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Hyperpolarized Solvatochromic Nanosensors towards Heparin Sensing in Blood

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Abstract: Heparin quantification at the patient's bedside has been of medical interest for years but a suitable point of care measurement method for whole blood is still elusive. Our group has recently developed a nanoparticle-based optical sensor for protamine that allows for heparin quantification in plasma. This work discusses the effect of the transducing-dye structure and the promise of embedding the sensors in an agarose gel to avoid red blood cell interference.

Keywords: Agarose · Heparin · Nanoparticles · Protamine · Solvatochromism

Robin Nussbaum studied chemistry at the University of Geneva and graduated with a Bachelor's degree in 2019. He pursued his studies with a Master's degree in chemistry during which he worked on electrochemical hydrogenase activity in the Milton group and on protamine optical sensors in the Bakker group for his Master thesis. In 2021, he started his PhD studies in the Bakker group, continuing his Master project while also beginning investigations into ultrasensitive pH electrochemical sensors.

1. Introduction

Heparin is an anionic glycosaminoglycan commonly extracted from natural sources for use during surgical procedures owing to its anticoagulant properties.[1] Typical target blood concentrations range from 0.1 to 5 IU/mL, and redosing is often required due to its clearance from the body resulting in a highly dynamic concentration.[2] Thus, it is crucial to be able to monitor the heparin level quickly at the patient bedside. Excessive heparin concentrations can induce uncontrollable bleeding while insufficient concentrations will not prevent thrombosis.[3] The effects of heparin are reversed *via* polyionic binding with protamine, a naturally occurring arginine-rich protein useful for neutralizing excessive heparin.[1]

Today, the gold standard for heparin quantification is the anti-Xa assay.[4] Unfortunately, this fluorescence-based method requires a significant amount of time as it cannot be performed in whole blood, resulting in long turnaround times from the central laboratory. Other clinical methods such as activated partial thromboplastin time (aTTP) or the Hepcon/HMS device also present drawbacks such as poor selectivity[2] or excessive costs for only minor improvements.[5] The need for a more convenient technique stimulated this research into developing more suitable heparin quantification methods.

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The first approach to quantify heparin was reported in the 1990s with ion-selective electrodes (ISEs) containing the protamine-selective lipophilic ion-exchanger $DNNS^-$.[6–8] The determination of heparin was achieved by quantifying unbound protamine after addition to the sample. The mechanism of response was investigated and found to be based on a bulk extraction process coupled to accumulation at the membrane surface.[9]

Later, chronopotentiometry was successfully used to achieve operationally reversible protamine quantification in blood.[10,11] Despite being well understood, electrochemical methods have not yet made their way into clinical practice, perhaps because they do not lend themselves to the production of disposable single use tests.

A convenient alternative to ISEs are ion-selective optical sensors which have low manufacturing costs and lend themselves to miniaturization.[12] The initial class of optical protamine sensors developed were based on cast films,[13,14] with recognition based on coextraction with a solution anion or formation of a protamine-ionophore complex at the surface of the film. Unfortunately, the equilibration time was found to be excessively long owing to the limited mass transport rate within the sensing film.[14]

Moving from a 2D film to emulsified nanoparticles (here in the order of 100 nm dia.) significantly increases the sensor surface area to volume ratio, allowing lower detection limits and significantly decreased response times.[15] Protamine and heparin sensitive emulsion-based nanoparticles containing a chromoionophore were subsequently developed but showed matrix interference in human serum.[16] Moreover, chromoionophore-based optodes are also often subject to undesirable pH cross-response.[17]

Recently, our group developed emulsion-based protamine sensitive particles utilizing a solvatochromic dye-based transducer.[18] This sensor showed no pH cross-response and was successfully used in human plasma to quantify heparin in hospital samples by heparin-protamine titration. In a classical ion-exchange mechanism (Fig. 1a), one would expect a blue shift when protamine enters the particles and the dye is expelled. However, with the particles studied here, the sensor experienced a red shift when binding to protamine (Fig. 1b). It has been previously hypothesized that the dyes experience a hyperpolarizing environment (meaning more polarizing than water) when no protamine is present due to the interaction between the sulfonate group of the $DNNS^-$ and the positively charged part of the dye.[18] When protamine binds to its ionophore, the dye is left with

