Polycationic calix[8]arenes able to recognize and neutralize heparin †‡

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A mutual induced fit mechanism is responsible for the exceptional complexation performances exhibited by calix[8]arene polycations towards heparin. The recognition process was studied in comparison with two other heparin antagonists: protamine and polylysine. The arrangement of multiple functional groups on the flexible macrocyclic scaffold of calix[8]arene, with respect to the conformationally rigid protamine and low ordered polylysine, allowed a mutual adaptability between calixarene polycations and heparin, significantly enhancing the recognition performances. Fluorescence, NMR titration, and activated partial thromboplastin time (aPTT) experiments confirmed that these calixarene derivatives have a very high specificity and affinity towards heparin neutralization as in aqueous solution as in blood. Analogous results were obtained with low molecular weight heparin (LMWH) whose effect protamine is unable to completely reverse.

Introduction

Heparin, a sulfated polysaccharide, is known as one of the most powerful anticoagulant drugs, based on its ability to accelerate the rate at which antithrombin, a naturally occurring serine protease inhibitor, inactivates several coagulation factors such as thrombin and factor Xa, whose action is essential in the blood coagulation cascade.[1] Heparin also interacts with a number of proteins involved in many basic biological processes like angiogenesis, tumour growth and infectious attack by bacteria, protozoa and viruses.[2]

To overcome the natural blood tendency to form clots,[3] systemic heparinization is the most common anticoagulation procedure in surgical practice and extracorporeal therapies such as heart–lung oxygenation and kidney dialysis. To avoid risk of bleeding, the excess of heparin needs to be balanced and, if necessary, carefully neutralized. Therefore, heparin, its analogues and inhibitors have attracted high interest in the therapeutic field.

Heparin is a mixture of helical polysaccharides with chains of different lengths, mainly composed of repeating disaccharide units of 1→4-linked sulfated iduronic acid and sulfated glucosamine residues (Fig. 1); sulfur-containing and carboxyl groups are displayed at defined intervals and orientation along the flexible polysaccharide backbone, and provide the highest negative charge density of any known biological macromolecule.[2] For these reasons the key features to consider for heparin neutralization are anion–cation interactions and conformational flexibility.

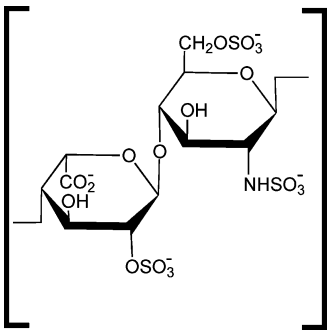

Fig. 1 Major heparin repeating unit.

One of the most used heparin antagonists is protamine sulfate, a low molecular weight protein bearing a high positive charge density due to the numerous arginine residues (*ca.* 20).[4] However, since protamine often causes severe side effects,[5] the finding of safe and efficacious heparin antagonists is currently a goal of great clinical importance.

With this aim, synthetic medium-sized peptides,[6] polypeptides (polylysine and polyarginine),[7–9] as well as low molecular weight protamine[10] and, very recently, foldamers[11] have been reported. Moreover, proteins such as lactoferrin,[12] histones[7] and antibodies[13] have been studied as heparin-neutralizing agents, but up to now, protamine, in spite of its well-known side effects, remains clinically the most extensively employed heparin antagonist.

Our strategy for the design of polyvalent heparin inhibitors with improved complexation properties was based on the use of a calix[8]arene[14] molecular scaffold having a high degree of functionalization, a well defined non-polymeric structure and elevated conformational adaptability, in order to achieve high affinity towards heparin through a mutual induced fit recognition mechanism. In fact, mutual adaptability amplifies immeasurably the scope and efficiency of the molecular complementarity on which molecular recognition is based, ultimately playing an essential role in most, if not in all, chemical and biochemical processes.[15] We expected that the mutual induced fit complexation mechanism would give rise to an improvement in the neutralization

