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Vesicular aptasensor for the detection of thrombin†

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Self-assembled phospholipid vesicles are functionalized with thrombin-binding aptamers using a thiol-click reaction. The resulting aptasensors signal the binding of the analyte to the vesicle surface by changes of the emission properties of membrane co-embedded reporter dyes.

The last two decades have seen considerable progress in the design of biopolymer-based receptors for high-affinity and -specificity analyte binding. The first reports on the evolutionary selection of RNA molecules binding to specific ligands in 1990[1] laid the foundation for the development of numerousness nucleic acid aptamers. A broad variety of ligands were targeted ranging from small organic molecules[2a] to proteins[2b] and pathogens,[2c] and the technique was applied in environmental and food analytics.[3] With an increasing number of available aptamers, suitable methods for transforming the microscopic binding event into a macroscopic signal were established.[4] Fluorescence-based[5a] and electrochemical[5b] detection methods are among the most important ones due to their high sensitivities. They both typically rely on covalent modification of the aptamers with a luminescent or an electroactive moiety. Another commonly used technique is based on hybridization of the aptamers with antisense oligonucleotides whose displacement by the target analytes induces a fluorescent or electrical response.[6] The requirement of covalent reporter attachment at the aptamer or the need of accordingly labeled complementary oligonucleotide strands can make such assays laborious and costly. For that reason, label-free strategies were investigated, but their general applicability is often limited as they rely on specific structural characteristics of the applied aptamers such as certain folding modes upon binding to the target. Formation of a double helix domain, for example, allows the intercalation of a suitable dye accompanied by a change of its emission properties.[7] Previously, our group reported on the modular construction of luminescent chemosensors by means of unilamellar vesicular membranes as self-assembled supporting frames for metal complex receptors and fluorescent reporter sites.[8] This strategy facilitates an effortless sensor preparation based on self-assembly by simple mixing of different functional amphiphiles in aqueous solution. As a consequence, our method allows an easy variation of the sensor composition, *i.e.*, of the binding and signaling units, their ratios and concentration on the surface and the physical properties of the supporting vesicle membranes determined by the nature of the phospholipids.[9] We now expand the pool of available binding sites to more complex structures based on molecules of biological origin. As a model system, we immobilized the very well established thrombin-binding 15-mer DNA aptamer[10] on fluorescent phospholipid vesicle surfaces. DNA-decorated liposomes have been used as signal amplification tags,[11] for controlled liposome adhesion or fusion[12] and for specific cell targeting with regard to drug delivery.[13] Moreover, the conjugated polymeric backbone of oligonucleotide-functionalized polydiacetylene vesicles was utilized as colorimetric sensing matrix for target detection.[14] These reports prompted us to combine the concept of liposome-anchored aptamers with our strategy of non-covalent receptor and fluorophore co-embedding into phospholipid membranes.

As attachment site for thiol-modified aptamers, we incorporated the amphiphilic maleimide **Mal-C_{16}** (Fig. 1, 5 mol%) into membranes of small unilamellar DSPC vesicles. Amphiphilic pyrene **Pyr-C_{18}** (→ vesicles **V-Pyr**) or rhodamine **Rho-C_{18}**[15] (→ vesicles **V-Rho**) were co-embedded as reporter dyes (1–5 mol%). The synthesis of **Mal-C_{16}** is described in the ESI,† preparation of **Pyr-C_{18}** was performed by *in situ* click reaction of commercially available *N*-(1-pyrenyl)maleimide and 1-octadecanethiol. The vesicle preparation was performed in 25 mM HEPES buffer at pH = 7.4 according to previously reported procedures (see ESI† for details).

Next, we functionalized the vesicle membrane covalently with the aptamer by nucleophilic thiol–maleimide addition. After addition of thiolated **Apt-Thr** (0.4–0.5 equivalents with respect to the total amount of **Mal-C_{16}**) to **V-Pyr**, we observed a time-dependent decrease of the fluorescence signal indicating the reaction progress on the vesicle surface. Phase separation processes in the membrane result in high local concentrations

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† Electronic supplementary information (ESI) available: Experimental details, synthesis of amphiphiles, preparation of functionalized vesicles, emission spectra and binding curves. See DOI: 10.1039/c4cc05221h