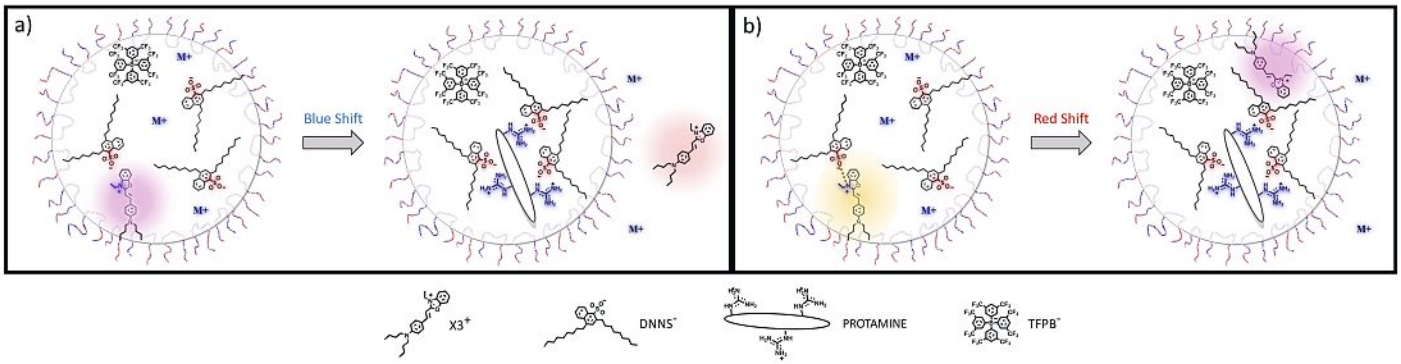

Fig. 1. DOS core nanoemulsion stabilized with the surfactant Pluronic F-127 with a) classical ion-exchange mechanism inducing a blue shift and b) proposed new mechanism inducing a red shift.

the $TFPB^-$ as counterion and experiences a much more non-polar environment because of the strongly delocalized negative charge.[19]

The strategy of freely mixing particle-based sensors with the sample solution is not applicable to whole blood because of the high background absorbance. As red blood cells are too large to effectively penetrate the pores of agarose gel,[20] we are exploring such gels to realize an optical whole blood assay for heparin. Such a gel-based nano-optode approach was already demonstrated by Xie and coworkers for the measurement of potassium or calcium in whole blood.[21,22] They were able to construct calibration curves for both ions in their respective physiological concentration range.

The work reported here investigates the solvatochromic properties of three different solvatochromic dyes that are potentially useful for the fabrication of emulsified ion sensors. One of the candidates is subsequently explored in protamine sensitive nanoparticles using hyperpolarization as sensor readout. The nanoparticles are subsequently embedded in an agarose gel to overcome the background absorbance of blood.

2. Experimental Section

2.1 *Chemicals*

Dioctyl sebacate (DOS) and sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB) of Selectophore™ grade, Pluronic F-127, boronic acid, citric acid, phosphoric acid, sodium hydroxide (NaOH) and agarose type-I were purchased from Millipore-Sigma (Burlington, USA). The three solvatochromic dyes were synthetized as previously reported.[19] Blood samples were provided in citrated tubes by the blood transfusion center of the Geneva University Hospitals (HUG).

2.2 Particle Preparation

5.46 µmol of DNNSH, 1.82 µmol of dye, 1.82 µmol of NaTFPB and 1 mg DOS were dissolved in 2 mL methanol. 200 µL of this cocktail was poured in 5 mL 0.02 wt % Pluronic® F-127 aqueous solution contained in a 10 mL vial. The vial was shaken under compressed air for 1 h to allow methanol evaporation. The particles were then buffered by a 5% v/v addition of 500 mM universal buffer (500 mM citric acid, boronic acid and phosphoric acid, pH=7.4) resulting in a 25 mM buffer concentration.

2.3 *Absorbance Measurements*

Absorbance measurements were performed in an Eppendorf Microplate (VIS, 96/F-PS) with an Infinite® M Nano Microplate Reader controlled with i-control 2.0 software. 100 µL of protamine solution in water was mixed with 100 µL of buffered particles. The injector of the instrument was used when precise time control was required.

The absorbance scans at different protamine concentrations were acquired between 400 and 700 nm with a 5 nm increment after complete reaction. The protamine calibration was obtained measuring the absorbance 460 and 530 nm two minutes after particles addition and performing a ratiometric analysis with Eqn. (1).

