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Effect of Buffer on Heparin Binding and Sensing in Competitive Aqueous Media

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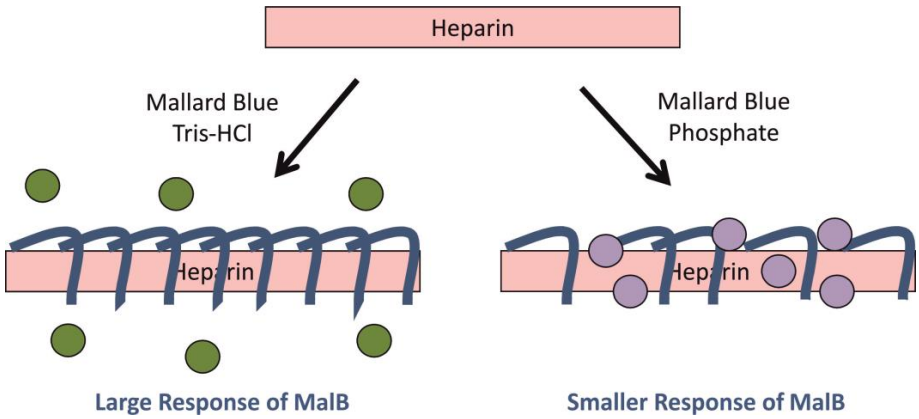
Graphical Abstract



Abstract. Although buffer-specific effects on molecular recognition are known in biological science, they remain rare in supramolecular chemistry. The binding between a cationic dye, Mallard Blue (MalB), and polyanionic heparin in aqueous NaCl (150 mM) is studied in three commonly-used buffers (Tris-HCl, HEPES, Phosphate, each 10 mM). Although MalB has a very similar UV-Vis spectrum in each buffer, the sensory response towards heparin was different in each case. This can be ascribed to differences in the complex formed. In Tris-HCl which has the least competitive chloride counter-anions, MalB exhibits a hypsochromic shift of 25 nm, assigned to strong binding and aggregation of the dye on heparin. In more competitive HEPES, containing a sulfonate anion, there is weaker binding and less aggregation of MalB along the heparin; the hypsochromic shift is only 15 nm. In phosphate buffer, MalB can interact quite strongly with buffer phosphate anions; although heparin binding is still observed, the hypsochromic shift associated with dye aggregation is only 5 nm. As such, specific buffer interactions with the MalB-heparin complex mediate host-guest binding and sensing. Buffer choice must be made carefully in studies of

molecular recognition – we would caution against using phosphate and sulfonate containing buffers when studying electrostatic binding.

Keywords: anion, buffer, molecular recognition, sensor, water

Introduction

When studying biologically relevant binding, or developing supramolecular systems which can mimic or intervene in such processes, it is necessary to work in highly challenging and competitive aqueous media (*1*). In addition to water, such binding events must also often remain robust in the presence of high levels of salt, and buffers which help control pH. It is well-known that electrolyte can impact on binding affinities either through charge-screening effects (*2*) or via the Hofmeister series (*3*). Ong and Kaifer explored the impact of the presence of ions on cucurbituril binding to viologens and found decreasing binding strengths as the ionic strength increased, with a dependence also on the charge of the competing cation (*4*). The importance of this was emphasised by Verboom and co-workers who studied the impact of counter-ions on electrostatic self-assembly (*5*). This type of effect has recently been revisited by García-Río and co-workers in the context of binding constant determination (*6*). We have also explored ionic strength effects on binding between cationic dendrons and DNA, and elucidated the impact of electrolyte on multivalent electrostatic interactions (*7,8*). However, the impacts of buffers on molecular recognition processes are less often explored.

As long ago as 1966, Good and co-workers discussed the appropriate choice of buffers and outlined a series of criteria for preferred buffers (*9-11*): (i) pH values between 6 and

8, (ii) maximum water solubility and minimum lipid solubility, (iii) minimal influence of temperature, ionic strength or buffer concentration on pKa, (iv) non-complexing towards metal ions, (v) stable, (vi) non-absorbing to light > 240 nm, (vii) cheap and easy to prepare. Furthermore, in biological studies, the potential for buffers to have specific effects is amplified. For example, certain buffers can induce protein folding or unfolding (*12*), which can have dramatic effects on protein-protein interactions as well as modifying other binding events. In addition, when working in living systems, buffers can interact with cell membrane components (*13*) and even have impacts on cell growth and survival (*14*).

