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Selective Chromofluorogenic Sensing of Heparin by using Functionalised Silica Nanoparticles Containing Binding Sites and a Signalling Reporter

Estela Climent, Pilar Calero, M. Dolores Marcos,* Ramón Martínez-Máñez,* Félix Sancenón, and Juan Soto[a]

An interesting approach for the preparation of novel sensory systems with enhanced properties is the combination of supramolecular and solid state chemistry concepts. In this field, the anchoring of molecular entities onto pre-organised structures results in the preparation of advanced hybrid systems that show remarkable synergic effects.[1] Prominent examples involving the combination of supramolecular tools with nanoscopic scaffoldings have been reported over the last few years. Among different nanosized solids the use of silica nanoparticles is appealing, owing to its easy preparation, straightforward surface functionalisation and large stability in water.[2] Silica nanoparticles functionalised with organic fluorophores that contain binding sites have been used for the fluorescent recognition of certain metal cations,[3] but anion detection using silica nanoparticles as supports is a scarcely studied field.[4]

Following our interest in the development of novel hybrid organic-inorganic materials as probes[5] we focused our attention towards the preparation of new optical chemosensors for heparin detection. Heparin is a highly sulphated polysaccharide formed by a heterogeneous mixture of diverse chain lengths, which consist of repeating copolymers of 1→4 linked iduronic acid and glucosamine residues in a semi-random order.[6] Heparin has been used extensively as clinical anticoagulant to prevent thrombosis[7] and the close monitoring and control of heparin blood levels during its application is of relevant importance. Several methods for heparin quantification have been traditionally used.[8] Additionally, very recently supramolecular chemistry concepts have been applied in order to prepare chromogenic and fluorogenic chemosensors for the selective detection and quantification of this important polyanion. Thus, colorimetric and fluorimetric displacement assays,[9] fluorescent molecular receptors,[10] organic polymers[11] and simple aggregation–deaggregation protocols using AuNps have been described.[12]

The new chromo-fluorogenic sensing paradigm we follow herein is shown in Scheme 1. It involves the use of silica nanoparticles decorated with two different moieties; thiol groups (R) and polyamines (H). The role of groups R is to react with the squaraine dye (D) through the nucleophilic attack of the thiol group to the electron deficient, central, four-member ring of the squaraine scaffolding. This is known to result in the bleaching of blue squaraine solutions. Additionally H is a suitable host for anion coordination.

The sensing protocol relies in the concept that interaction of H with a suitable guest (G) would inhibit of the reaction between R and D, resulting in a chromo-fluorogenic signalling. In this Scheme the group H is able to control (via coordination or not with suitable guests) mass transport of certain molecules (in our case D) from the solution to the surface of the nanoparticle. Similar procedures have been reported by Umezawa for the electrochemical detection of certain guests.[15]

Squaraines are popular dyes that show favourable spectroscopic properties. These dyes possess typically narrow and very intense absorption bands ($\varepsilon > 10^5$) at the red end of the visible spectral window and fluorescence bands of a mirror-image shape with high fluorescence quantum yields $\Phi > 0.1$. In fact, attracted by these features, we and others have utilised squaraine derivatives in a number of chemical signalling systems for cations, anions and neutral molecules.[13] Also, squaraines have been used for the development of detection protocols in biological samples.[14] On the other hand, polyamines (and polyammonium groups) are expected to display interactions with charged polysaccharides through hydrogen bonding and electrostatic coulombic attractive forces.