2.4 *Cuvette Preparation and Pictures*

100 mg of agarose were dissolved in 5 mL of 25 mM univeral buffer, pH = 7.4, heated for 1 h and mixed with buffered particles solution (50:50). 750 µL of this warm solution was poured into a polystyrene cuvette using an Eppendorf Multipette® M4, covered with parafilm and cooled down for 1 h.

Pictures were taken every 60 s for a period of 30 min with a Canon EOS 5D Mark III camera in RAW format with a MP-E 65mm f/2.8 1-5x Macro Photo objective, using an acquisition time of 1/2000 s, an aperture size of f/5 and an ISO of 6400. The camera was mounted on a tripod and the sample was placed in a shooting tent with two white LED arrays behind the cuvettes as light sources.

3. Results and Discussion

3.1 *Sensor Characterization with Several Dyes*

Our sensor is composed of a solvatochromic dye (Fig. 2), a protamine recognition unit $DNNS^-$ (at a molar ratio to dye of 3:1) and NaTFPB as counter ion in a core containing the organic solvent (plasticizer) DOS that is stabilized by Pluronic F-127.

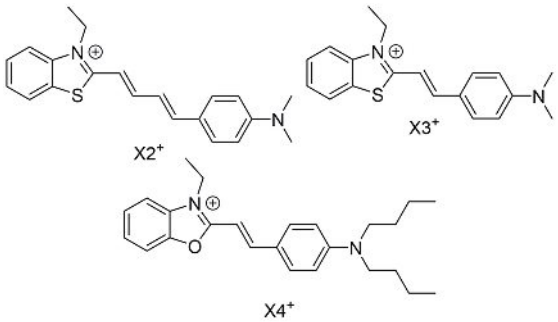

Fig. 2. Structures of the solvatochromic dyes studied in this work.

Since nanoparticles are used, the response time is expected to be quite short.[15] However, when studying the absorbance kinetics of the sensors containing $X2^+$ and $X3^+$, the response was in the order of minutes (data not shown). This suggests that there were additional rate-limiting processes occuring than just diffusion limited ion-exchange which would be expected to take place much faster. One might argue that the high concentration of components in the particles made it difficult for the protamine to disturb the dye-$DNNS^-$ interaction. The consequent size of protamine may indeed limit its diffusion in a very concentrated medium.