In an outstanding review article from 2015, Soares and co-workers grappled further with this topic, considering data for a wide range of commercially available buffers and their relative (un)suitability for studies of biological, biochemical and environmental systems (*15*). In particular, they noted that even amongst Good's buffers, significant differences could occur, and that this was a key factor in metal-dependent binding processes as a consequence of metal-buffer interactions. Indeed, such factors have long been recognised to be important in metal-coordination chemistry, particularly when binding constants are being derived. With metal binding in mind, there have recently been reports in which changing the buffer can alter the metal selectivity of supramolecular metal sensors (*16-18*). However, these buffer effects have not been explored systematically.

It is likely that buffer choice can also have significant impacts on many processes which are not metal dependent, and it is fair to say that in general terms, the importance of buffers is less often discussed in the area of supramolecular chemistry than in the fields

of bioscience or metal coordination chemistry. Indeed, the choice of buffer for studies of molecular recognition in water is rarely commented on, other than by way of a simple statement of which buffer was used. Influential reviews from 2007 and 2010 dealing with supramolecular chemistry in water (*19*) and aqueous anion binding (*20*), and a recent review from 2015 describing anion binding in water (*21*) indicate that a very wide range of buffers are used in binding studies. However, there were no specific comments in these reviews addressing the impact this may have.

A rare example of a buffer effect in supramolecular chemistry was reported by Sirish and Schneider in 2000, who demonstrated that for the interaction of cationic porphyrins with a variety of small phosphate anions, the presence of phosphate buffer at increasing (and high) concentrations 0.1 M and 0.3 M had a significant impact on binding affinity and mode, with lipophilic interactions gaining in importance as the concentration of phosphate buffer increased (*22*). However, this was primarily due to the impact of ionic strength, and as noted above, such effects on molecular recognition are relatively well-known, particularly in the field of electrostatic binding (*2, 4-8*). In very rare cases, authors have commented on specific buffer effects, such as Rebek and co-workers, who noted in passing in 2004 that for reasons which were 'not readily understood' their hydrophobic hosts showed different binding strengths and selectivities towards adamantane guests in pure water, tris buffer and phosphate buffer (*23*).

As noted earlier, in biomolecular recognition, such effects are much better recognised – the impact of this has been recently highlighted by Salis and Monduzzi, in an excellent review discussing the importance of buffer-specific effects in the biosystems analysis (*24*). To exemplify this, Baaske and co-workers developed a specific assay to determine

the buffer dependence of aptamer binding (*25*). Given the emerging importance of fusing supramolecular chemistry with biology (*1*), and the fact that biological chemists are increasingly exploring specific ion and buffer effects at binding interfaces (*26*), it is clear that supramolecular chemists need to increasingly think very carefully about the environment in which molecular recognition is taking place. Any assumption that all aqueous solutions behave similarly is clearly insufficient – as such, it is perhaps surprising that supramolecular chemists have focussed relatively little attention on the roles which buffers can play.

Electrostatic binding is of key importance in biological processes, as it can deliver relatively large amounts of binding affinity even in competitive aqueous media (*27*). Interestingly, it has been noted that much of biology is a 'polyanion world', with a wide range of polyanionic species being of interest – including nucleic acids, glycosaminoglycans, proteoglycans, microtubules and filaments and cell membranes (*28*). Remarkably, biology is able to control these different nanoscale polyanionic species with a good degree of precision. Furthermore, biology has to achieve this in the presence of many potential competitor species.

