A naked-eye sensing ensemble for the selective detection of citrate—but not tartrate or malate—in water based on a tris-cationic receptor

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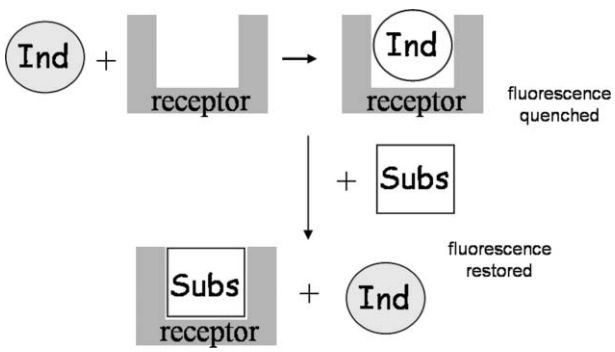
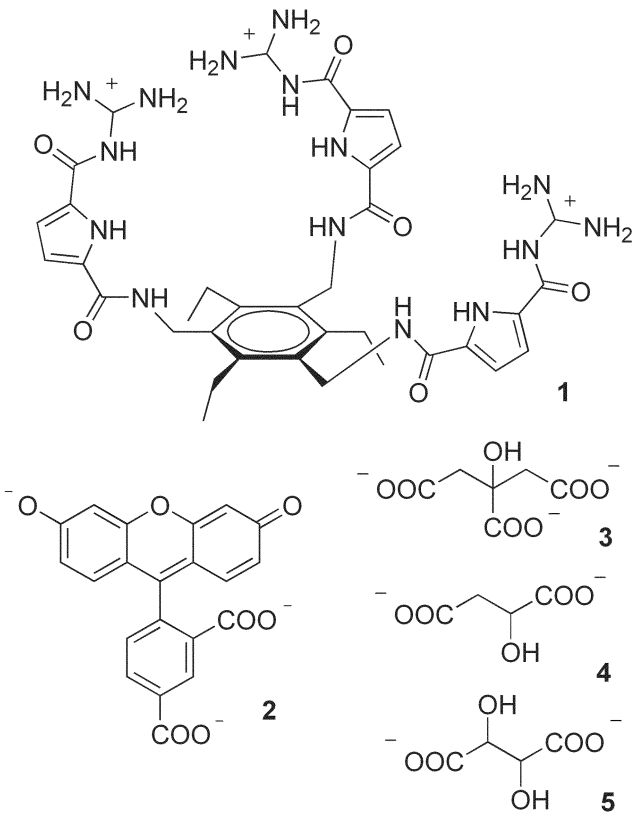
Citrate **3** can be selectively detected in aqueous solvents even in the presence of malate or tartrate using a naked-eye detection system based on the complex between tris-cation **1** and carboxyfluorescein **2**.

The specific detection of small biomolecules is of considerable interest. The design of a chemosensor requires a receptor unit, that selectively interacts with the substrate of choice, and a method to read-out the binding using a change in a physical signal.[1] In most cases an additional reporter unit (*e.g.* a fluorescent chromophore) is covalently attached to the receptor for this purpose. Another possibility is to use a "silent" receptor without an attached read-out device and to employ the indicator displacement method (Scheme 1) as elegantly demonstrated in recent years by Anslyn *et al*.[2] The most convenient method is the so called "naked-eye" detection, in which the binding event is directly visible in a colour change of the system and which does not require any further analytical instruments. In recent times a variety of such naked-eye detection systems for different analytes has been reported, but the majority of them require either strong metal–ligand interactions or covalent bond formation for efficient binding of both the analyte and the indicator.[3] Furthermore, their selectivity for different analytes with similar recognition elements is often rather modest. We want to report here a new naked-eye detection system for citrate[4] based on a tris-cationic receptor **1** that we recently introduced, which solely relies on weak and reversible non-covalent interactions.[5] Using an indicator displacement assay with carboxyfluorescein **2** a remarkably selective naked-eye detection of citrate **3** with respect to even closely related substrates such as malate or tartrate in water is possible.