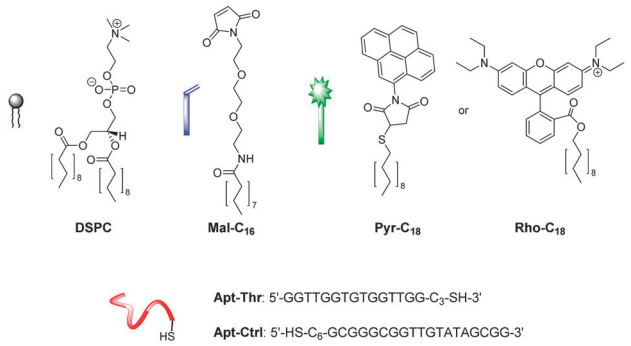

Fig. 1 Structures of the functional amphiphiles used for vesicle preparation and of the immobilized aptamers.

of the embedded functional amphiphiles as it is evidenced by the presence of the pyrene excimer signal of **V-Pyr** at 470 nm (Fig. 2A, inset). We assume that the surface-anchored oligonucleotides strongly impact the physical properties at the membrane interface by their high negative charge and steric demand. This local interference results in a partial fluorescence emission quenching of the nearby pyrene aggregates (Fig. 3, top). Moreover, it is well documented that nucleotides are able to quench the fluorescence of fluorophores, such as pyrene[16] or rhodamine[17] derivatives, by photoinduced electron transfer. We believe that this mechanism also contributes to the observed effect in our case.

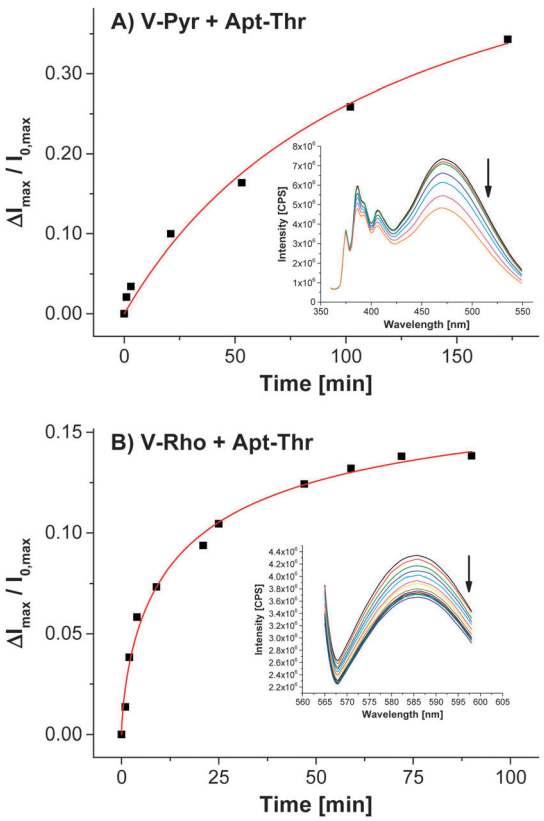

Fig. 2 Fluorescence response monitoring the reaction progress of pyrene- (A) and rhodamine-modified (B) vesicles during surface functionalization with aptamer **Apt-Thr**.

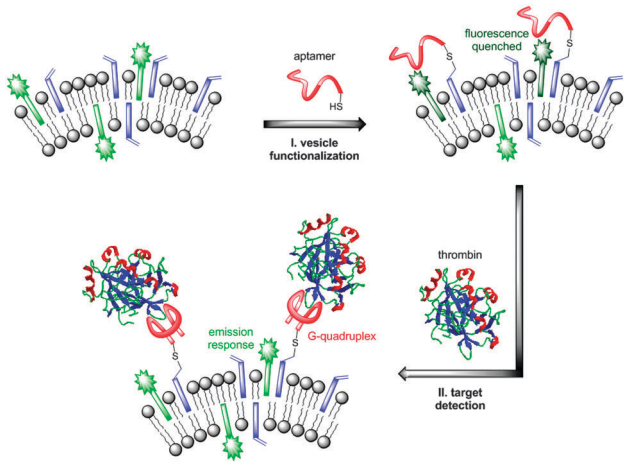

Fig. 3 Principle of vesicle surface functionalization (I) and fluorescence response after analyte binding (II).

Rhodamine-modified liposomes **V-Rho** exhibit a similar behavior during functionalization with the aptamer **Apt-Thr** (Fig. 2B).

