Recognition of isozymes *via* lanthanide ion incorporated polymerized liposomes†

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We report the selective recognition of carbonic anhydrase isozymes based on the excited-state lifetimes of chelated Eu^{3+} ions incorporated in polymerized liposomes.

In the current proteomics era, the protein expression profiles of various diseases are being unraveled rapidly.[1] Usually, the disease states are characterized by the over-expression of many enzymes.[1] Often these enzymes have several isozymes and some of the isozymes are selectively over-expressed in the diseases.[2] For example, carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitously distributed, Zn^{2+} containing metalloenzymes with 16 isozymes.[3] Of the 16 isozymes, CA II and XII are found to be over-expressed in the brain of epileptic mice.[4] CA II and IV are the target enzymes for the treatment of increased intraocular pressure in glaucoma.[3] CA IX and XII are overexpressed in hypoxic tumors and the expression levels correlate with poor prognosis of the disease.[3] Clearly, the selective detection of CA isozymes is needed to characterize these disease states.

Since the isozymes are evolved to catalyze the same chemical reaction, their active sites are very similar. In addition, many isozymes display a high degree of sequence homology.[5] Hence, the design of active site probes for selective recognition of different isozymes of the same enzyme is a major problem in medicinal chemistry.[6] Usually, selective detection of the isozymes requires the use of biological antibodies.[7]

In contrast to the marked similarity of the active sites, the distribution of solvent-exposed amino acid residues varies across isozymes and hence, luminescent probes that bind to the protein surface should interact with the isozymes in different spatio-temporal orientation. Herein, we report our results demonstrating the proof-of-concept for this principle by employing the recombinant human carbonic anhydrases as the model isozymes and polymerized liposome-incorporated Eu^{3+} ions as the luminescent probes.

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† Electronic supplementary information (ESI) available: Synthesis of polymerizable inhibitor lipid **3**. Preparation and polymerization of the liposomes. Estimation of Eu^{3+} concentration on the outside surface of the liposomes. Cloning, expression and purification of carbonic anhydrase isozymes. Luminescence titration and determination of excited state lifetime for Eu^{3+}. Luminescence decay measurements. Determination of dissociation constants. Fig. S1: Time-resolved emission spectra of Eu^{3+}-incorporated polymerized liposomes as a function of increasing concentrations of CA I, CA II, CA XII and bovine erythrocyte CA. See DOI: 10.1039/b709815d

The lanthanide ions are extensively used as luminescent probes in bioassays, including in the detection of proteins.[8] These metal ions show several advantages over organic fluorophores in the bioassays, *e.g.*, narrow emission bands, large Stokes' shifts, long lifetimes *etc.* These advantages are offset by the weak molar extinction coefficients for the lanthanide ions. Consequently, the reported lanthanide-based bioassay protocols require the use of organic fluorophores to sensitize the emission.[8] We have demonstrated that the conjugated ene-yne backbone of polymerized liposomes (prepared from conjugated diacetylenic lipids) can be used to efficiently sensitize the emission of Eu^{3+} ions.[9] During the current studies, we used this strategy to enhance the luminescence emission intensity of the liposome-incorporated Eu^{3+} ions.

Liposomes were prepared incorporating the commercially available, mixed-chain polymerizable phosphatidylcholine **1** (Fig. 1) as the major lipid (85% by weight). This lipid is reported to polymerize efficiently upon UV irradiation (254 nm).[10] The previously-reported[9] polymerizable lipid **2**.Eu^{3+} complex was used as the luminescent reporter lipid (5% by weight, Fig. 1) in the liposomes. In order to facilitate the interactions between the liposomes and the CA isozymes, small amounts of the lipids **3** (5% by weight) and **4** (5% by weight) were incorporated in the liposomal formulations (Fig. 1). Lipid **3** has a weak inhibitor of CA (benzenesulfonamide, K_i = 2 μM)[11] as the head group. Lipid **4** incorporates iminodiacetate (IDA) chelated Cu^{2+} ions to interact with the surface exposed histidine residues of CA isozymes.[12] Scheme 1 shows the synthesis of the polymerizable lipid **3** (synthetic details are provided in ESI†). Lipid **4** was prepared following a reported procedure.[13]