One of our specific polyanionic binding targets of interest is heparin (Fig. 1), a key glycosaminoglycan used during major surgery to prevent blood clotting (*29*). Effective sensors for this polyanion have potential clinical importance so that anesthetists can rapidly determine how much heparin is present in a patient to ensure optimal clotting behaviour throughout and after surgery *(30*). We developed the sensor Mallard Blue (MalB, Fig. 1), a thionine unit functionalised with arginine amino acids, which responds to heparin through a distinctive change in its UV-visible spectrum (*31,32*). This sensor

relies on well-organised electrostatic interactions between its cationic groups and the anionic sites on the heparin, and can operate at clinically relevant concentrations in highly competitive conditions, such as the presence of 150 mM NaCl and/or human serum. In this field of research, there has been considerable focus on developing systems which operate effectively in competitive media (*33-39*) as replacements for simple dyes such as Azure A (*40*) which are unable to function under such challenging conditions. As such, the robustness of heparin sensors to environmental conditions is of key importance.

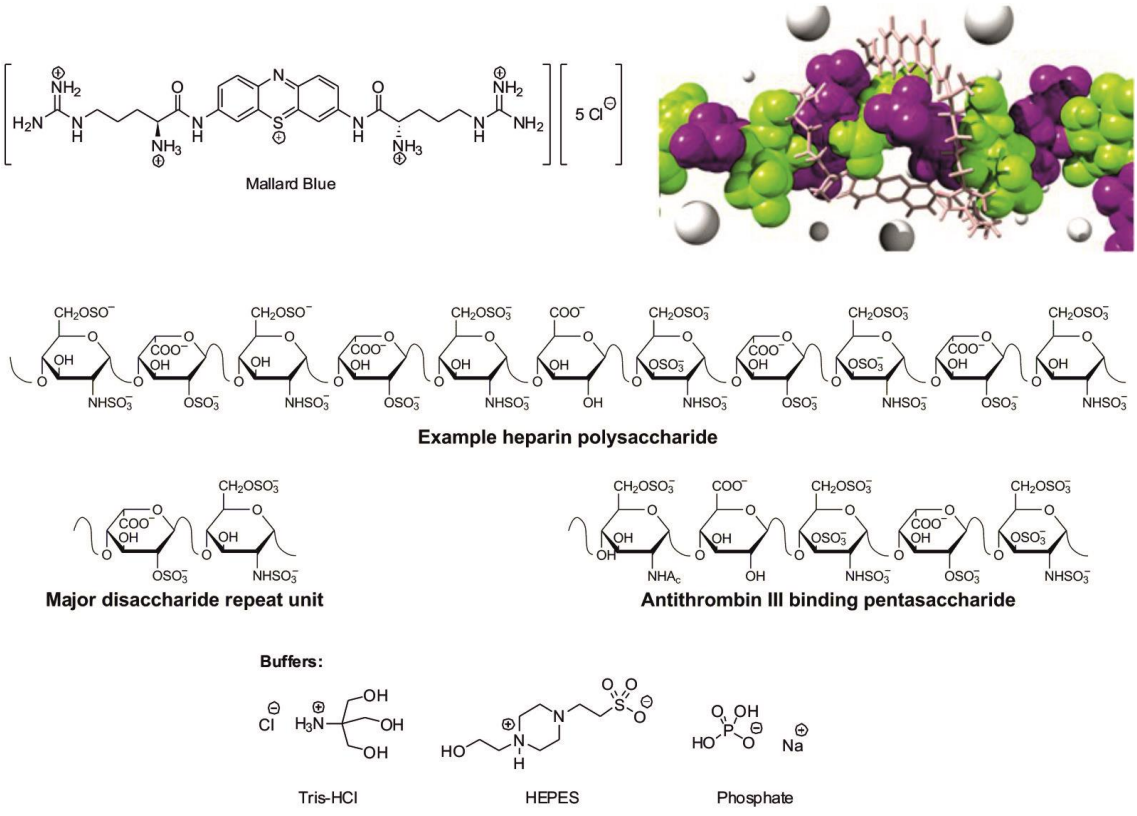


Figure 1. Structures of Mallard Blue, heparin (highlighting the major disaccharide repeat unit and the active pentamer which binds antithrombin III), and buffers used in this study. Molecular dynamics model of Mallard Blue binding to heparin reproduced from reference (*31*) with kind permission of American Chemical Society.