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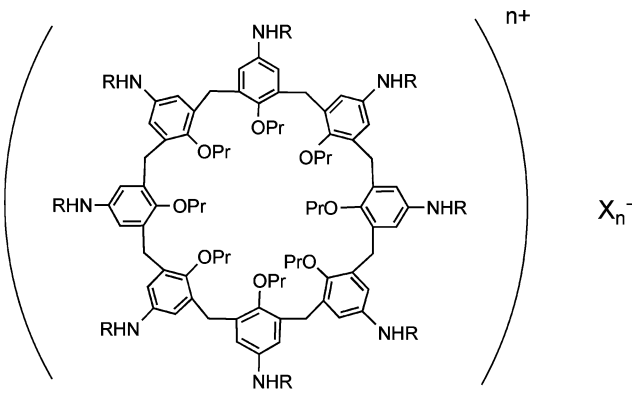
† Electronic supplementary information (ESI) available: Spectral data of compounds **1a** and **2a**. Kinetic model for heparin–polycation complexation. Graphics of competitive titration experiments between **2a** and protamine, and **1a** and polylysine. Low molecular weight heparin NMR titration and activated partial thromboplastin time (aPTT) calibration curve for low molecular weight heparin. See DOI: 10.1039/b608887b

‡ Dedicated to Professor Mario Piatelli on the occasion of his 80th birthday.

performance of our derivatives towards heparin with respect to the relatively rigid protamine or a low-ordered polymeric substance like polylysine.[16]

Calix[4]arenes,[17] widely studied compounds in supramolecular chemistry, have been effectively used as a scaffold in the synthesis of derivatives for the recognition of protein surfaces.[18] Calix[8]arenes are similar in reactivity but the larger dimension of the macrocycle ring and the higher conformational mobility make them ideal candidates for our purposes. For instance, a *p*-sulfonated calix[8]arene displayed a mutually induced fit formation of host–guest complexes with photolabile cholinergic ligands.[19]

We designed and synthesized two polycationic calix[8]arene derivatives (Fig. 2) having similar structures but different charge density in order to verify the influence of the charge density on the heparin recognition phenomenon. The shape and the charge disposition of these compounds also allowed the recognition and the inhibition of the enzyme tryptase, a serine protease involved in several allergic and inflammatory disorders.[20]



1a R = L- Lys, X = CF_3COO^-
2a R = $CO(CH_2)_6NH_2$, X = CF_3COO^-
1b R = L- Lys, X = Cl^-
2b R = $CO(CH_2)_6NH_2$, X = Cl^-

Fig. 2 Polycationic calix[8]arene derivatives.

Discussion

Preliminary molecular dynamic studies on the binding of compound **1a** to an octasaccharide sequence of heparin in aqueous solution suggest a very strong interaction between the two compounds. The calix[8]arene moiety adopts a pinched conformation having two almost identical subunits each defined by three aromatic nuclei in a cone conformation, and two opposite aromatic rings in 1,5-positions in an out orientation. This conformation is similar to the *syn* conformer of 1,5-intrabridged calix[8]arenes.[21] The cationic arms of the calixarene completely surround heparin in an "octopus-like" structure[22] in which the chelate effect is maximized (Fig. 3).[23]

The calix[8]arene derivative and heparin are mutually adapted so as to allow a complete charge neutralization: every charge present is surrounded and neutralized by two charges of opposite sign. The formation of this compact mutually adapted structure could be the reason for the very high neutralization power of calix[8]arene derivatives towards heparin.