Scheme 1 Indicator displacement assay.

The tris-cation **1** binds citrate in pure water with an association constant of $K_{ass} = 1.6 \times 10^5$ M^{-1}.[5] This is the largest affinity ever observed for citrate by an artificial chemical receptor solely based on weak non-covalent interactions. For example, the affinity of **1** for citrate **3** is around two orders of magnitude larger than Anslyn's receptor system which is based on simple guanidinium cations instead of the guanidiniocarbonyl pyrrole cations used here. Therefore, Anslyn's receptor required the use of methanol as a solvent to achieve strong complexation of citrate in a competitive indicator displacement assay. Our receptor should be capable to detect citrate even in water based on the improved binding affinity. There is only one receptor recently reported by Fabbrizzi *et al.* which shows an even slightly higher affinity for citrate ($K_{ass} = 3.9 \times 10^5$ M^{-1} in buffered water at pH = 7). But in this case the binding is due to much stronger metal ligand interactions between three Cu(II)-cyclams in the receptor and the carboxylate groups of citrate,[6] whereas our receptor uses only ion pair formation for substrate binding.



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Carboxyfluorescein **2**, an aromatic tris-anion, also interacts with receptors of type **1**. As the fluorescence activity of **2** significantly depends on the local environment around the chromophore,[7] binding of **2** within the hydrophobic cavity of **1** should alter its fluorescence (*e.g.* by changing the pK_a's of the carboxylates or π-stacking interactions with the aromatic system). And indeed upon addition of **2** (3×10^{-4} M) to a solution of **1** (1.2×10^{-5} M) in 10% DMSO in water (2 mM bis-tris-buffer, 10 mM NaCl, pH 6.3) both the fluorescence of **2** at $\lambda = 518$ nm as well as the fluorescence of the receptor at $\lambda = 335$ nm was completely quenched (Fig. 1).

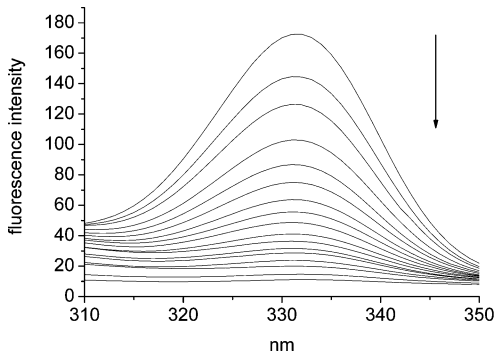

Fig. 1 Change in the fluorescence spectrum of receptor **1** upon the incremental addition of 8 equivalents of **2** in 10% DMSO–water (2 mM bis-tris-buffer, 10 mM NaCl, pH = 6.3).

A quantitative analysis of the quenching of the pyrrole fluorescence of **1** at $\lambda = 335$ nm using a titration experiment was therefore performed. To a solution of **1** (1.2×10^{-5} M) in 10% DMSO in water (2 mM bis-tris-buffer, 10 mM NaCl, pH 6.3) aliquots of a stock solution of **2** (3×10^{-4} M) were added. The fluorescence spectrum was recorded after each addition (synchronous excitation with $\Delta\lambda = 20$ nm) and the change in the emission maximum at $\lambda = 335$ nm was used to calculate the binding constant using a non-linear regression method based on a 1 : 1-complexation model. This 1 : 1-complex stoichiometry was independently confirmed using Job's method of continuous variation (Fig. 2; bottom). Based on the observation that only the receptor and the complex but not the carboxyfluorescein have a significant emission at this wavelength, the data analysis was done using the following equation implemented in a standard mathematical curve fitting program:

$$A = \varepsilon_R [\mathbf{1}]_0 + \frac{K\Delta\varepsilon [\mathbf{1}]_0 [\mathbf{2}]}{1 + K[\mathbf{2}]}$$

with

$$[\mathbf{2}] = [\mathbf{2}]_0 - \frac{K[\mathbf{1}]_0 [\mathbf{2}]_0}{(1 + K[\mathbf{2}]_0)^2 + K[\mathbf{1}]_0}$$

and:

