalB had ~1:1 ratio between cationic:anionic charge in Tris-HCl and HEPES, although in HEPES the EOT value was slightly larger. The EOT in PBS, however, was significantly larger, suggesting more MalB was required to saturate heparin.

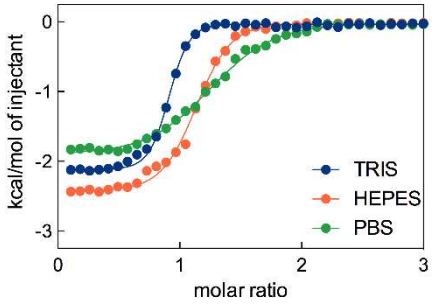

Figure 2. Comparison of ITC curves for titration of MalB into heparin in three different buffers at ca. 150 mM salt, pH 7.4, 25°C. ITC raw data are reported in Figure S1 (see ESI).

The binding of cationic MalB to anionic heparin in different buffers can be understood in terms of the ability of the anionic species in the buffer to compete for binding to the cationic groups on MalB. This is in-line with expectations from supramolecular chemistry of anion binding[14] based on the charge density of each of the buffer anions – phosphate has a higher 2-/1- charge, sulfonate has a 1- charge delocalised onto directional oxygen atoms, while chloride has its surface 1- charge dispersed over a large non-directional spherical surface. As such, binding to the buffer anion would be expected to follow the trend: phosphate > HEPES > Tris-HCl. Competition in this order therefore limits the binding of MalB to heparin.

In the absence of NaCl, binding in Tris-HCl was largely unaffected, but in HEPES, binding strengthened. The low ionic strength experiment could not be performed in PBS, as the buffer itself contains salts. At low ionic strength, in Tris-HCl, ΔH increased slightly, suggesting stronger electrostatic interaction as expected due to less charge screening, and ΔS decreased a little, indicating less desolvation, with enthalpy-entropy compensation leading to similar overall ΔG. Tris-HCl is ion-matched to the background electrolyte (150 mM NaCl), and we therefore propose the 10 mM chloride provided by Tris-HCl offers a less concentrated, but similar ionic environment. In HEPES, ΔH again increased a little in the absence of NaCl, but ΔS increased very significantly suggesting greater 'desolvation'. HEPES will interact more via competitive interactions between its sulfonate anion and cationic MalB, with HEPES release increasing ΔS.[25] This also supports the slightly larger EOT value in HEPES. The difference between Tris-HCl and HEPES was somewhat surprising, given they are both 'Good' buffers.

We then investigated the impact of buffer on cationic SAMul nanosystems. We selected C_{16}-DAPMA (Fig. 1),[24d,e] as it combines simple synthesis with effective heparin binding, and is assembles into well-defined cationic micelles under the micromolar regime of heparin binding. We performed a Nile Red assay (Figs. S1-S3)[26] and ITC (Fig. S5) to determine critical micelle concentrations (CMCs, Table 2). Within error, C_{16}-DAPMA had the same CMC in each buffer.

To characterize the SAMul nanostructures further, we used dynamic light scattering (DLS, Table 2, Figs. S7-S18). This was performed at high C_{16}-DAPMA concentration (1 mg/mL, 2.2 mM) with ca. 150 mM electrolyte. Under these conditions, significant further hierarchical aggregation of the SAMul systems occurred. At 70°C, in Tris-HCl and HEPES, relatively well-defined assemblies were observed (ca. 6.8 nm) with equivalent ζ-potentials (ca. +40 mV), consistent with the formation of simple spherical micelles. However, in PBS, the species formed were larger (ca. 21 nm), with greater dispersity, and lower ζ-potentials (ca. +25 mV). We suggest that interactions with the phosphate anions in PBS occur at the cationic micellar surface, causing charge neutralisation and some aggregation. Indeed, we know from previous work that anions can induce hierarchical assembly of these cationic micelles.[24e] On lowering the temperature to 25°C, further assembly was observed. In PBS, the diameter was >1 μM and the ζ-potential was lowered to effectively zero. However, even in HEPES and to a lesser extent Tris-HCl, aggregation was

Table 1. Thermodynamic parameters obtained from ITC measurements for MalB titrated into heparin in different buffers (10 mM). ΔH_{obs}, -TΔS and ΔG are in kcalmol^{-1}, EOT is the end of titration point and K_d is the effective dissociation constant