Given the fact that different aqueous solutions can be very different, and buffer may have significant impacts on binding, we therefore wanted to explore the impact of buffer on MalB. In particular we wished to compare the binding and sensing in different buffers. Further, we aimed to do this in the presence of a high concentration of NaCl (150 mM) such that the impact of buffer could not simply be assigned to changes in ionic strength – in our approach the ionic strength is kept approximately constant by the large excess of salt. In this way, we hoped to determine whether buffers were having particular effects on binding and sensing as a result of specific interactions with the host and/or guest. Given the relative paucity of reports in which buffer choice has a direct impact on supramolecular binding, we report the results here, and consider their general implications and significance.

Results and Discussion

A binding study of MalB with heparin in different buffer systems was performed using UV-Vis spectroscopy. Specifically, we chose the three most commonly used buffers in supramolecular and biological science – Tris-HCl, HEPES and Phosphate buffer (Fig. 1). In each case, the pH of the solution was 7.0 and buffers were applied at 10 mM concentration – sufficient to control pH during the titration experiment, but well below the 150 mM concentration of NaCl which was also present in the assay to control ionic strength. The concentration of MalB was 25 μM and heparin was titrated into the sample. MalB was also present in the titrant, so there were no dilution effects during the titration. Calculating the concentration of heparin is relatively challenging, as it is a polydisperse anion with a variety of repeat units, and there is sample-to-sample variability in terms of its make-up. We assumed for the calculations, a molecular mass

which corresponds to the most commonly occurring dimeric saccharide heparin repeat unit. All titrations were repeated in triplicate on separate samples and showed very good reproducibility.

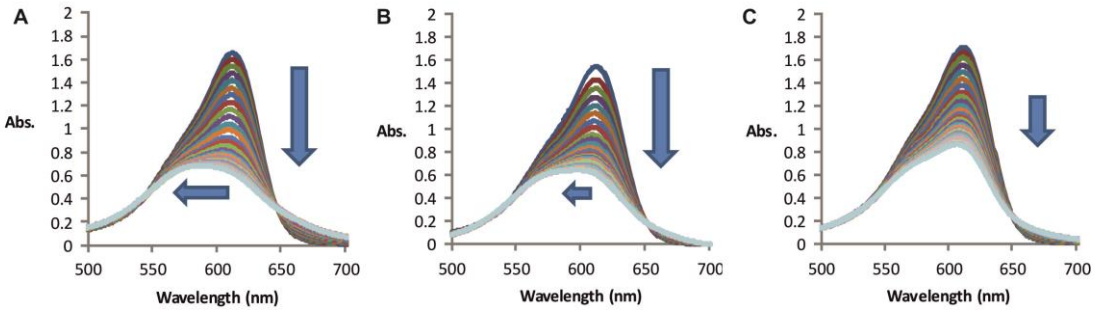


Figure 2. UV-Vis absorbance of Mal-B with increasing heparin concentration in (A) Tris-HCl buffer, 10 mM, pH 7, 150 mM NaCl; (B) HEPES buffer, 10 mM, pH 7, 150 mM NaCl; (C) Phosphate buffer, 10 mM, pH 7, 150 mM NaCl.

Studies of MalB binding heparin in Tris-HCl buffer have previously been reported by us (*31*) and our results here were in agreement with them (Fig. 2A). As can be seen, there is a decrease in absorbance on addition of heparin to MalB, and the max absorbance wavelength (λ_{max}) shifts from 612 to 587 nm as the concentration of heparin increases – a hypsochromic shift of 25 nm. The reasons for this are discussed in more detail below. A similar effect was observed on the titration of heparin into MalB in HEPES buffer (Fig, 2B), however in this case the λ_{max} only shifted from 612 to 598 nm – a hypsochromic shift of just 14 nm. In the case of the titration performed in phosphate buffer, the UV response to heparin binding was more significantly different. Although the absorbance decreased, this was a much less significant decrease than in the other two buffer systems. Furthermore, the λ_{max} value only shifted from 612 nm to 607 nm –

just a 5 nm hypsochromic shift. There are clearly some subtle and important differences between the way in which MalB binds heparin in the different buffer systems.