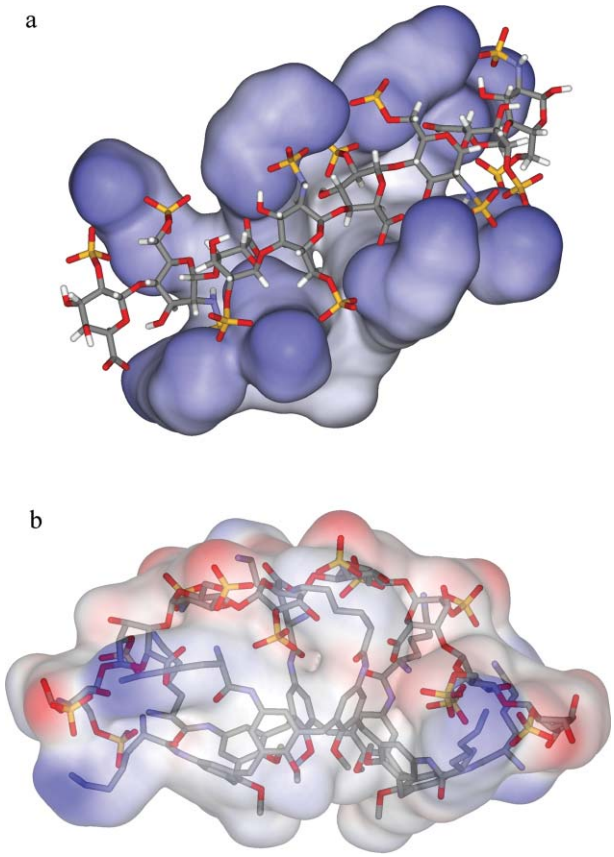


Fig. 3 Computer model of heparin–**1a** complex: a) top view of heparin octasaccharide fragment (stick model), bound to the calixarene derivative **1a** (solid-surface); b) side view of an overlay representation (stick model and solid surface). In the solid surface model hydrophobic patches are coloured in white, acidic patches in red and basic patches in blue.

The absolute value of the association constant (K_a) for the heparin–polycation interaction in solution is particularly difficult to assess due to the almost complete irreversibility of the reaction, the formation of precipitate, and the often incomplete knowledge of the nature and number of binding sites on biopolymers involved in the complexation.[24] Therefore the heparin–protamine[25] or heparin–polycation[7,26] recognition process has always been studied in comparison with other different but totally ionic binding processes. Therefore, our study to estimate the binding strength of the calixarene derivatives towards heparin has been carried out in comparison with protamine and polylysine in both aqueous solution and in blood. Moreover, as two forms of heparin are in clinical use, unfractionated heparin (UFH, average molecular weight 15 000 Da, average negative charge −75) and low molecular weight heparin (LMWH, average molecular weight 3000 Da, average negative charge −15), recognition experiments were performed with both kinds of heparin.

The first evidence of the recognition phenomenon between heparin and calixarene polycations **1a** and **2a** was revealed with indicator displacement experiments[27] by fluorescence spectroscopy, as similarly described for protamine.[25] Dye displacement experiments were carried out in both water and buffer solution (2 mM Hepes, pH 7.2), assuming for calixarene polycations **1a** and **2a** a nominal number of +16 and +8 positive charges, respectively. The binding of eosin Y by **1a** and **2a** was monitored by the quenching of the

dye emission at 540 nm. Subsequent displacement of the dye by UFH, with the accompanying reappearance of the emission at 540 nm, gave evidence of the UFH–**1a** interaction, indicating a final stoichiometry of 17–18 UFH sites per calixarene **1a** molecule (Fig. 4).

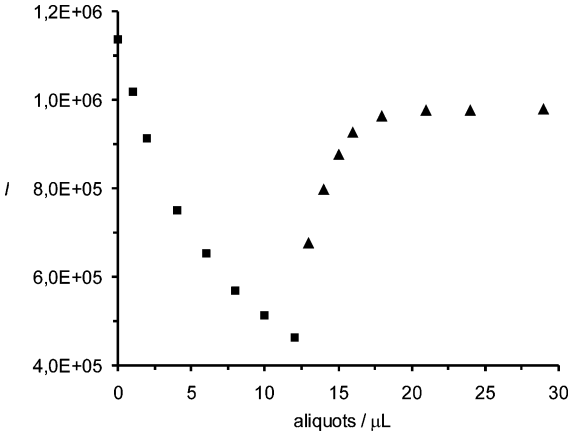


Fig. 4 The interaction between UFH and eosin Y–**1a** complex. Titration was carried out in a 3 mL volume using an eosin Y solution (5.2×10^{-9} M). The fluorescence emission was monitored at 540 nm (excitation wavelength 516 nm). Sequential addition of 7 aliquots of **1a** (4.5×10^{-5} M, 12 μL in all) (■) was followed by 8 aliquots of UFH (3.9×10^{-5} M, 17 μL in all) (▲). Addition of 3.5 μL of UFH was sufficient to restore ninety per cent of the fluorescent emission.