Buffer	[salt], mM	EOT	ΔH_{obs}	-TΔS	ΔG	K_d, μM
Tris	150	1.1 ±0.1	-2.15 ± 0.04	-6.36 ± 0.06	-8.51 ± 0.06	0.58 ± 0.06
HEPES	150	1.4 ± 0.1	-2.37 ± 0.12	-5.50 ± 0.17	-7.87 ±0.05	1.72 ± 0.15
PBS	140	2.0 ±0.2	-1.74 ± 0.11	-5.57 ± 0.19	-7.31 ±0.08	4.41 ±0.25
Tris	0	1.2 ±0.1	-2.46 ± 0. 11	-5.78 ±0.12	-8.24 ±0.0.2	0.91 ±0.03
HEPES	0	1.3 ±0.2	-2.63 ±0.13	-6.82 ±0.24	-9.45 ±0.10	0.12 ±0.02

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observed at 25°C. In HEPES, the observed diameter was ca. 300 nm and the ζ-potential was lowered compared to Tris-HCl, in which the observed diameter was ca. 50 nm. This suggests interactions between cationic C_{16}-DAPMA and anionic buffer components are in the order PBS > HEPES > Tris-HCl.

Table 2. CMC values of assemblies formed by C_{16}-DAPMA as assessed by Nile Red assay and ITC in different buffers (10 mM, pH 7.4), and Z-average hydrodynamic diameter and ζ-potential of C16-DAPMA derived by DLS at 70°C (10 mM buffer, 150 mM NaCl).

Buffer	CMC[a] (μM)	CMC[b] (μM)	Diameter (nm)	ζ-Potential (mV)
Tris	40 ± 1	35 ± 2	6.9 ± 0.1[c] 51 ± 10[d]	+40.2 ± 1.9[c] +57.2 ± 2.6[d]
HEPES	36 ± 2.5	39 ± 3	6.7 ± 0.9[c] 300 ± 20[d]	+39.9 ± 3.3[c] +50.8 ± 1.2[d]
PBS	38.5 ± 0.5	32 ± 2	20.9 ± 2.2[c] 1930 ± 400[d]	+24.8 ± 3.5[c] -0.3 ± 0.9[d]

[a] Determined by Nile red assay; [b] Determined by ITC, [c] Measured at 70°C, [d] Measured at 25°C.

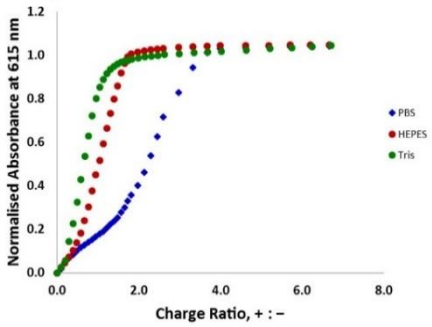

Figure 3. Titration curves for MalB displacement assays on titration of C_{16}-DAPMA into an aqueous solution of MalB (25 μM), heparin (27 μM – based on disaccharide repeat unit with a charge of -4), 10 mM buffer and ca. 150 mM salt, at pH 7.4.

Competition assays rapidly tested the relative heparin binding of C_{16}-DAPMA in each buffer. In this assay,[22b] the ability of the SAMul nanosystem to displace MalB from its complex with heparin was monitored by UV-Vis. This yields charge excess (CE_{50}) of the binder, the number of positive charges per heparin negative charge to obtain 50% MalB displacement, effective concentration (EC_{50}) at the same point and the 'dose', i.e., mass of binder required to bind 100 'international units' of heparin (Table 3, Fig. 3). In Tris-HCl, C_{16}-DAPMA binds heparin very well, displacing MalB at low loadings (EC_{50} 34 μM) similar to the CMC. Binding is slightly less effective in HEPES, with more C_{16}-DAPMA required to displace MalB (EC_{50} 55 μM), and very much less effective in PBS (EC_{50} 121 μM), significantly above the CMC, suggesting competition to heparin binding at the charged nanosurface. This competition assay is referenced to the binding affinity between MalB and heparin. In each buffer, the reference complex also has a different strength as described above (Table 1). As MalB is most effective in Tris-HCl, and least effective in PBS, it might have been expected that C_{16}-DAPMA would be less-able to displace strongly-bound MalB in Tris-HCl than weakly-bound MalB in PBS – the inverse of what was observed. The enhanced ability of C_{16}-DAPMA to displace MalB in Tris-HCl (vs. PBS) is therefore even more remarkable.