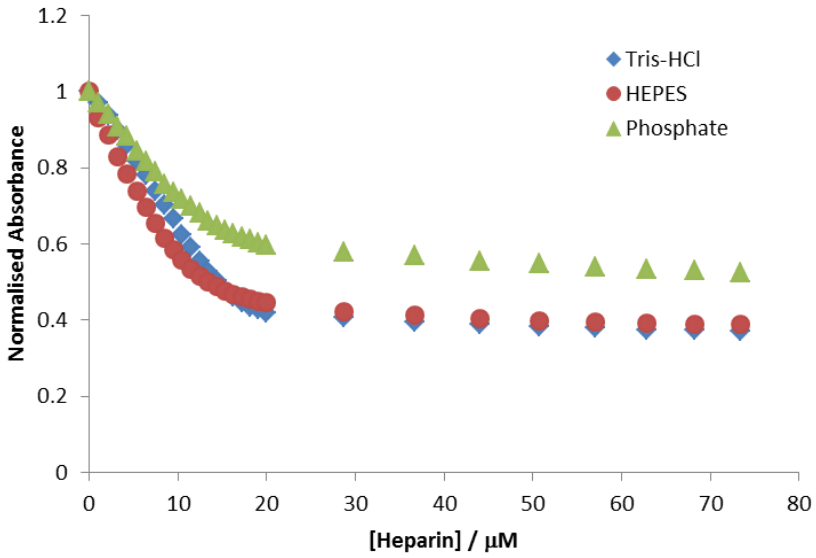


Figure 3. Binding profiles for MalB titrated with heparin, extracted at 615 nm, in different buffer systems – Tris-HCl, HEPES and Phosphate – all in the presence of 150 mM NaCl.

Figure 3 presents the binding profiles for each of these titration events in the different buffers with average normalised data extracted from the spectra at 615 nm. It is clear from the binding profiles that the binding curves have similar endpoints when the titration is performed in Tris-HCl or HEPES, but a significantly different one when carried out in phosphate buffer. As such the titration curves suggest that the phosphate buffer is having the most significant impact on the binding event (see below) – in agreement with the observed hypsochromic shifts.

More detailed inspection of the binding curves between MalB and heparin in Tris-HCl and HEPES would suggest that the binding profile is sharper in Tris-HCl, and hence the

binding is stronger, with the binding profile being shallower in HEPES, suggestive of weaker binding. Furthermore, it appears by inspection that the binding event in phosphate buffer is weaker again, with a shallower titration profile. It is, however, somewhat problematic to assign numerical binding constants to these processes for a number of reasons:

(i) Binding is taking place to a polymer, and it is unclear what the precise stoichiometry of binding is – furthermore, it appears this is slightly different in each buffer (see below),

(ii) Heparin is polydisperse, with different repeat units, and hence chemically different sites on the polymer, each of which will have different affinities for MalB, varying through the titration,

(iii) When more MalB is bound to the polymer (at low heparin concentrations) each binding event will have a different affinity owing to MalB-MalB interactions when the MalB units are in close proximity on the heparin chain – once again, the binding affinity will vary through the titration dependent on the degree of loading.

As such, it can be difficult to extract meaningful binding constants – and we were unable to do so in a meaningful way. In particular, we noted that in each buffer, the apparent binding stoichiometry was slightly different. Finding the approximate saturation point in each case, it became clear that in Tris-HCl, more heparin had to be added to saturate the response of MalB than in either of the other buffers. Approximately 20 μM of heparin (concentration reported as the typical dimer) saturates MalB in TrisHCl, whereas this falls to 15 μM in HEPES or phosphate buffer. This means that one equivalent of MalB binds to every 2.5 saccharide units on heparin in Tris HCl, falling to only one MalB to every 3.3 heparin saccharide units in the other

buffers. This suggests that in some way, the buffer is mediating the interactions between MalB and heparin, and as a result modifying the loading of MalB onto the polyanion and hence the apparent stoichiometry of the complex (see below for further discussion).