Similarly, **1a** showed a quasi equimolar neutralization power towards LMWH. The neutralization ability of **2a** proved to be lower than **1a**, suggesting that the nominal number of positive charges was the crucial element in the recognition phenomenon.

These results were validated by NMR titration experiments, performed under physiological conditions (10 mM PBS, pH 7.2, NaCl 150 mM), following the disappearance of UFH signals upon the addition of **1a**, **2a**, protamine or polylysine due to the formation of insoluble complexes. In these experiments, neutralization of UFH solution (171 USP per 0.5 mL) required 1.43, 2.25, 1.62 and 1.05 mg of **1a**, **2a**, protamine and polylysine, respectively. The analysis of the end-point of the titrations showed that polylysine was unable to totally remove heparin from the solution; about 4% of heparin always remained soluble, and this amount increased in the presence of an excess of polylysine. In transforming these amounts from weight to charge, it was apparent that under these conditions (pH, concentration and saline content), the complex formation was mainly driven by the one-to-one pairing of acidic with basic functions.

In order to evaluate the strength of binding of derivatives **1a**, **2a**, protamine and polylysine towards heparin, a determination of the "limiting salt concentration" (the concentration of NaCl in the medium required to completely dissociate the complex) was made.[26] In fact, assuming that the interaction between two macromolecules is a totally ionic process, the resistance of the complex to the dissociation by simple electrolytes provides a method for the comparison of the binding strength.[26] The experiments were performed monitoring the reappearance of the NMR signals of polycations or heparin by addition of small aliquots of a buffered saturated NaCl solution to a suspension of a preformed heparin–polycation complex in deuterated PBS buffer, pH 7.2, until complete dissolution of complex was reached. The limiting salt concentration for **1a**–UFH, **2a**–UFH, protamine–UFH and polylysine–UFH complexes was >3, 2.2, 1.9 and 1.7 M respectively. These results indicated a stronger binding for calixarene–UFH complexes than protamine–UFH or polylysine–UFH, underlining that the mutual induced fit complexation mechanism effectively enhanced the affinity of **1a** and **2a** for heparin. The reversibility of the complexes was also tested for pH variation. At pH values >13, the complexes were destroyed and UFH as well as derivatives **1a** and **2a** could be separated and recovered.

The complexation process under physiological conditions can be considered to be irreversible. In fact, a preformed heparin–protamine complex in the presence of an excess of **1a** remains unchanged after a week, despite the fact that the stability of the heparin–**1a** complex is considerably higher. Similarly, a preformed heparin–**1a** complex remains undissociated in the presence of an excess of protamine. This result shows that the equilibrium condition for this recognition process will be reached after a period of months if not years. For this reason a valid value of K_a for these heparin–polycation complexes cannot be easily found.

Due to the irreversibility of the complexation process, competitive NMR experiments give us the possibility to estimate the relative rate of formation of our heparin–polycation complexes. Knowledge of this parameter is useful if these heparin receptors are to be tested in such a complex medium as blood, because a faster interaction with heparin could improve the recognition selectivity. The experiments were accomplished by adding heparin to a solution containing both protamine or polylysine and **1a** or **2a** (Fig. 5).

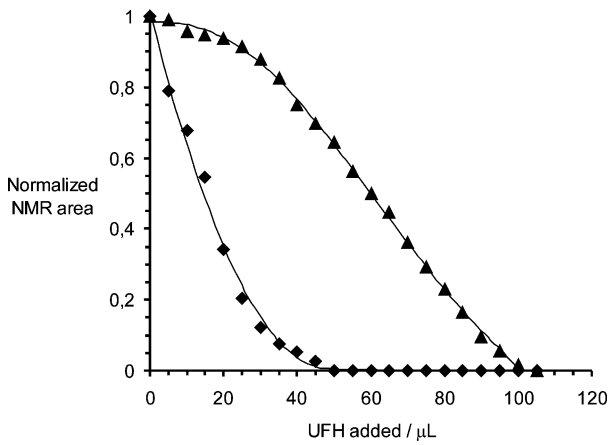


Fig. 5 NMR competitive titration between **1a** (◆) and protamine sulfate (▲). Small aliquots of UFH solution (20 mg mL^{-1}) were added to an NMR sample containing 500 μL PBS buffer (10 mM, pH 7.2, NaCl 150 mM), **1a** (1.43 mg), protamine sulfate (1.62 mg), and *t*-butanol (0.516 μL) as a standard for NMR area calculation.

