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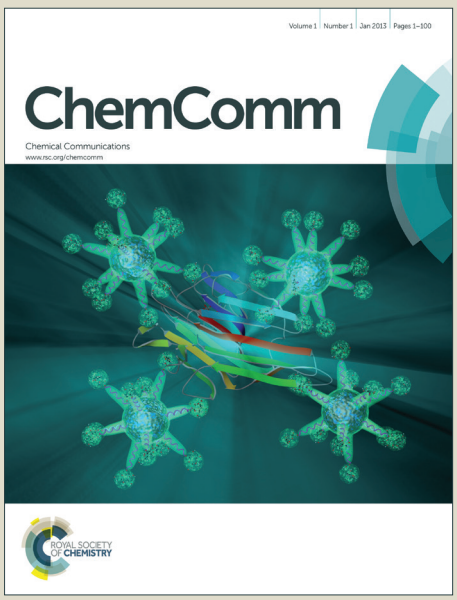

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Monitoring Clinical Levels of Heparin in Human Blood Samples with an Indicator-Displacement Assay†

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We report that a "tree-like" polymer of lysine is able to form a multi-ligand complex with a fluorescently labelled peptide, leading to the almost complete extinction of the optical signal that can be restored upon the introduction of heparin. This simple system allows, for the first time, the turn-ON fluorescent sensing of the anticoagulant in human blood at clinically relevant levels.

Heparin is a polysulfated glycosaminoglycan (GAG) that is extensively used as an intravenous anticoagulant, with for example approximately 12 million Americans treated with this molecule each year (Fig. 1).[1] It is highly desirable to quantify heparin – administrated at dosing levels ranging from 2-8 USP.mL^{-1} (15-58 µM) during surgery to 0.2-1.2 USP.mL^{-1} (1.5-9 µM) during prophylaxis – in order to prevent thrombophilia or bleeding disorders.[2] In clinical settings, heparin quantification relies mainly on clotting time-based assays.[3] However, these methods are not fully satisfactory due to their inaccuracy and poor implementability at point-of-care.[4] To address this issue, the use of fluorescent/colorimetric sensors based on synthetic heparin binders has emerged over the last decade as an interesting alternative for its monitoring.[5,6] Still, only detection and quantification of the anticoagulant in serum was reported in previous studies, imposing therefore additional centrifugation steps from whole blood samples. In order for the sensors to be able to operate in such a competitive and complex medium (*i.e.,* blood), highly efficient and selective binders for heparin are required.

Recently, Smith *et al.* have revealed the ability of cationic dendritic structures to mimic protamine, a heparin neutralizing protein, by binding the polyanionic anticoagulant in a multivalent manner.[7] We thus decided to design new fluorescent heparin sensors based on Dendri-Graft Poly-*L*-Lysine polymers (DGLs, Fig. 1).[8] The advantages of these architectures are numerous: i) straightforward and green synthesis on multi-gram scale, ii) strong positive electrostatic potential[9] for interactions with anionic species, iii) conformational flexibility, allowing the structure to organize its total charge for binding, and iv) chemical flexibility for further functionalization[10] (*e.g.*, immobilization on surfaces). We envisioned that polycationic DGLs should be able to bind several units of a same anionic fluorescein derivative by electrostatic interactions,[11] leading eventually to the extinction of the optical signal.[12] Indeed, because their emission spectrum (λ_{em}=535 nm) sufficiently overlaps with their absorption spectrum (λ_{ex}=485 nm), fluorescein derivatives have a high potential for proximity-dependent fluorescence self-quenching that can mainly be attributed to the resonance energy transfer between close fluorescein molecules.[13,14] Then, removal of the indicator from the host with the highly negatively charged heparin molecule should lead to the restoration of fluorescence, in a typical Indicator-Displacement Assay (IDA).[15]