We then measured the UV-Vis spectra of MalB on changing the ratio of Tris-HCl:phosphate buffers – the two extreme cases. For MalB alone, in the absence of heparin, the λ_{max} and absorbance did not significantly shift as the amount of phosphate anion increased from 0 to 10 mM (Fig. 4A). Given that at pH 7.4, phosphate anions are a mixture of $H_2PO_4^-$ and more highly charged HPO_4^{2-}, it might have been expected that they bind more strongly to MalB than the surrounding Cl^- ions in the NaCl. However, Figure 4A clearly demonstrates that if phosphate does bind to MalB, this does not have any impact on the UV-Vis spectroscopic properties.

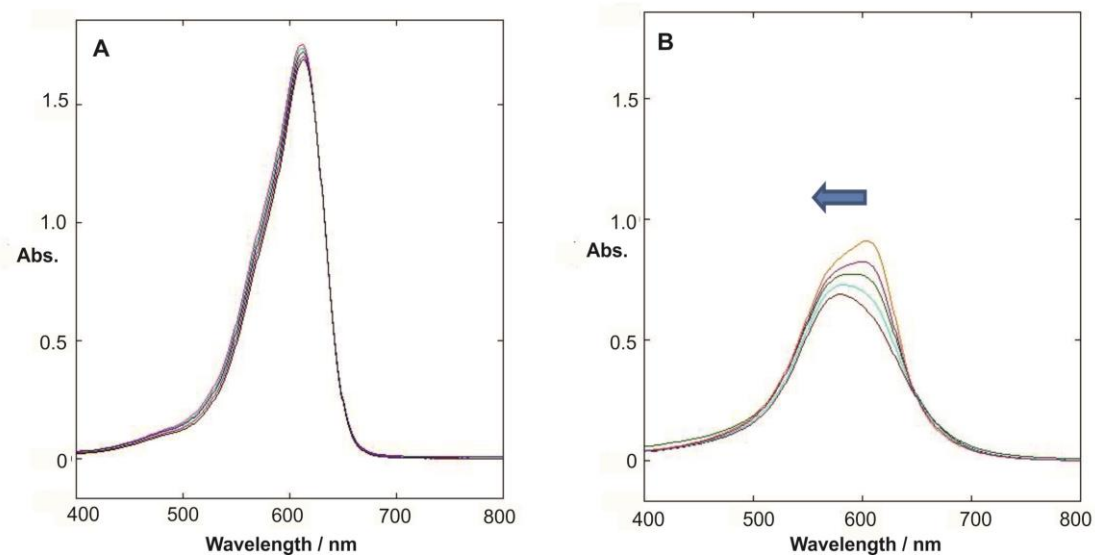


Figure 4. UV absorbance in different ratios of buffer (1:0 to 0:1 phosphate:Tris-HCl) of (A) MalB (25 μM) indicating little impact on the UV-Vis spectrum, and (B) MalB (25

μM) fully complexed to heparin (25 μM), indicating that in Tris-HCl the complex absorbs at shorter wavelength and with lower intensity.

However in the presence of heparin, switching the buffer between phosphate and Tris-HCl had a major effect on the spectroscopic properties. As the level of Tris-HCl was increased (and the phosphate content decreased), there was a shift in λ_{max} from 612 to 587 nm, and a decrease in the absorbance (Fig. 4B). It is therefore clear, that the differences in the titration responses discussed above are a direct result of the differing optical properties of the MalB-heparin complexes in the different buffers, and do not reflect differences in the optical properties of MalB alone. Furthermore, based on the titration data, HEPES lies in-between the extremes of Tris-HCl and phosphate buffers in terms of its impact on the MalB-heparin complex optical properties.

Given that we are performing the studies in an aqueous solution of 150 mM NaCl, and studying all three buffers at the same concentration (10 mM) we can rule out any possibility that the effect of buffer is a result of ionic strength. We can also rule out cation-derived effects associated with the phosphate buffer, as the cation is sodium – the same as present in the supporting electrolyte (NaCl). As such, it seems likely that the impact of the buffer is associated with its anionic character – phosphate, sulfonate (HEPES) or chloride (Tris-HCl).