Table 3. CE_{50}, EC_{50} and doses obtained for C_{16}-DAPMA using MalB competition assay (10 mM buffer, 150 mM NaCl, pH 7.0). [MalB] = 25 μM, [Heparin] = 27 μM (based on a typical disaccharide repeat unit with an assumed -4 charge).

Sample	CE_{50}	EC_{50} / μM	Dose / mg 100 IU^{-1}
Tris	0.64 ± 0.02	34 ± 1	0.46 ± 0.01
HEPES	1.02 ± 0.02	55 ± 1	0.73 ± 0.02
PBS	2.24 ± 0.03	121 ± 18	1.60 ± 0.24

We used ITC to characterise binding between SAMul C_{16}-DAPMA and heparin (Table 4 and Fig. S6). Heparin binding was exothermic – more so than for MalB – as expected for a multivalent electrostatic process. The entropies were positive, suggesting solvent and ions are released from the binding interface. The ΔG values (Table 4) clearly show that in PBS SAMul/heparin binding is much less effective (ΔG = -6.31 $kcalmol^{-1}$) than HEPES (ΔG = -7.45 $kcalmol^{-1}$) than Tris-HCl (-8.08 $kcalmol^{-1}$). In more detail, ΔH_{obs} is greater in Tris-HCl than PBS (or HEPES), presumably because competitive interactions of the latter buffers with the cationic micelle limit the enthalpic gain. Further, ΔS increases from Tris-HCl to PBS, suggesting greater displacement of bound ions/solvent. However, the increase in ΔS on changing to PBS in no way offsets the loss of ΔH, and as such, significant differences in free energy arise. Interestingly, the data show that for these nanoscale SAMul systems, the impact of buffer is greater than for MalB. The difference in ΔG for heparin binding between Tris-HCl and PBS increases from 1.20 $kcalmol^{-1}$ (MalB) to 1.77 $kcalmol^{-1}$ (C_{16}-DAPMA). The adverse effect of competitive buffers on the larger enthalpic term, which results from the highly charged multivalent SAMul

Table 4. Thermodynamic parameters obtained by ITC for C_{16}-DAPMA SAMul micelles titrated into heparin in different buffers (10 mM). ΔH_{obs}, $-T\Delta S$ and ΔG are in $kcalmol^{-1}$, EOT is the end of titration point and K_d is the effective dissociation constant.

Conditions	[salt], mM	EOT	ΔH_{obs}	$-T\Delta S$	ΔG	K_d / μM
Tris	150	0.8 ±0.1	-4.31 ± 0.03	-3.77 ± 0.06	-8.08 ± 0.05	1.2 ± 0.1
HEPES	150	0.9 ± 0.1	-3.91 ± 0.08	-3.54 ± 0.11	-7.45 ± 0.06	3.5 ± 0.4
PBS	140	2.1 ± 0.1	-2.18 ± 0.06	-4.13 ±0.06	-6.31 ±0.07	24 ± 3

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system, primarily drives the greater buffer sensitivity of C_{16}-DAPMA. Our study therefore suggests highly charged nanoscale binding interfaces are more sensitive to buffer competition.

In summary, the binding of MalB to heparin decreases in the order Tris-HCl > HEPES > PBS. We conclude buffer effects result from interactions between anionic buffer component (phosphate/sulfonate/chloride) and cationic binder. Such interactions occur in 10 mM buffer, even in the presence of 150 mM electrolyte, and even a 'Good' buffer such as HEPES competes. In the absence of salt, binding becomes stronger in HEPES than Tris-HCl suggesting ionic strength mediates these competitive interactions. SAMul nanostructures show the same overall order of binding (Tris-HCl > HEPES > PBS), but the effect of buffer on the multivalent interactions between the highly charged SAMul binding array and heparin is even greater. In conclusion, when studying electrostatic binding, it is initially desirable to use a non-competitive buffer such as Tris-HCl in background electrolyte. However, the biological medium itself contains many anions, including phosphates and other highly competitive anionic species. The specific effects of these anions on electrostatic (and other) binding processes must be carefully considered when developing recognition systems for use *in vivo*. We emphasize the need to consider the impact of all species in solution – even apparently inert ones like buffers can significantly affect binding.

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Notes and references

# These authors contributed equally to the work.

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Effect of buffer on the electrostatic binding of biological polyanions

Graphical abstract

The electrostatic binding of polyanionic heparin by cationic receptors is highly dependent on the buffer in which the binding assay is carried out.