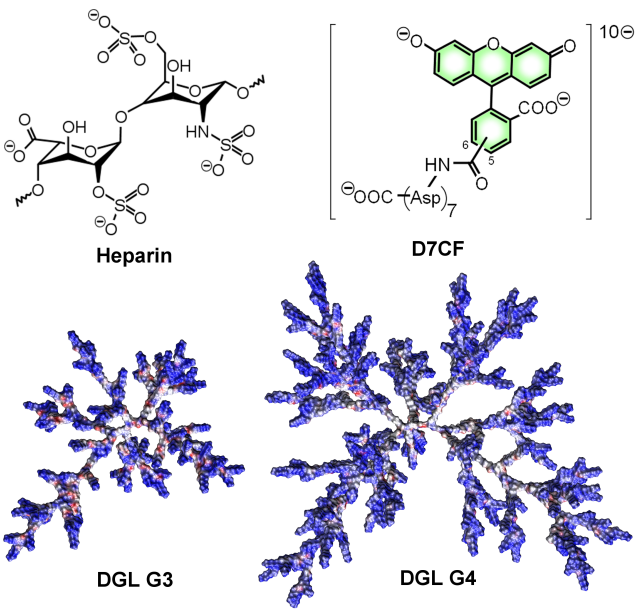

Fig. 1 *Top left*: major repeat unit of heparin. The average molecular weight of most commercial heparin preparations is in the range of 12 to 15 kDa. *Top right*: bioconjugate between a homopolymer of aspartic acid and 5(6)-carboxyfluorescein, D7CF. *Bottom*: minimized structures with electrostatic potential surfaces of third- and fourth-generation Dendri-Graft Poly-L-Lysine (DGL) polymers G3 and G4. For calculation details, see Supporting Information.

Upon addition of the simple 5(6)-carboxyfluorescein (CF) to the third-generation Dendri-Graft Poly-*L*-Lysine polymer (G3) in aqueous solutions containing 10 mM HEPES and buffered to pH 7.8, only a moderate quenching of the fluorescent signal occurs (Fig. 2, blue curve, as compared to CF alone, orange curve). We attributed this incomplete quenching to the presence of free CF in solution along the entire titration experiment, as a result of the partial dissociation of the CF/G3 complex in the low-concentration conditions that are necessary for the detection of heparin in clinical levels (*vide supra*). In a sensing context, one would definitively desire to get a true OFF-ON response toward a target analyte. Thus, a new fluorescent reporter was designed, consisting of a bioconjugate between a homopolymer of

aspartic acid and 5(6)-carboxyfluorescein (Figure 1, D7CF).[16] We expected that additional negative charges would lead to a much more efficient binding event. Stepwise addition of D7CF to G3 in the previous conditions led to a quite different titration profile (Fig. 2, cyan curve, as compared to D7CF alone, yellow curve). A first bell-shaped phase (Fig. 2, A→D) is observed until a fluorescence intensity (FI) minimum (fluorescence quenching yield $(1\text{-}FI_{G3/D7CF}/FI_{D7CF})\times100=95\%$). Upon further increase of the indicator concentration, a portion of the quenched fluorescence emission was recovered (Fig. 2, D→E). A similar titration profile was observed when using the fourth-generation Dendri-Graft Poly-*L*-Lysine polymer (G4), but with a less intense bell-shaped phase (Fig. 2, red curve).

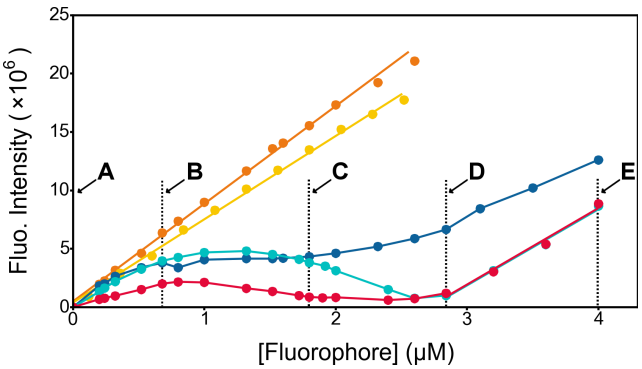

Fig. 2 Fluorescence titration of DGLs with CF and D7CF. *Orange*: CF alone, *yellow*: D7CF alone, *blue*: CF in presence of G3, *cyan*: D7CF in presence of G3, *red*: D7CF in presence of G4. Conditions: 10 mM HEPES buffer, pH 7.8, [G3]=163 nM, [G4]=55 nM, λ_{ex}=485 nm, λ_{em}=535 nm. Both DGLs are at same concentration with respect to monomeric lysine residues, [lysine]=20 µM.