The observations agree with the expectations we may have of these buffers as phosphate>sulfonate>chloride in terms of potential for interaction with cationic sites. This is a result of (i) the higher charge of phosphate at physiological pH making it the most strongly binding anion, followed by (ii) the greater propensity of both phosphate and sulfonate to form directional hydrogen bonds and hence out-compete the supporting

electrolyte (chloride). We would expect that Tris-HCl should have effectively no anion-mediated effect on binding, as the chloride of this buffer is matched with the supporting electrolyte.

We therefore need to understand why these anions have an impact on the UV-vis spectra of MalB, but only when complexed to heparin. Naively, it might be expected that on binding to heparin, any interactions with the surrounding buffer will be displaced as a result of heparin's greater charge density. However, the concentration of the heparin in these experiments is only ca. 20 μM at the saturation point (60-80 μM per negative charge), whereas the concentration of the buffer anion is 10 mM; >100-fold excess. In this context, it is actually remarkable that MalB still shows highly effective heparin binding, even in the presence of such a large excess of phosphate anions. It does, however, allow us to propose their direct involvement in the overall complex which is formed.

It is well-known that this type of dye exhibits absorbance bands associated with the monomer at longer wavelength, but on the formation of dimers has a shorter wavelength absorbance (*41*). Indeed, it has been known for many years that this dimerisation process and spectroscopic shift is encouraged, even for simple cationic dyes such as unfunctionalised thioine, on binding to polyanions – a process referred to as metachromasia (*42-44*). In buffered solution, irrespective of buffer choice, MalB is therefore present as a monomer with an emission band of 612 nm. The monomer may be bound to anionic constitutents in the buffer (such as phosphate or sulfonate), but this does not cause aggregation of the dye, and as such, has no impact on the optical properties. On binding to heparin, MalB dyes are brought into closer proximity with

one another, and effectively form H-type dimers (*41*), pre-organised on the polyanionic heparin chain (Fig, 5, left). This explains the hypsochromic shift of 25 nm when heparin is added to MalB in Tris-HCl. However, in the presence of phosphate anions, binding of phosphate to MalB can limit the extent of MalB binding along the heparin chain, and hence the extent of dimer formation (Fig. 5, right) – as such, the hyposchromic shift is therefore limited to just 5 nm, and the change in absorbance spectrum is much smaller. This model is in agreement with the apparent stoichiometries observed in the titration curves (Fig. 3) and discussed above. In HEPES, interactions between MalB and sulfonate somewhat, but not completely, limit heparin binding and MalB dimersiation, and hence the change in optical properties is intermediate between the extremes observed in Tris-HCl and Phosphate buffers, with a hypsochromic shift of 14 nm.

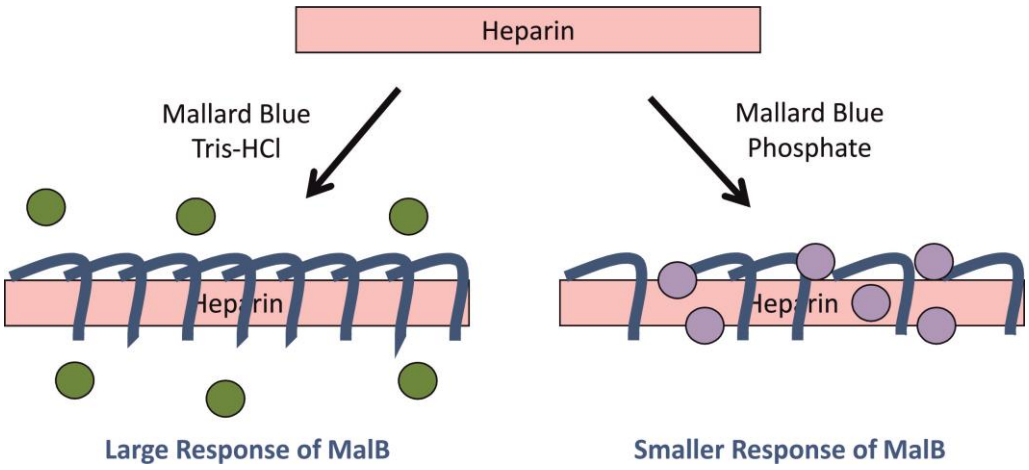


Figure 5. Schematic of Mallard Blue (crescent-shaped) binding to heparin in different buffers. In Tris-HCl, the chloride anions act as innocent spectatators, and enable effective binding and aggregation of MalB, while in phosphate buffer, the phosphate anions are intimately involved in interactions with MalB in the complex, and hence limit the sensory response of the dye.