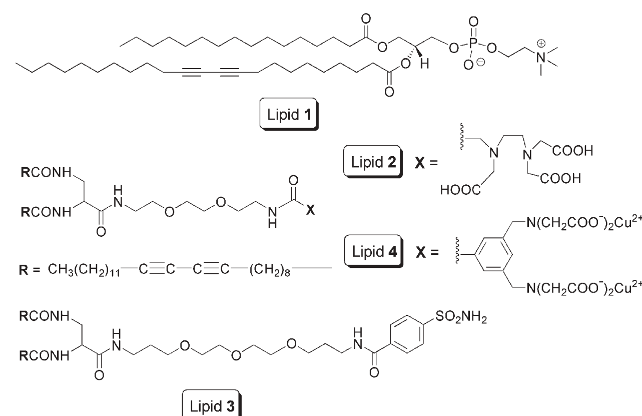
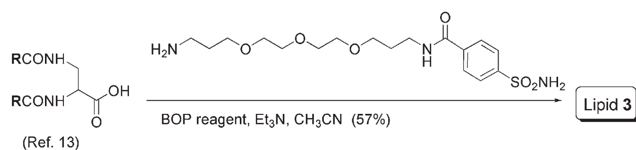


Fig. 1 Structures of the polymerizable lipids used in the liposomal formulations.



Scheme 1 Synthesis of the polymerizable lipid **3**.

The liposomes (average diameter: 50 nm, ESI†) were polymerized by exposure to UV radiation at 254 nm for 30 min.[10] The emission from the chelated Eu^{3+} ions in the resultant polymerized liposomes were efficiently sensitized by exciting the ene-yne backbone of the liposomes at 320 nm. A delay of 70 μs removed the emission from the polymerized liposome backbone completely. The concentration of Eu^{3+} ions on the outer surface of liposomes was estimated to be 131 nM (see ESI†).

When we titrated the polymerized liposomes with the recombinant CA isozymes, the emission intensity of the Eu^{3+} ions was found to increase with increasing concentrations of the added proteins. The lipid **2**.Eu^{3+} complex has three water molecules coordinated to the Eu^{3+} ions.[9] The enhancement in emission intensity is possibly due to the replacement of the H_2O molecules from the primary coordination sphere of Eu^{3+} ions with functional groups from the protein surface (carboxylate moieties from Asp and Glu).[14,15] The enhancements in the sensitized emission intensity of the liposome incorporated Eu^{3+} ions were different in the presence of the recombinant human CA I, II, VII, XII and the bovine erythrocyte CA (Sigma Chemical Company). As an illustrative example, the emission intensities of the Eu^{3+} ions at 615 nm ($^5D_0$ to $^7F_2$ transition, λ_{ex} = 320 nm)[15] as a function of different concentrations of added CA VII are shown in Fig. 2 (similar plots for the other CA isozymes are provided in ESI†).

Encouraged by the differential increases of the Eu^{3+} emission intensity by the different CA isozymes, we proceeded to determine the excited state lifetimes of the Eu^{3+} ions in the presence of the CA isozymes. A biexponential decay profile was observed for the polymerized liposome incorporated Eu^{3+} ions at 615 nm, (λ_{ex} = 320 nm) in the absence of protein, with lifetimes of 57 and 158 μs (Fig. 3). This reflects that the Eu^{3+} ions on the liposomes are experiencing two different types of chemical environments with different numbers of coordinated H_2O molecules.[15] Currently, we are investigating the origin of these two chemical environments for the polymerized liposome incorporated Eu^{3+} ions. Both of these lifetimes increased in the presence of saturating concentrations of CA isozymes, indicating that the Eu^{3+} bound H_2O molecules are being replaced by the surface-exposed functional groups from the enzymes (carboxylate moieties from Asp and Glu).[15] The lifetimes (especially τ_2) were different in presence of the different isozymes of CA (Table 1). For example, while in the presence of CA II, the excited state lifetimes increased to 94 and 455 μs, the lifetimes with CA VII were 55 and 301 μs, respectively. The excited state decay profiles for the Eu^{3+} ions in the presence of CA II and CA VII are also shown in Fig. 3 (details are provided in ESI†).

The different excited state lifetimes of Eu^{3+} in the presence of different CA isozymes indicated that the isozymes are interacting differently with the Eu^{3+} ions on the liposomal surface. In order to test this hypothesis further, we determined the dissociation constants of the CA isozymes for the polymerized liposomes (assuming 1 : 1 stoichiometry) by non-linear regression analyses of the titration curves (Table 1, ESI†).