$[\mathbf{1}]_0$ = total concentration of receptor **1**

$[\mathbf{2}]_0$ = total concentration carboxyfluorescein **2**

$$\Delta\varepsilon = \varepsilon_{RL} - \varepsilon_R$$

Furthermore, the total concentrations of receptor $[\mathbf{1}]_0$ and ligand $[\mathbf{2}]_0$ in the actual sample can be calculated from the initial concentrations of the stock solution used ($[\mathbf{1}]_0$* and $[\mathbf{2}]_0$*), by accounting for the change in volume caused by each substrate addition using a dilution factor x.

$$[\mathbf{1}]_0 = \frac{[\mathbf{1}]_0^*}{1+x} \quad [\mathbf{2}]_0 = \frac{[\mathbf{2}]_0^* x}{1+x} \quad \text{with } x = \frac{V_{\text{added}}}{V_{\text{initial}}}$$

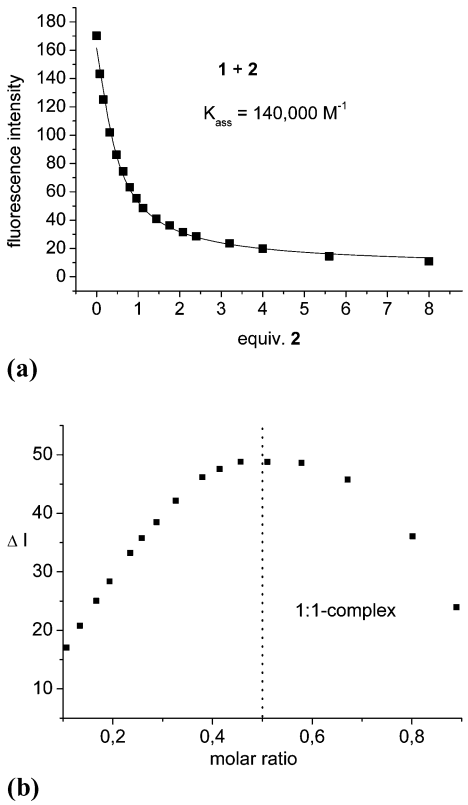

Fig. 2 *Top*: Binding isotherm at $\lambda = 335$ nm obtained from a fluorescence titration of **1** (1.2×10^{-5} M) with **2** (3×10^{-4} M) in 10% DMSO in water (2 mM bis-tris-buffer, 10 mM NaCl, pH 6.3). *Bottom*: Job plot obtained from the titration confirming the 1 : 1-complex stoichiometry.

The binding constant K together with the emission coefficients of the receptor ε_R and of the complex ε_{RL} were used as fitting parameters for the non-linear regression.

Analysis of the binding isotherm shown in Fig. 2 (top) provided an association constant of $K_{ass} = 140\,000$ M^{-1} for the complexation of carboxyfluorescein **2** by tris-cation **1** in buffered water (Fig. 2).[8] This is a surprisingly strong association. Most likely the flat aromatic ring system of **2** provides additional hydrophobic or π–π-interactions stabilizing the complex of **2** with receptor **1**. But as expected for the binding of a tris-anion by receptor **1**, the affinity of **2** is similar to that of citrate **3** (the association constants are both $K_{ass} \approx 10^5$ M^{-1}) making this an ideal combination for a sensing ensemble. The selectivity of an indicator displacement assay is most significant when the indicator has the same or a slightly lower affinity than the substrate of choice, but a larger affinity than other competing analytes.[9]

Therefore, upon the addition of citrate **3** the carboxyfluorescein **2** is displaced from the binding cavity and its fluorescence is restored. However, substrates that are less efficiently bound by **1** than citrate **3** are not capable to displace **2**. Hence, even

substrates such as malate **4** or tartrate **5**, which are closely related to citrate both in terms of their biological occurrence as well as their recognition elements (carboxylates and OH groups), do not interfere with the detection of citrate by this sensor ensemble. They are both bound by receptor **1** with $K < 10^4$ M^{-1},[5] hence at least one order of magnitude less efficiently compared to **2** or **3**. They are therefore not capable to displace carboxyfluorescein **2** from the binding site. Also acetate or other monoanions such as chloride (even at an excess of more than 20 eq.), which are bound even worse than tartrate, have no influence.