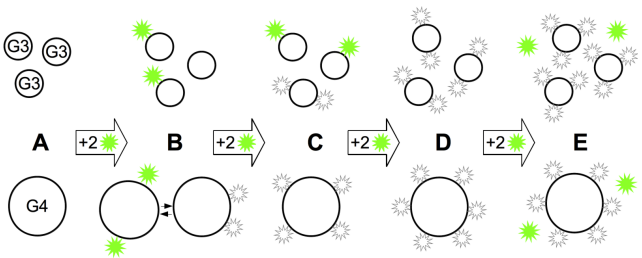

Fig. 3 Proposed model for the binding behaviours of D7CF to G3 (*top*) and G4 (*bottom*).

A simple model to explain such binding behaviours is proposed, starting from the same amount of G3 and G4 with respect to the number of lysine residues (Fig. 3, A).[17,18] As a first step, the addition of D7CF leads to either the absence or a partial quenching of the fluorescent signal, depending on the dendrigraft size (Fig. 2 and 3, B). Upon further dye loading, the fluorescence quenching occurs earlier for the fourth-generation dendrigraft as a result of a higher surface ligand density (Fig. 2 and 3, C). Finally, both receptors saturation is reached for the same [lysine]/[D7CF] ratio=7.04 (Fig. 2 and 3, D), followed by the linear reappearance of the optical signal (Fig. 2 and 3, D). It should be noted that the regime of this second straight phase is identical to the one of the D7CF alone, meaning that extra dye content – beyond the FI minimum that corresponds to the saturation of the dendrigraft with D7CF – is actually totally free in solution. Signal maximum quenching is reached at far less than 1 equiv. of dendrigraft, which means high binding stoichiometries. In 10 mM HEPES buffer at pH 7.8, the maximum number of D7CF ligands per dendrigraft was determined to be 17 and 52 for G3 and G4, respectively. For subsequent heparin sensing experiments, the G4/D7CF complex was preferred since the redistribution of the ligand density on the dendrigraft surface during IDAs perturbs to a lesser extent the fluorescent signal (Fig. 2 and 3, D→C→B→A).

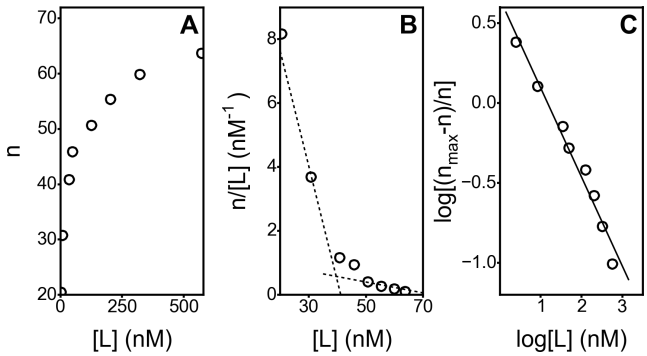

Fig. 4 Binding isotherm (**A**) of the D7CF ligand with the DGL G4 polymer and the corresponding Scatchard (**B**) and Hill (**C**) plots. n is the mean number of ligands per polymer, n_{max} is the maximum number of ligands per polymer, and [L] is the free ligand concentration. *Dotted lines*: Scatchard plot fitted with a two binding sites model.[19a] Conditions: 10 mM HEPES buffer, pH 7.8, [G4]=195 nM.

Quantitative analysis of the interactions between G4 and D7CF was performed by analytical centrifugation at different G4/D7CF ratios. Experiments were realized using Vivacon® 500 concentrators (10 kDa membrane cut-off) to selectively retain receptor/fluorescent probe complexes but not free D7CF; the concentration of which being determined by fluorescence spectroscopy. A binding isotherm representing the number of bound ligands n per G4 was plotted as a function of free ligand concentration [L] (Fig. 4, A). The isotherm displays a rapid increase of n with [L] suggesting a rather high ligand/substrate association constant. Surprisingly, the corresponding Scatchard plot appeared to be non-linear (Fig. 4, B), which is indicative of either: i) multiple non-equivalent independent binding sites, or ii) multiple equivalent non-independent binding sites. To test both hypotheses, the binding isotherm can be directly fitted according to the corresponding models (see Supporting Information).[19] Whereas a two sets of binding sites model turned out to be in poor agreement with the experimental data (Figure 4, B, dotted lines), a linear Hill plot of the experimental data was obtained (Fig. 1C). The slope of the straight line (*i.e.*, Hill coefficient n_H=0.56) suggests negative cooperativity of D7CF binding to G4. By negative cooperativity we mean here the alteration in the affinity of a binding site, when other sites are occupied by a ligand. To put it simply, the more D7CF ligands are bound to G4, the less the incoming dyes have available positives charges for further binding. From the Hill plot, a global dissociation constant K_D=14.8 nM was also determined between G4 and D7CF in 10

mM HEPES buffer at pH 7.8.