Coated silica nanoparticles **N1** were prepared by using the trialcoxysilyl derivatives mercaptopropyltrimethoxysi-

[a] E. Climent, P. Calero, Dr. M. D. Marcos, Prof. R. Martínez-Máñez, Dr. F. Sancenón, Dr. J. Soto
Instituto de Química Molecular Aplicada
Universidad Politécnica de Valencia
Camino de Vera s/n, 46022 Valencia (Spain)
Fax: (+34) 96-387-9349
E-mail: rmaez@qim.upv.es

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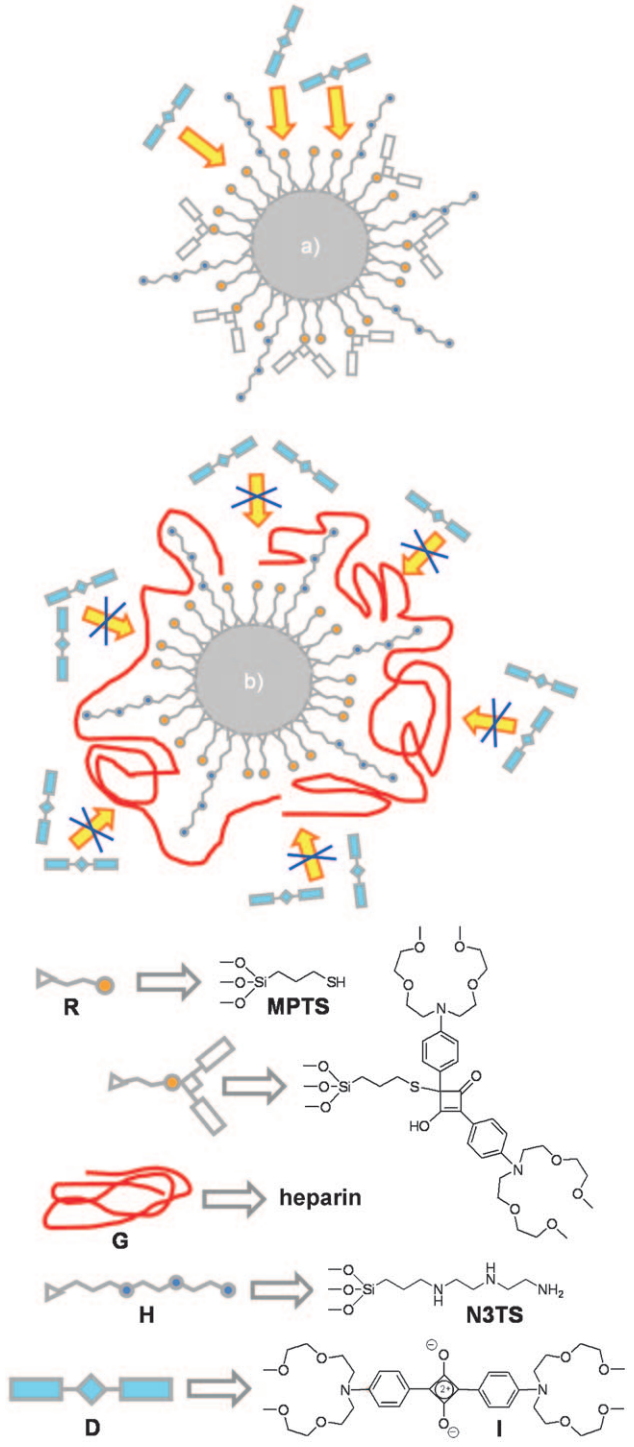

Scheme 1. Colorimetric protocol for heparin signalling. a) bi-functionalised nanoparticles **N1** without the presence of heparin are able to react with squaraine **I**, and b) heparin coordinate with the polyamines on **N1** inhibiting the thiol-squaraine reaction.

lane (**MPTS**) and 3-[2-(2-aminoethylamino)ethylamino]-propyl-trimethoxysilane (**N3TS**) following reported procedures by Montalti and co-workers (see the Supporting Information).[16] In this synthetic protocol, commercially available silica nanoparticles were heated at 70 °C in a mixture of water/ethanol/acetic acid (1:2:1, v/v/v) in the presence of the coating subunits.