Analyses of the excited state lifetime data and the dissociation constants indicate that the CA isozymes are interacting differently with the polymerized liposomes. The polymerized liposomes with Eu^{3+} ions act as surface probes for the CA isozymes and are able to distinguish them. The limits of detection for the various CA isozymes were also determined following a literature procedure[16] and are shown in Table 1. To the best of our knowledge, the other reports of distinguishing the surfaces of CA isozymes rely on the use of antibodies for these enzymes.[7] The luminescent liposomes reported herein provide the proof-of-concept for the non-biological discrimination of the isozymes. In addition, the polymerized liposomes are very stable compared to the biological antibodies and can be stored at room temperature for several weeks without any loss of the recognition and luminescence properties. Studies are currently in progress to test the ability of our polymerized

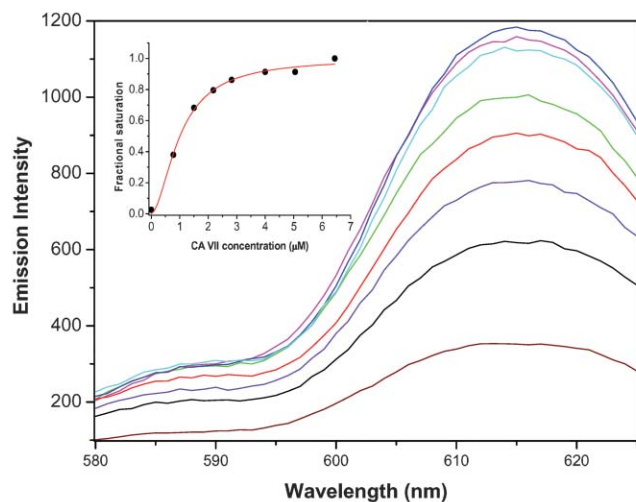


Fig. 2 The luminescence emission intensities of polymerized liposome incorporated Eu^{3+} ions (λ_{ex} = 320 nm) are shown as a function of added CA VII (0–7 μM). The inset shows the fractional saturation of the liposomes as a function of added CA VII concentration.

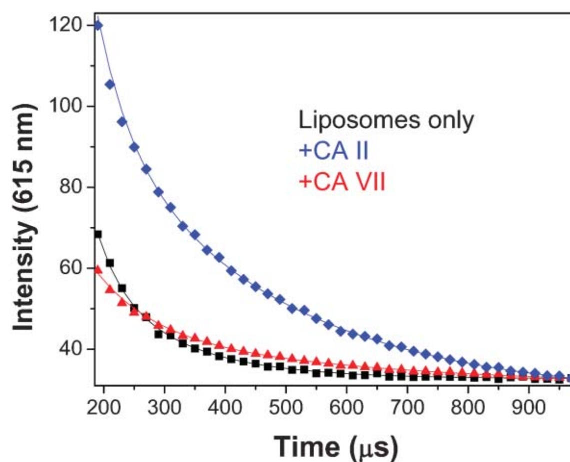


Fig. 3 Excited state decay curves for polymerized liposome incorporated Eu^{3+} ions (monitored at 615 nm, λ_{ex} = 320 nm) in absence (black squares) and in the presence of CA II (blue squares) and CA VII (red triangles) are shown. The smooth lines are the fitted curves for determining the lifetimes.

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Table 1 Excited state lifetimes (τ_1 and τ_2, μs) of Eu^{3+} ions incorporated in polymerized liposomes (monitored at 615 nm, λ_{ex} = 320 nm), the dissociation constants (K_d, μM) and the limit of detection (LOD, mg L^{-1}) in the presence of various CA isozymes are shown

Enzyme	τ_1/μs	τ_2/μs	K_d/μM	LOD/mg L^{-1}
—	57 ± 8	158 ± 11		
CA I	93 ± 4	399 ± 10	5.6	4.3
CA II	94 ± 2	455 ± 4	0.35	0.22
CA VII	55 ± 7	301 ± 8	0.58	1.2
CA XII	85 ± 0.3	426 ± 0.7	4.9	4.4
Bovine CA	83 ± 1.3	232 ± 19	1.1	1.1

liposomes to distinguish isozymes of other enzymes and to increase the sensitivity of this detection method.

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