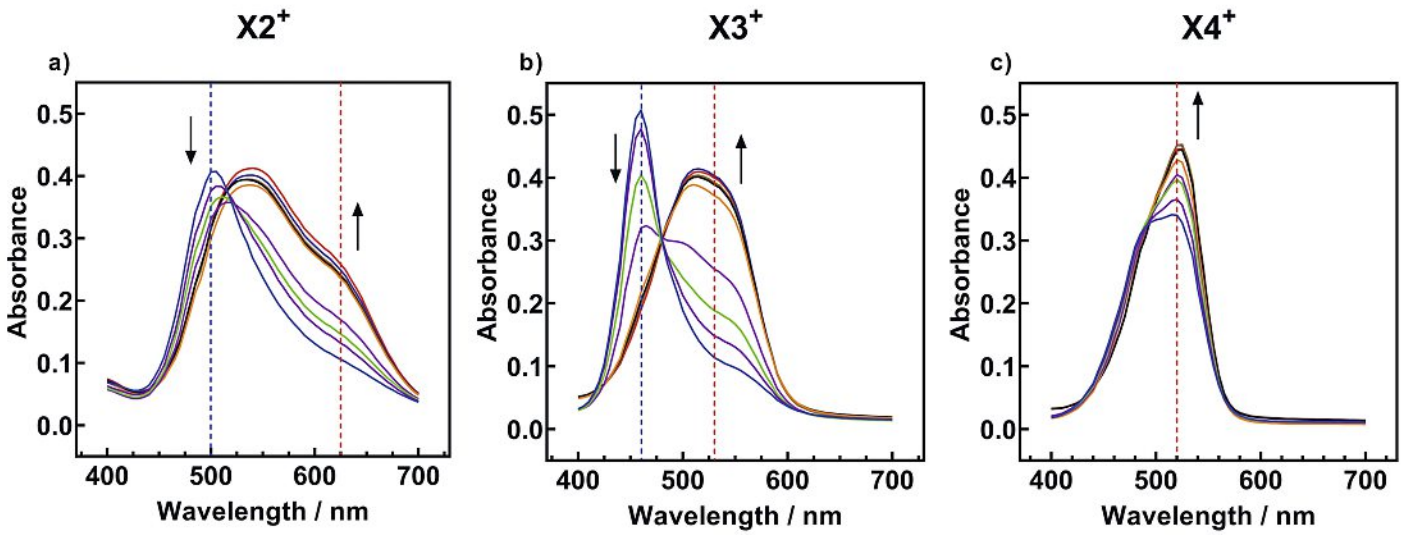

Fig. 3. Absorbance shift of different particle emulsions with increasing protamine concentration (0,1,2,3,4,5,6,7,8,10 µM). The arrows indicate the direction of the spectral changes. The blue dotted line corresponds to the hyperpolarized state while the red one corresponds to the non-polarized state.

Three solvatochromic dyes with varying hydrophobicity ($X4^+ > X2^+ > X3^+$, Fig. 2) were investigated.[19] The spectra can be separated in three regions: a hyperpolarized state at lower wavelengths, a non-polarized state at higher wavelengths and a hybrid state in between.

The most hydrophobic dye showed a weak solvatochromic response when in the nanoparticles and in presence of protamine (Fig. 3c). There was no hyperpolarized signal and the non-polarized state increased with protamine concentration. This could be due to the presence of the oxygen atom in the benzoxazolium moiety of $X4^+$, resulting in less electronic density in the conjugated system and in a smaller absorbance shift.

$X2^+$ showed a large absorbance shift when in presence of protamine (Fig. 3a). However, there was significant overlap between the hyperpolarized and hybrid states and the non-polarized peak was weaker than the intermediate peak. This could be due to its longer conjugated system. In fact, the charge is more delocalized in the pi system. Since the negative charge of the sulphonate group of the DNNS is very localized, its binding with $X2^+$ would be weaker than with a smaller pi system such as in $X3^+$.

As expected, $X3^+$ experienced a greater absorbance shift along with a sufficient separation between hyperpolarized and the other states (Fig. 3b). Therefore, we proceeded with this dye for further protamine sensing. Studies are ongoing to investigate the kinetic effects and to better understand the response mechanism of the sensor containing $X3^+$.

When performing a protamine calibration (Fig. 4), the sensor exhibited a signal increase of up to 4 µM followed by a plateau. This indicates that all the dye became dissociated from $DNNS^-$ to give a saturated sensor response. This type of sensor is called 'exhaustive'.[17] The absorbance data were analyzed with Eqn. (1), which provided a signal independent of the light intensity, background color and pathlength,[23]

$$\alpha = \frac{\log\left(\frac{I_{P2}I_1}{I_{P1}I_2}\right)}{\log\left(\frac{I_{P2}I_{NP1}}{I_{NP2}I_{P1}}\right)} \quad (1)$$

where α is the response ratio, I_1 is the light intensity at 460 nm, I_2 at 530 nm, I_P when fully polarized (0 µM protamine) and I_{NP} when fully non-polarized (after the saturation point, here 10 µM).

In the presence of heparin, the protamine response curve shifts to higher protamine concentrations due to protamine complexation by heparin present in the sample. Previous reports have shown that quantifying this shift allows the determination of heparin concentration in patient plasma samples but in this work the high absorbance of blood limited further progress.[18]

3.2 *Distance-based Analysis in Agarose Gel*

Devices using optical detection can take several forms such as microplates containing an optode film,[13] coated capillaries combined with distance-based reading on paper[24] or nano-optodes embedded in agarose gel. Emulsion-based particles were found to disassemble on cellulose leading to a loss of the hyperpolarized signal and a rise of non-polar signal either due to interaction with cellulose or $TFPB^-$. The stability of the particles and thus the hyperpolarized signal could be preserved by embedding the particles in a buffered agarose hydrogel. When protamine is present, it diffuses through the gel, penetrates the particles and induces a similar color change as in solution (Fig. 5). With this type of device, one cannot use simply absorbance measurements. Previous works have analyzed pictures of gel-based sensors using different methods: RGB color channels ratio,[21] gray value profile[22] or a