Solid **N1** was characterised using standard procedures. The content of polyamine and thiol were determined by elemental analysis and thermogravimetric measurements and amounts to 0.49 and 2.87 mmol/gSiO_2, respectively. The resulting nanoparticles were characterised by transmission electron microscopy (TEM, see the Supporting Information). The final silica particles have a mean diameter of 20 nm. Analysis of the ^{1}H NMR spectra revealed signal broadening, typical of anchored subunits on nanoparticles. Taking into account the contents and the value of the specific surface of the silica nanoparticle support (195 m^2g^{-1}), the average coverage on solid **N1** by triamine and thiol groups was 8.80 molecules/nm^2, resulting in an average distance between anchored molecules of about 3.50 Å. By approximation of a single monolayer of subunits on a smooth sphere, a total of 11 000 subunits per nanoparticle are calculated.

One advantage of using silica derivatives as nanoparticles, is the possibility of prepare dispersible stable suspensions. This is especially appealing when using silica supports in sensing protocols because the monitoring of colour or fluorescence changes can be easily made, avoiding the need of filtering as when using micro-metric silica particles. Thus, **N1** nanoparticles were found to form stable suspensions in DMSO containing water up to 50 %. Additionally the squaraine **I** is stable in acetonitrile solutions for weeks without any noticeable decomposition. Consequently, studies were carried out using suspensions **N1** in DMSO to which water containing a certain anion and an acetonitrile solution of **I** were consecutively added. This gives the final water/DMSO/acetonitrile (45:45:10, v/v/v) mixtures in which the following experiments were carried out.

A range of pH values were tested of which pH 7 was selected, because the system displayed a higher selectivity.[17] The studies were carried out in the presence of anions (fluoride, chloride, bromide, iodide, sulfate, phosphate and nitrate), monosaccharides (sucrose, glucose, fructose, arabinose), charged disaccharides (calcium lactobionate) and charged polysaccharides (chondroitin-4-sulfate and heparin).[18] A clear guest control of the squaraine–thiol reaction can be seen in Figure 1, which plots the absorption at

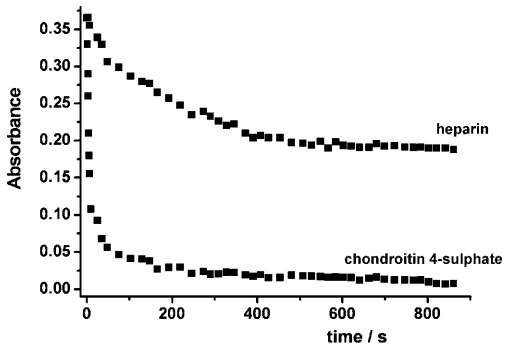

Figure 1. Absorbance at 643 nm (squaraine band) vs. time at pH 7 for mixtures of water/DMSO/acetonitrile (45:45:10, v/v/v) containing **N1**, squaraine **I** and heparin or chondroitin-4-sulfate.

643 nm (squaraine band) versus time for solutions containing **N1** and **I** at pH 7 in the presence of a certain guest. As it can be seen, the reaction of the dye with the thiols is highly inhibited in the presence of heparin, whereas it is still effective and very fast in the presence of chondroitin-4-sulfate. Other guests (vide infra) display similar reaction profiles to that found for chondroitin-4-sulfate (data not shown). To study the effect of the guest concentration, the following studies were carried out. In a typical assay a DMSO suspension (1.35 mL) of **N1** (80 mg in 150 mL of DMSO) were mixed with water containing a certain concentration of a given anion/saccharide at pH 7 (1.35 mL) and then an acetonitrile solution of squaraine **I** (0.3 mL, $C_{squaraine} = 5 \times 10^{-5}$ mol dm^{-3}) was added. After 15 min the absorbance of the squaraine band (643 nm) was measured.