In this way, the more interactive buffer anions effectively 'solvate' the MalB and limit its binding to, and organisation on, the heparin polyanion, which is responsible for the sensing response. This hypothesis is in agreement with the stoichiometric observation that more heparin binds to MalB in Tris-HCl than in the other two buffers – in the other buffers, MalB is also interacting with the buffer anions present – hence limiting the interactions with heparin and leading to its saturation at lower heparin loadings.

Conclusions

The results of this study demonstrate that the choice of buffer is essential when considering host-guest interactions in aqueous systems. This binding event was based on electrostatics, and these results particularly highlight the ability of anionic components in buffer to mediate complexation events and modify host-guest complexes as a result of their specific ability to interact with the complex.

Interestingly, although both Tris-HCl and HEPES are both classified as Good's buffers (*9-11*), in this case, Tris-HCl is effectively non-interactive, and acts as an innocent spectator, whereas HEPES becomes somewhat involved in the recognition event, lowering the binding strength (see above) and changing the sensory response. This would support the recent review article which noted that not all of Good's buffers can be considered equally good for understanding biological and biochemical processes (*15,24*). Furthermore, for phosphate, which is not one of Good's buffers, this effect is even more significant and the difference in the sensory response of MalB is indeed quite dramatic. The low suitability of phosphate buffers for metal-binding processes is well-

known, but this has not clearly been stated for supramolecular electrostatic binding events. Furthermore, phosphate buffers remain the first choice in many biochemical studies. As a result of this study, we would caution against the use of phosphate buffers (and to some extent sulfonate buffers) when investigating binding processes with electrostatic character.

All of the buffers used in this study were very standard buffers, frequently considered for use in binding assays – as such, we encourage supramolecular chemists to select buffers carefully, and remember they may play active roles in the molecular recognition event of interest. Although, as outlined in the Introduction, such factors are well-known in biological chemistry, they are less well appreciated in supramolecular chemistry, and given the current drive to further extend the study of synthetic recognition processes into aqueous media (*19-21*), and to have impacts on biomedical processes (*1*), we believe these observations will be of general interest and importance to the community.

Experimental

Mallard Blue was synthesised according to published methods (*30*), and analytical data were in agreement with previous reports. Solutions were always made up fresh and incubated for 24 hours at 50ºC prior to use, being stored in the dark. Sodium salt heparin from porcine intestinal mucosa with a molecular weight between 15,000 ± 2,000 Da (1 kU = 1000 units) was obtained from Calbiochem.

Binding of Heparin to Mal-B. A cuvette was charged with 2 mL of a stock solution of MalB (25 μM) in NaCl (150 mM) and Tris-HCl (10 mM), HEPES (10 mM) or phosphate

buffer (10 mM). This solution was titrated with a stock solution of heparin (200 μM) in MalB (25 μM), NaCl (150 mM) and Tris-HCl (10 mM), HEPES (10 mM) or phosphate buffer (10 mM) to a final cuvette volume of 3 mL. The absorbance at 615 nm was recorded after each addition. Experiments were performed in triplicate. Samples containing mixtures of buffers were made by mixing suitable aliquots of the appropriate buffered solutions.

For the purpose of calculations, the molecular weight of heparin is assumed as that of the sodiated analogue of the heparin repeat unit shown in Figure 1: namely 665.40 g mol^{-1}. It should be noted that as supplied, heparin only contains ca. 30-40% of material with the active pentamer sequence of repeat units. However, all of the sample contains anionic saccharide units which can bind, even if they are in the wrong sequence. Hence to best evaluate binding stoichiometries, we report the total concentration of the anionic disaccharide – irrespective of whether it is present in the active form of heparin or not.

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