The concentration of the two receptors was fixed in such a way that the number of positive charges for each competitor was nominally equal. The experiments showed that the rate of formation for the **1a**–UFH complex was 10 and 30–35 times higher than the protamine–UFH and polylysine–UFH complexes respectively.[28] This rate ratio was almost constant throughout the titration experiment, indicating a good approximation model for this system.[28] It seems that the relative rigidity of protamine

influences negatively the heparin complexation, while the lack of a compact and ordered structure makes the recognition process even slower for polylysine. These results highlight that the conformational mobility and adaptability of the calix[8]arene scaffold makes the complexation process faster, potentially enhancing the selectivity of the recognition process. Interestingly, the same experiment for **2a** showed an even greater rate ratio than for **1a**, suggesting that conformational features of the calix[8]arene derivatives (mobility and adaptability) affect the complexation rate more than the simple charge density.[28]

To further validate the heparin neutralization ability of the calix[8]arene derivatives in a strictly biological medium, activated partial thromboplastin time (aPTT) clotting assays in blood were performed. At first, aPTT experiments were performed with **1b** and UFH in the range of therapeutic dosing levels of 8–0.2 USP mL^{-1} (3.2–0.08 μM). These concentrations cover the therapeutic administration range both during cardiopulmonary surgery, emergency deep vein thrombosis (8–2 USP mL^{-1}) and post-operative as well as long-term anticoagulant therapies (2–0.2 USP mL^{-1}).

Using a **1b**/UFH neutralization ratio of 6.2 μg/USP, as suggested by ^{1}H-NMR and fluorescence titration experiments, a complete reversal of the aPTT was reached over the whole concentration range tested. In the same way, titrations by polycations **1b**, **2b** and protamine at fixed 0.3 USP mL^{-1} heparinized plasma were performed. The results shown in Fig. 6 also confirmed the high neutralization efficiency of **1b** and **2b** towards UF heparin in blood, revealing a better activity for derivative **1b** than protamine. Polylysine was not tested due to its well-known toxicity.[29]

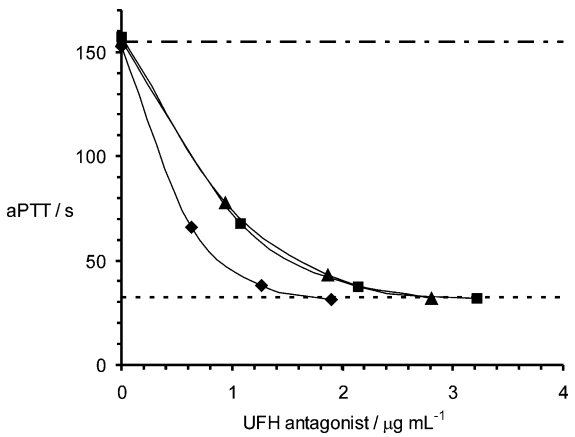


Fig. 6 aPTT clotting assay of UF heparinized blood (0.3 USP mL^{-1}) after addition of antagonist **1b** (◆), **2b** (■), and protamine sulfate (▲). aPTT normal blood (· · ·), aPTT UF heparinized blood (—·—).

Extremely interesting results were obtained by LMWH neutralization with our calixarene derivatives. It is known that protamine is unable to completely reverse the anticoagulant effect of LMWH,[30] a fact of great relevance due to the increasing use of LMWH in clinical practice.