It can be seen how the coordination of heparin to the binding structure (H) results in the modulation of the squaraine-thiol reaction that is function of the heparin concentration (Figure 2). The charged polysaccharide chondroitin-4-sulfate partially hinders the thiol-squaraine reaction, but only at concentrations higher than 400 μM. The presence of other species; that is, fluoride, chloride, bromide, iodide, sulfate, phosphate, nitrate, lactobionate, sucrose, glucose, fructose and arabinose did not induce any effect even at relatively high concentrations. This full accessibility of squaraine to the thiol-functionalised nanoparticle surface is a consequence of the poor interaction of small anions and mono- and disaccharides with the polyamine network at neutral pH.

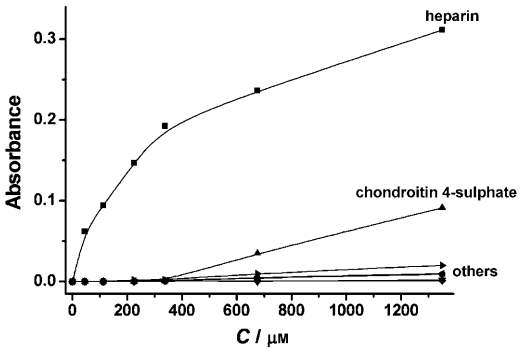

Figure 2. Absorbance at 643 nm (squaraine band) at pH 7 in water/DMSO/acetonitrile (45:45:10, v/v/v) mixtures containing **N1**, squaraine **I** and a certain concentration of heparin, chondroitin-4-sulfate and other guests (fluoride, chloride, bromide, iodide, sulfate, phosphate, nitrate, sucrose, glucose, fructose, arabinose and calcium lactobionate).

The degree of inhibition (heparin > chondroitin-4-sulfate) is clearly related to the anionic charge density of the respective polysaccharide, which suggests that the electrostatic interactions play a dominant role in binding with the polyamine network (H).[19] The overall result is a highly selective colorimetric signalling of heparin, as seen in Figure 2.

By using simple UV-visible measurements a detection limit of ≈50 μM for heparin detection has been achieved. Bearing in mind that a therapeutic dosing level of heparin ranges from 67 μM to 1.7 μM[20] the chromogenic assay would only be able to cover the upper limit. To increase the range of applicability, we made use of the well known fluorescent properties of squaraine **I**. Upon addition of increasing quantities of heparin into different water/DMSO/acetonitrile (45:45:10 v/v/v) suspensions of **N1**, containing **I**, and monitoring the emission band of **I** centred at 679 nm (λ_{exc} = 649 nm) the titration curve depicted in Figure 3 was obtained. Using emission measurements a detection limit for heparin as low as 2.0 μM was determined.

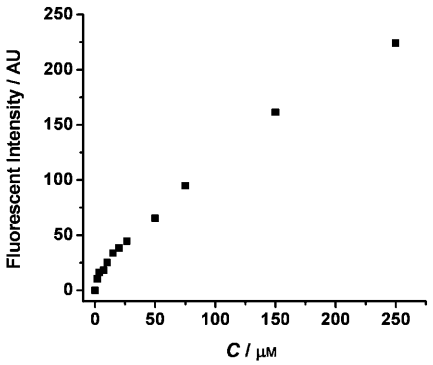

Figure 3. Intensity emission at 679 nm vs. concentration of heparin at pH 7 in water/DMSO/acetonitrile (45:45:10, v/v/v) mixtures containing **N1**, squaraine **I** and a certain heparin concentration.

In relation to the chemical stability of **N1** nanoparticles toward possible thiol oxidation with time, it is important to note that the sensing features remained unaltered for months. Thus a five month old sample of **N1** showed similar recognition features against heparin at pH 7.0 than that found for a freshly prepared sample.