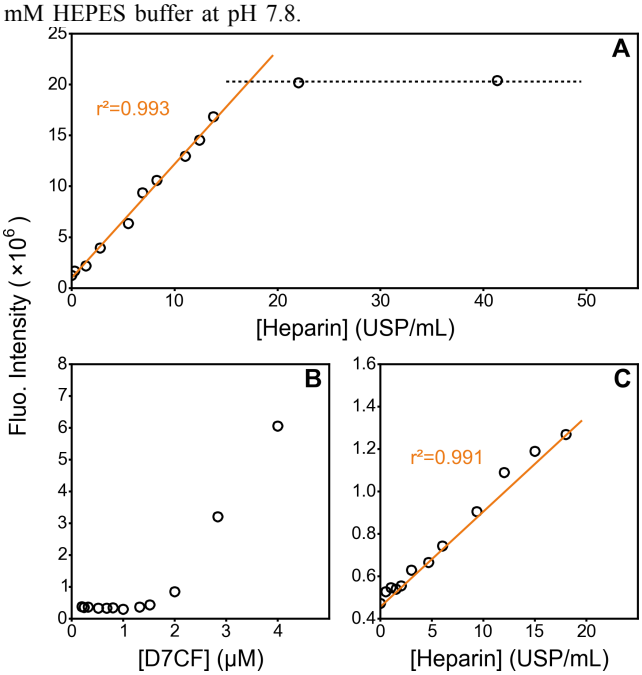

Fig. 5 (**A**) Fluorescence titration of heparin with a G4/D7CF complex in 10 mM HEPES buffer, pH 7.8. [G4]=55 nM, [D7CF]=2.84 μM. (**B**) Fluorescence titration of G4 with D7CF in 10 mM HEPES buffer, pH 7.8 and in presence of 5% human blood. [G4]=55 nM. (**C**) Fluorescence titration of heparin with a G4/D7CF complex from heparinized human blood samples.[20] Conditions: 5% human blood, 10 mM HEPES buffer, pH 7.8, [G4]=55 nM, [D7CF]=1.52 μM, λ_{ex}=485 nm, λ_{em}=535 nm. Heparin concentrations given in abscissa are the actual heparin levels in blood samples.

In presence of our G4/D7CF sensor, increased levels of heparin in buffered water correlated linearly with FI response (Fig. 5, A). The following plateau indicates the complete displacement of the indicator by heparin from the dendrigraft surface (FI=20.3×10⁶ corresponding to [D7CF]=2.8 μM in solution, see: Fig. 2, yellow calibration curve). These observations validate the proof-of-principle of our IDA for heparin monitoring. Before testing the assay from heparinized human blood samples, a titration of G4 with D7CF in 10 mM HEPES buffer, pH 7.8 and in presence of 5% human blood was realized to determine the optimal receptor/indicator ratio (Fig. 5, B). Two differences from previous titrations in pure buffer can be spotted: i) the lower amount of D7CF that is necessary to reach dendrigraft saturation ([lysine]/[D7CF]=13), and ii) the absence of the bell-shaped phase. Such differences could be explained by the partial complexation of G4 with blood components (*e.g.*, proteins), resulting in both less available dendrigraft surface (=faster saturation) and tighter indicators confinement (=more efficient quenching). In a typical sensing experiment, a sample of human blood containing heparin of a known concentration was added to a buffered aqueous solution of G4 and D7CF (final blood/buffer ratio=0.05). The fluorescent emission at 535 nm (excitation at 485 nm) was used to detect and quantify heparin within the range of clinically relevant concentrations (0-18 USP.mL^{-1}, Fig. 5, C). Limits of detection (LOD) and quantification (LOQ) of heparin in human blood were determined as 0.12 USP.mL^{-1} and 0.30 USP.mL^{-1}, respectively. Because blood has a strong absorption band with a maximum centred around 410 nm, fluorescence intensities are significantly lower in comparison with measurement in pure buffer.