Explorative NMR experiments, carried out by titrating a buffered solution of LMWH with **1b**, **2b** or protamine, confirmed the inability of protamine to completely neutralize LMWH. In contrast, **1b** and **2b** were able to totally eliminate it from the solution, as demonstrated by the complete disappearance of the NMR signals of LMWH.[28] Furthermore, neutralization with derivative **2b** was reached before theoretical charge neutralization. Validation in plasma was gained by an aPTT assay. As this test is considered relatively insensitive for measuring the activity of LMWH, a standard calibration test was performed in order to verify the reliability of the experiment in a heparinized plasma concentration range of 0.25–2.0 UI mL^{-1}.[28] The aPTT test showed 96, 91 and 75% of LMWH inhibition by **2b**, **1b** and protamine respectively, confirming the superior ability of calixarene derivatives towards LMWH neutralization (Fig. 7).

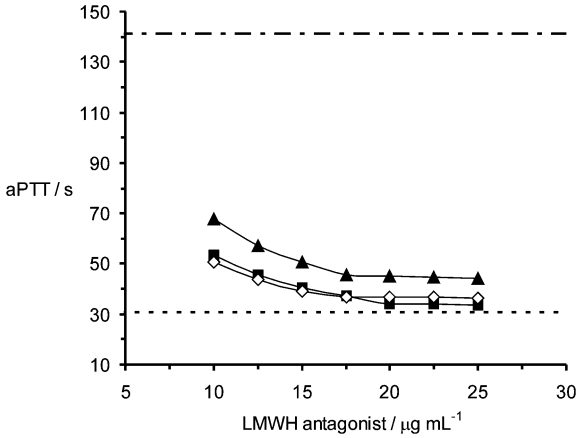


Fig. 7 aPTT clotting assay of LMW heparinized blood (1.5 UI mL^{-1}) after addition of antagonist **1b** (◇), **2b** (■), and protamine sulfate (▲). aPTT normal blood (· · ·), aPTT LMW heparinized blood (—·—).

The protamine-resistant fractions in LMWH are composed of very low-molecular-weight chains with low sulfate charge density.[31] The neutralization of these fractions requires not only a high positive charge density but mainly a high conformational adaptability of the neutralizing agent that allows a better mutual induced fit all around the LMWH interacting groups. For this reason the affinity of these fractions towards the relatively rigid protamine is reduced, while it remains high for the mobile calix[8]arene derivatives. The finding that **2b** neutralizes LMWH better than **1b**, even though **2b** has half the charge density of **1b**, confirms that this recognition phenomenon is deeply affected by other important factors rather than the simple charge density of the antagonists.

Finally, we measured the hemolytic activity of the compounds against human erythrocytes. Hemolytic activity is typically used as a measure of cytotoxicity and is known to increase with the hydrophobicity of the structure.[32] The results, in agreement with this consideration, indicated an HC_{50} of 45 μM (130 μg mL^{-1}) and 20 μM (50 μg mL^{-1}) for derivatives **1b** and **2b** respectively. These HC_{50} values are higher than the necessary amount to neutralize heparin in the highest range of use (8–2 USP mL^{-1}). This finding supports the assumption about the potential therapeutic employment of these calixarene derivatives as promising heparin antagonists.

Conclusion

We have described polycationic calix[8]arene derivatives able to neutralize UF and LMW heparin faster and more efficiently than protamine and polylysine, in aqueous solution and in blood. The results highlight that even for this kind of recognition where the

electrostatic interactions seem to be prevalent, other factors like mutual induced fit and conformational adaptability of the receptor could influence significantly the affinity and specificity of the host–guest association.

These novel heparin antagonists could be exploited in some biomedical applications as protamine substitutes. With this in mind, more in-depth studies on their toxicity and immunogenicity are in progress. Furthermore, in order to obtain new materials for the design of extracorporeal devices as dialysis membranes, the possibility of immobilization on biocompatible substrates is currently under investigation.