Finally we have made an attempt to quantify the interaction of the binding structure with the guest and have developed a model that is able to describe the observed behaviour in Figures 1 and 2. The proposed model suggests that the apparent rate of the reaction between the squaraine and the thiols follows a second order kinetics and is proportional to both, i) the concentration of squaraine dye and ii) the fraction of binding centres (H) that are not occupied by the corresponding guest at a certain concentration [Eq. (1)]:

$$-\frac{d[Sq]}{dt} = k_v(1-\Theta)[Sq]^2 \quad (1)$$

in which k_v is the absolute rate constant of the squaraine-thiol reaction, Θ is the number of occupied binding centres at the surface and [Sq] is the squaraine concentration (in mol dm^{-3}). The product $k_v(1-\Theta)$ is the "apparent rate constant" that reflects that the mass transport of the squaraine from the solution to the thiol-functionalised nanoparticle surface is dependent on the interaction between H and G (see Scheme 1). Our model assumes that the isotherm coverage can be expressed in terms of the Langmuir adsorption constant (K) and the concentration C (in mol dm^{-3}) of the

charged polysaccharide at the equilibrium. From these initial hypotheses, the following final Equation (2) used for fitting was obtained:

$$\frac{1}{A}-\frac{1}{A_0}=\frac{k_v}{\varepsilon}\left(1-\frac{KC}{1+KC}\right)t$$

in which [Eq. (3)]:

$$c=\frac{-(K(\frac{n_M}{V}-C_0)+1)}{2K}+\sqrt{\left(\frac{(K(\frac{n_M}{V}-C_0)+1)}{2K}\right)^2+\frac{C_0}{K}}$$

In this equation, A_0 and A are the absorbances of the squaraine band ($\lambda=643$ nm) at $t=0$ and at time t, ε is the molar extinction coefficient of the squaraine **I**, C_0 is the added concentration of charged polysaccharide (in mol dm^{-3}), n_M is the maximum concentration of charged polysaccharide that can be adsorbed in the solid by interaction with the amines and V is the volume (in litres) in which the experiment was carried out. The formalism that leads to the deduction of this equation is given in the Supporting Information.

To determine *kv* (the absolute kinetic rate for the squaraine-thiol reaction on the surface) and *K* (the interaction between a given charged polysaccharide and the polyamines at the surface) the reaction between the solid **N1** and the squaraine **I** was studied by using different concentrations of charged polysaccharides and a fixed concentration of squaraine. *log K* values of 5.55 and 4.38 for heparin and chondroitin-4-sulfate were determined respectively. Log *kv* was found to be 2.88.

Finally, we were concerned with the possibility that the observed effect was not caused by the protocol shown in Scheme 1, but a result of some simple change in the reactivity of the squaraine dye with the thiol groups anchored on the silica surface in the presence of heparin. To eliminate this possibility we prepared solid **N2**, which is similar to **N1**, but lacking the presence of the binding structure (H in Scheme 1); that is, **N2** are silica nanoparticles functionalised only with thiol groups. Interestingly this solid shows a very-rapid anion-independent decolouration of the dye, as consequence of the reaction of the squaraine with the SH groups, which indicates that the control of the reactivity observed in Figure 1 is a result of the coordination of the protonated amines (H) with the anionic heparin (G).

In summary, we have applied here a new approach on nanoparticles for the chromo- fluorogenic detection of heparin. The system we show here is one of the few reported heterogeneous molecular probes for anion sensing in water-based solutions. Additionally, this protocol shows enhanced features and displays potential that can not be considered when using classical receptors.[20] For instance, it would be possible to modify with a minimum effort the dye (including size and charge) and the binding structures (H groups). Additionally, the ratio R/H (see Scheme 1) can also be easily modulated to give rise to solids with different sensing features and dynamic ranges. Additionally, the anchoring of independent groups on silica nanoparticles in close proximity to the surface support leads to cooperative effects without the need of a direct covalent link between them, avoiding complex synthetic procedures. We also believe that the general protocol shown in Scheme 1 can be of general application to other systems and may lead to new functionalised nanoparticles that show enhanced sensing selective response towards target bio-guests.

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Keywords: chromogenic sensors · heparin · hybrid materials · ion channels · molecular recognition

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