The strong and optically visible fluorescence of **2** allows a direct naked eye detection of citrate using a standard UV-lamp (Fig. 3). As can be seen in Fig. 3, the green fluorescence of **2** is completely quenched upon binding to the receptor **1**. The addition of a 20 fold excess of either acetate or malate does not restore the green fluorescence despite the large excess of substrate. However, the same amount of citrate **3** completely displaces carboxyfluorescein **2** from the binding site and hence restores the fluorescence as can be nicely seen by the recurrence of the green emission of **2** under UV light.

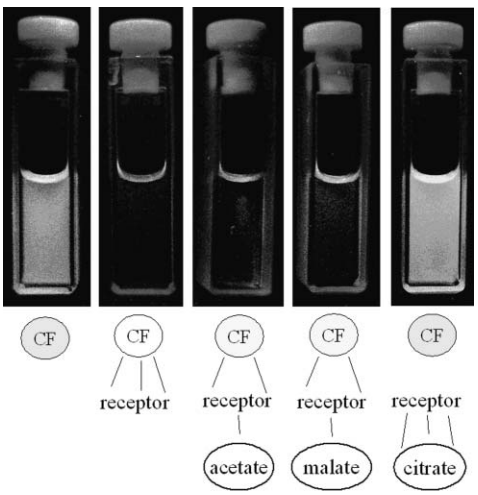

Fig. 3 Naked-eye detection of acetate, malate and citrate (all 10 mM) with a 1 : 1 mixture of receptor **1** and carboxyfluorescein **2** (0.5 mM) in aqueous DMSO. Only citrate **3** is capable to displace **2** from its complex with **1** thereby restoring its fluorescence.

Besides this qualitative demonstration of the substrate selectivity, also a quantitative analysis of the selectivity of this sensing ensemble was performed as shown in Fig. 4. The addition of citrate **3** to the 1 : 1-complex of **1** and **2** in buffered aqueous solution causes a significant increase in the fluorescence intensity of **2** at $\lambda = 518$ nm (the fluorescence maximum of carboxyfluorescein) compared to the addition of either acetate, malate or tartrate which only cause a slight increase in the fluorescence. Upon the addition of 1 equivalent of analyte this sensor ensemble is more than 10 times more selective for citrate than for the next best binding substrate (malate). Even after the addition of a large excess of 14 equivalents of each substrate the selectivity for citrate over malate or tartrate is still 4 : 1 and 9 : 1, respectively.

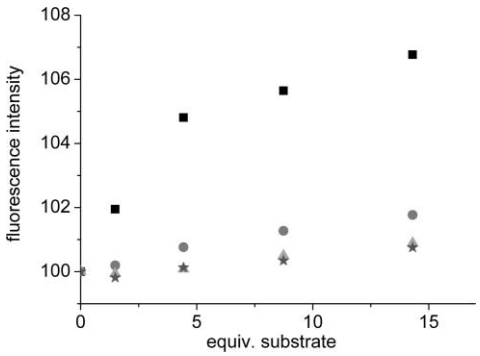

Fig. 4 Change in the fluorescence intensity of **2** at $\lambda = 518$ nm (excitation at $\lambda = 495$ nm) upon the addition of either acetate (△), malate (●), tartrate (*) or citrate (■) to a 1 : 1 mixture of **1** and **2** in 50% DMSO in water (20 mM Hepes buffer, pH = 7.4).

Conclusions

In conclusion, we have presented here a new naked-eye sensor ensemble for the detection of citrate in water. This sensor shows the highest affinity so far reported for the binding of citrate by receptors which are solely relying on weak non-covalent interactions. Furthermore, a remarkable selectivity for citrate of >10 : 1 relative to even closely related substrates such as malate or tartrate is achieved.

Acknowledgements

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