Experimental

General methods

Synthesis of compounds **1a** and **2a** has been previously described.[20] Derivatives **1b** and **2b** were obtained by dissolving **1a** and **2a** in 0.5 M HCl and drying in vacuum several times. UF heparin sodium salt from porcine intestinal mucosa (H-9399, 171 USP units mg^{-1}), LMW heparin sodium salt from porcine intestinal mucosa (H-3400, 70–130 UI units mg^{-1}), and protamine sulfate from herring Grade III (P-4505), were purchased from Sigma. Poly-L-lysine hydrobromide 5000–10 000 (81331) was purchased from Fluka. All other chemicals were reagent grade and used without further purification. NMR experiments were made on a Bruker Avance™ 400 (400.13 MHz) instrument. Buffer solutions for NMR titration were prepared in D_2O. Fluorescence experiments were executed on a FluoroMax-3 spectrometer (HORIBA JOBIN YVON). UV spectra were acquired on a Agilent 8453 UV–vis spectrophotometer. aPTT activity was measured with an LAbor Fibrintimer model FI (Hamburg, Germany). The aPTT commercial kit was purchased from Futura System S.r.l., Formello (Roma), Italy.

aPTT activity measurements

For all aPTT activity measurements and samples incubation the temperature was 37 °C. All tests were performed in triplicate. The reported coagulation time represents the mean of the three experiments. The following procedure was carried out for heparin neutralization in blood: a 500 μL test sample was taken from 2.5 mL of citrated blood (2.25 mL blood + 0.25 mL sodium citrate 3.8%) and used to measure the aPTT of the normal blood. UF heparin (4.39 μL of a 1 mg mL^{-1} solution) or LMW heparin (30.0 μL of a 1 mg mL^{-1} solution) was added to the remaining 2.0 mL of citrated blood, incubated for 2 min and fractioned into 400 μL samples. One of these samples was used to measure the aPTT of the heparinized blood, then the appropriate amount of heparin antagonist solution was added to the remaining samples. The samples were incubated for 4 min and centrifuged at 2500 rpm for 5 min. The plasma (100 μL) was added to 100 μL of aPTT reagent solution, incubated for 4 min, then 100 μL of 0.02 M $CaCl_2$ solution was added; the timer on the fibrometer was started simultaneously. Once a clot formed the timer was stopped and the clotting time was recorded.

Hemolysis assay

Hemolytic activity measurements were performed with a 0.25% suspension of freshly drawn human red blood cells (HRBC) in PBS buffer (10 mM PBS, 150 mM NaCl, pH 7.2). A 10 mM buffered solution of **1b** or **2b** was prepared and different amounts (0–50 μL) were added to 450 μL of the erythrocyte suspension. Buffer was added to reach a final volume of 500 μL. The resulting suspension was incubated for 1 h at 37 °C, then centrifuged for 5 min at 1500 rpm. The supernatant was diluted 3 times and the absorbance at 415 nm was measured. The absorbance of the sample containing only erythrocytes was considered as 0% hemolysis, while total hemolysis (100%) was achieved with 2% of triton-X 100. The percentage hemolysis was calculated by:

$$[(A_{415}\text{ of the calixarene-treated sample} - A_{415}\text{ of buffer-treated sample})/(A_{415}\text{ of triton-X 100-treated sample} - A_{415}\text{ of buffer-treated sample})] \times 100\%$$

The HC_{50} values, which represent the concentrations of **1b** or **2b** at which 50% hemolysis was observed, were then determined.

Molecular dynamic simulation

Molecular modelling was performed with Macromodel 7.0.[33] The octasaccharide heparin fragment was deduced from the dodecasaccharide structure taken from the PDB database (1HPN); the calix[8]arene starting structure was manually constructed and minimized in a pleated-loop conformation using the MMFFs force field[34] with a convergence on gradient criterion with a threshold of 0.05 kcal mol^{-1}. The structure of the complex was obtained as follows: heparin was placed on the top of the calix[8]arene, then a first minimization using the MMFFs force field was performed using water as solvent. A molecular dynamics simulation with a stochastic dynamics method[35] was performed at $T = 550$ K to allow the complex to explore a wide range of conformations. The system was equilibrated for 10 ps and the analysis was performed based on a subsequent 10 ns production run and a time step of 1.5 fs. The conformations obtained were stored every 50 ns for a total of 200 structures. Finally, these latter structures were minimized and the lowest energy one was reported.

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