The thrombin-binding aptamer folds into a highly compact G-quadruplex structure upon binding to thrombin.[18] This chair-like conformation is stabilized by a metal cation, particularly potassium cations. Analogously to the sensing mechanism for the membrane functionalization process, we believe that binding of the target to the surface-bound oligonucleotide, accompanied by its conformational change, has a drastic impact on the membrane structure which triggers then the dynamic emission response of the fluorophore molecules in close proximity (Fig. 3, bottom). At last, rather the presence of the "aptamer bound" analyte at the membrane–water interface causes the change of the emission signal than a defined conformation of the immobilized oligonucleotide molecules.

High concentrations of potassium cations are known to induce the transition of the oligonucleotide from random coil to the folded form with a reported binding constant of $\lg K \approx 2.1$.[19] Therefore, we first tested the suitability of the thrombin aptamer-functionalized vesicles **V-Pyr-Thr** for K^+ detection. The fluorescence titration revealed an excimer signal change of almost 60% (Fig. 4, red squares). Due to the relatively low binding affinity of the aptamer

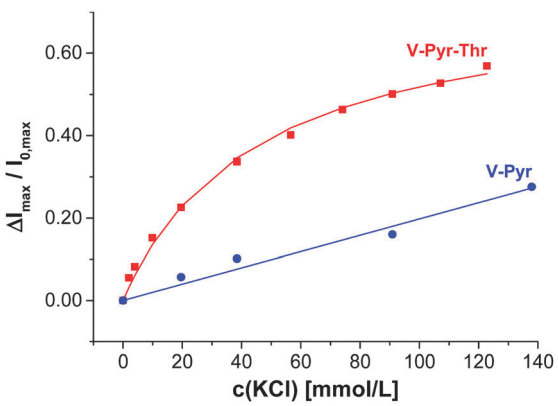

Fig. 4 Fluorescence emission titrations of KCl *versus* aptamer-functionalized (red squares) and non-functionalized (blue dots) vesicles **V-Pyr**.

Table 1 Binding affinities of fluorescent, aptamer-functionalized vesicles to different analytes

Vesicles	Analyte	lg K	Reference
V-Pyr-Thr	KCl	1.3 (±8%)	2.1[19]
V-Pyr-Thr	Bovine thrombin	6.3 (±3%)	7.1[23]
V-Rho-Thr	Bovine thrombin	7.2 (±8%)	7.1[23]
V-Rho-Thr	Human thrombin	7.7[a]	6.7–7.6[24]

[a] Single experiment.

to potassium cations, KCl concentrations of up to 140 mM were required to obtain a reasonable binding isotherm. In the same concentration range, non-functionalized vesicles showed a smaller non-specific response with linear relationship (Fig. 4, blue dots). We explain this effect by the huge change of ionic strength in the aqueous buffer. It is well documented that high salt concentrations have a strong influence on the membrane's physical properties such as surface polarity and hydration and thus can alter the emission of membrane-embedded fluorophores.[20] Non-linear curve fitting of the obtained binding isotherm resulted in a logarithmic binding constant of 1.3 (Table 1). The lower value of the potassium cation binding constant compared to the reported one can be attributed to the interference of specific binding and non-specific salt effects.

Next, we investigated the detection of thrombin. Pyrene-functionalized vesicles **V-Pyr-Thr** showed a relatively small fluorescence increase upon addition of thrombin (≈ 20% at a 2.0 μM concentration of thrombin, see Fig. S4, ESI†), whereas rhodamine-modified liposomes **V-Rho-Thr** exhibited a considerable luminescence response (Fig. 5, red squares). As expected, non-functionalized vesicles **V-Rho** did not significantly alter their emission properties upon the addition of thrombin (Fig. 5, blue dots).

Table 1 summarizes the apparent binding constants of the vesicular aptasensors to different analytes. All calculations were performed by non-linear curve fitting using the equation for 1:1 binding.[21] The affinity constants of **V-Rho-Thr** to both bovine and human thrombin are in good agreement with reported literature values. The limit of detection was determined to be 25 nM (on basis of a signal to noise ratio of 3), while the sensors span a dynamic range of up to 2 μM concentration of thrombin (see ESI† for fluorescence titration data). The analytic results are reproducible as shown by repeated measurements. Since thrombin is a crucial enzyme in the blood coagulation cascade, its concentration in circulating blood is low and often undetectable. During clotting initiation, it becomes significantly elevated at a low nanomolar concentration level and peaks at concentrations in the low micromolar region during the propagation phase.[11b,22] Thus, the dynamic recognition range of our sensor matches the physiological concentration range.