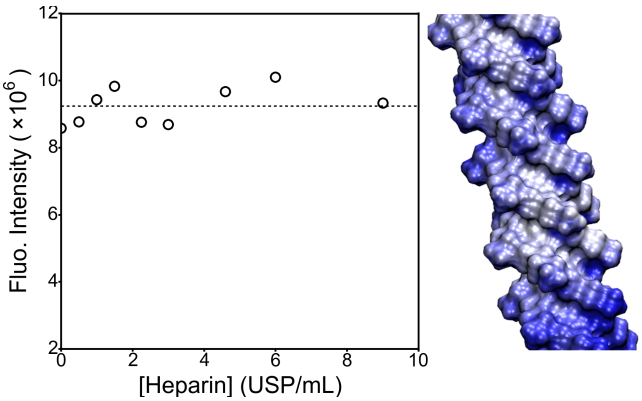

Fig. 6 *Left*: Fluorescence titration of heparin with a PLL_{400}/D7CF complex. Conditions: 10 mM HEPES buffer, pH 7.8 in presence of 5% serum, [PLL_{400}]=50 nM ([lysine]=20 μM), [D7CF]=1.52 μM, λ_{ex}=485 nm, λ_{em}=535 nm. *Right*: Electrostatic potential surface of a poly-L-lysine fragment in a α-helix conformation.[21]

Finally, a heparin sensing assay with α-poly-*L*-lysine (PLL_{400}) – as a linear equivalent of DGLs – in presence of serum was unsuccessful (Fig. 6). The D7CF molecules are fully displaced from the polymer even in absence of heparin. This definitely highlights the importance of the receptor topology for a strong association event between the binding partners when working in complex media. Additional control experiments in serum with others GAGs were also performed (Fig. 7). Whereas hyaluronic acid (one negative charge by dimeric unit) was not able to displace the indicator, it turned out the chondroitin sulfate A (two negative charges by dimeric unit) was almost as efficient as heparin in this role; suggesting that electrostatic interactions are the predominant interactions between GAGs and DGLs. It must be noted that the mean concentration of endogenous total GAGs in serum is about 15 nM,[22] which is about two orders of magnitude below heparin therapeutic concentrations, thus excluding possible interferences during the monitoring of the exogenous anticoagulant.[23]

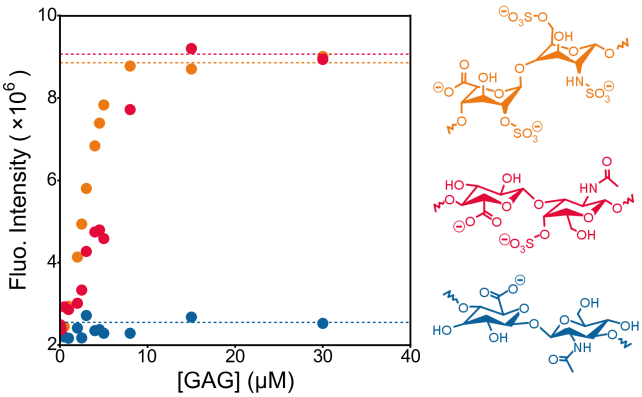

Fig. 7 Fluorescence titration of heparin (*orange*), chondroitin sulfate A (*red*), and hyaluronic acid (*blue*) with a G4/D7CF complex.[20,24]

Conditions: 10 mM HEPES buffer, pH 7.8 in presence of 5% serum, [G4]=55 nM ([lysine]=20 μM), [D7CF]=1.52 μM, λ_{ex}=485 nm, λ_{em}=535 nm.

In conclusion, we reported a simple indicator-displacement assay, involving a dendrigraft and a new designed dye, which allows for the first time the turn-ON fluorescent detection and quantification of heparin in human blood and at actual clinical levels. Qualitative and quantitative analyses of the involved supramolecular interactions were proposed. Also, this study revealed the ability of dendrigrafts to bind very efficiently some GAGs. By intervening in GAG-mediated biological processes, such accessible and non-immunogenic[25] materials may find new biomedical applications. We are currently working in this direction.

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† Electronic Supplementary Information (ESI) available: Synthetic procedure for D7CF; Details for the analytical and computational methods. See DOI: 10.1039/b000000x/

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