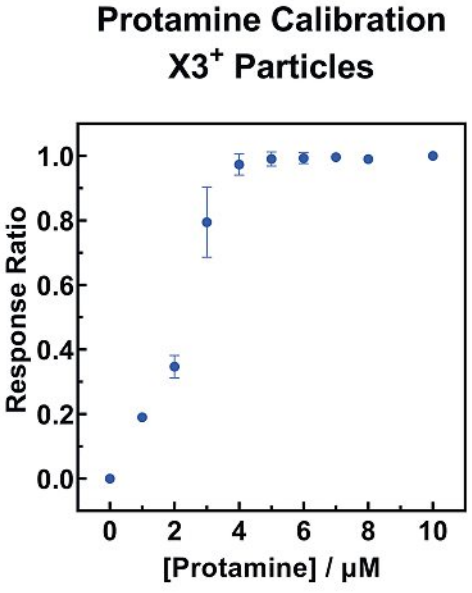

Fig. 4. Protamine calibration curve in buffer. The absorbance was recorded at 460 and 530 nm at 2 minutes after particles addition. The response ratio was obtained with Eqn. (1) using 0 µM protamine as hyperpolarized and 10 µM as non-polarized signal.

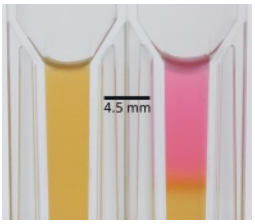

Fig. 5. Picture of the cuvettes after 18 h in contact with water (left) and 1 mM protamine (right).

modified version of Eqn. (1).[25] The latter approach provides a signal independent of brightness, RGB spectral sensitivity and gamma correction.[25]

In the previously cited examples of embedded optodes, the detected concentrations were in the order of millimolar, and allowed for a distance-based readout.[21,22] In the case of protamine sensing, the concentration of interest is much smaller and an intensity-based readout method is required to quantify the signal.

Blood samples were analyzed to demonstrate the suitability of the sensing principle for this matrix (Fig. 6, top). The ratio of the blue and green channels from the sRGB color space was used to construct a vertical profile of the pictures showing an increased height in the presence of protamine (Fig. 6, bottom).

Interestingly, the particles showed a response even without protamine being present. This was also previously demonstrated in human plasma using Eqn. (1).[18] The interfering species has not yet been identified. Nevertheless, the protamine-induced signal was clearly visible, which holds promise for eventual heparin quantification in whole blood.

4. Conclusion

A recently presented sensing mechanism using dye hyperpolarization was interrogated with various solvatochromic dyes and applied to protamine sensing in an agarose gel. The different solvatochromic dyes were investigated to optimize the particles' recipe. The dye with the largest and clearest absorbance shift was chosen. These nano-particles were successfully embedded in buffered agarose gel and showed suitable response towards portamine. This technique was demonstrated to be promising for the quantification of protamine and heparin in whole blood. Further image analysis and experimental improvements are underway.

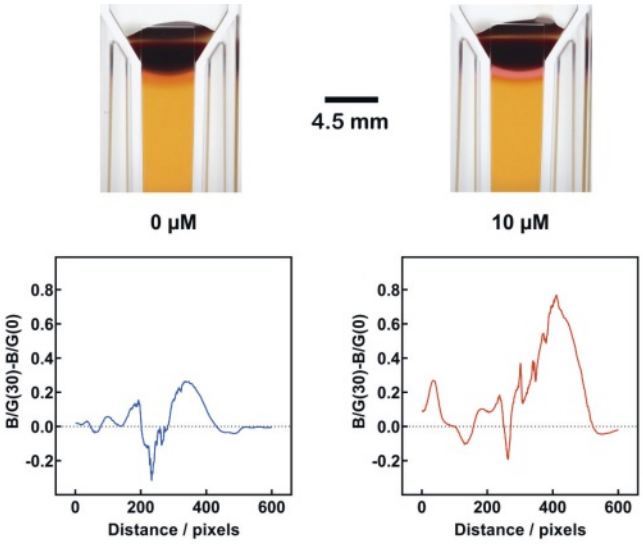

Fig. 6. Pictures of cuvettes containing blood (top) in contact with agarose gel containing nanosensors after 30 min. without (left) and with (right) 10 µM protamine with their corresponding vertical profiles (bottom). The profile at t=0 was subtracted from the one after 30 min. to minimize the blood background.

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