To demonstrate that the interaction of thrombin with the covalently membrane-attached aptamer is responsible for the change of the rhodamine emission intensity, we prepared fluorescent vesicles lacking the amphiphilic binding anchor **Mal-C_{16}** and incubated them with the thiolated thrombin-binding aptamer **Apt-Thr** leading to no significant response upon analyte addition (Fig. 5, orange diamonds).

We tested the specificity of our aptasensors by immobilizing a non-binding control oligonucleotide **Apt-Ctrl** (Fig. 1) with comparable length on the vesicle surface. The slight response after thrombin addition can be attributed to non-specific binding (Fig. 5, magenta triangles). Although thrombin has a small negative overall charge in the used buffer system (pH: 7.4, isoelectric point of thrombin: 7.05[25]), its numerous negatively and positively charged residues are not distributed uniformly over the whole molecule.[26] They are clustered in a sandwich-like structure of two highly positive poles, whose electrostatic fields spread far into the extramolecular space. We suppose that the electropositive exosites are capable to displace counterions from the negatively charged oligonucleotide backbone by electrostatic attractions.[27]

Emission titration using the protein elastase, which is besides thrombin a member of the serine proteases enzyme family, *versus* **V-Rho-Thr** indicates the aptamer–protein non-specific interaction. The fluorescence intensity decreases during titration (Fig. 6), which is in contrast to the emission intensity increase observed with thrombin. Elastase has an isoelectric point of 9.5,[28] and it is assumed that its high positive net charge possibly results in strong non-specific oligonucleotide–protein interactions,[27] leading to different fluorescence emission intensity changes.

In conclusion, we have developed a novel type of aptasensor based on self-assembled, luminescent vesicle membranes, which

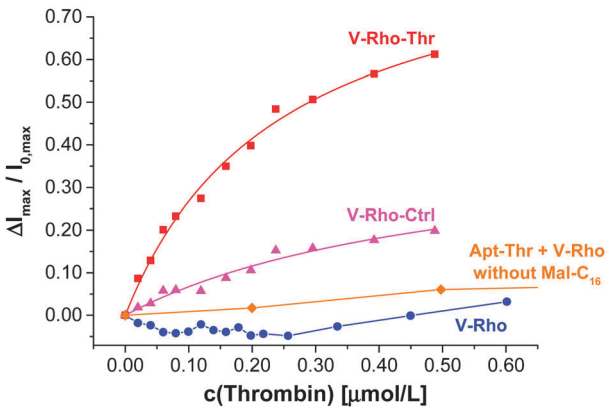

Fig. 5 Binding isotherm of thrombin to aptamer-functionalized vesicles **V-Rho-Thr** (red squares) and fluorescence response of control experiments: non-functionalized vesicles **V-Rho** (blue dots), maleimide-lacking **V-Rho** vesicles incubated with **Apt-Thr** (orange diamonds) and vesicles **V-Rho-Ctrl** functionalized with non-binding oligonucleotide **Apt-Ctrl** (magenta triangles).

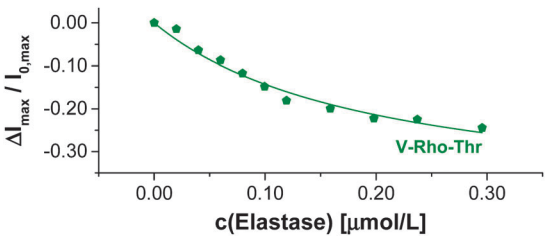

Fig. 6 Fluorescence emission titration of control protein elastase to **V-Rho-Thr**.

are functionalized with the thrombin-binding aptamer by nucleophilic thiol–maleimide addition. The high-affinity binding of the aptamer to thrombin is indicated by a dynamic response of the co-embedded fluorescent reporter molecules in the physiological concentration range. The obtained binding constants are in good agreement with the reported values. Despite of the observed non-specific interactions in case of mismatched aptamer–protein combinations, the specific binding to thrombin can be clearly distinguished in the fluorescence output. As the reporter group is neither covalently attached nor coordinated to the aptamer, an inexpensive and easy variation of aptamer–fluorophor combinations is possible. The signaling mechanism is independent of specific conformational changes of the receptor molecules, which may allow a broader application compared to previously reported label-free aptasensors. An extension to other aptamers, enzymes or proteins and the immobilization of the vesicular aptasensors on solid supports can be readily envisaged.

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