A combinatorial approach to surface-confined cation sensors in water†

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Received 9th February 2005, Accepted 21st March 2005
First published as an Advance Article on the web 14th April 2005
DOI: 10.1039/b502102b

A novel material for sensing cations in water *via* fluorescence spectroscopy is presented. The material consists of a glass substrate functionalized with a series of fluorescent self-assembled monolayers. Parallel modification with pairs of fluorophore-binding molecules of monolayers formed on glass yield a library of sensitive glass substrates. Measurements of the changes in fluorescence intensity of the layers upon addition of aqueous solutions of Cu^{2+}, Co^{2+}, Ca^{2+} and Pb^{2+} confirmed the ability of the monolayer library to produce a ''fingerprint'' response for separate analytes with a high reproducibility. This new protocol for fabrication of sensitive probes in glass is suitable for array fabrication in small size substrates. Additionally, the covalent attachment of the fluorophore moieties to the monolayer allows monitoring of the integrity of the monolayer in time in contact with solutions. To the best of our knowledge this is the first example of sensing of cations in water by a self-assembled monolayer on glass.

Introduction

Surface-confined sensing in water is of interest for a number of fields including medical diagnostics and detection of environmental contaminants.[1–3] As sensing surfaces, self-assembled monolayers (SAMs) offer advantages such as a unidirectional responding surface, minimization of analyte sorption time to the receptors, and fast response times.[4] We present here the first example of ion sensing in water by fluorescence on a SAM on a glass surface. Such a system has potential applications for use in microarrays.

Thus far, the majority of molecular recognition in water on SAMs has been reported for gold substrates,[5] utilizing mainly electrochemical methods,[6] surface plasmon resonance,[7,8] or atomic force microscopy to monitor the interaction. Fluorescence spectroscopy is a highly desirable measurement technique due to its sensitivity, ease of data collection, and minimization of impact on the sample (no use of electrolytes),[9,10] but it is not well suited to measuring interactions on top of gold surfaces due to quenching of the fluorescence by the gold.[4,11] Glass, however, is a suitable substrate, offering the additional advantage compared to gold of covalent attachment of the sensing monolayer to the surface.[12] The field of surface-confined biosensing, which often uses glass as a platform for the generation of microarrays, has widely employed fluorescence to monitor biological interactions such as enzyme activity,[13] recognition of DNA sequences,[14] and protein–protein interactions.[15] Recently, the use of fluorescence to sense molecular recognition processes on SAMs on glass[16–18] and silica particles[19,20] has started to emerge. A new methodology for the recognition of cations by fluorescent SAMs in organic solvents[21,22] has been reported recently by our group.

Sensing in water on SAMs covalently bound to glass can be hampered by hydrolysis of the Si–O bond, particularly in basic media.[23,24] However sensing in water is particularly important due to its applicability to real-world analyses. It is often not possible to monitor in time the layer stability, and most sensing studies on surfaces are reported without consideration of the highly important integrity of the monolayer. While an interaction can still be monitored without knowledge of the stability of a monolayer, it becomes problematic to perform reliable quantitative studies.

Recently, our group developed a new methodology involving a series of glass-confined sensing systems for detecting inorganic cations and anions in organic solvents.[21,22] Once the proof of principle was established the extension of the methodology to aqueous media was imperative. Here, the methodology is expanded to sense ions by SAMs on a glass platform in aqueous solutions and at the same time it is shown that this methodology provides a simple method to study the stability of the sensitive material. The protocol is an unprecedented parallel combinatorial approach to the deposition of different complexing functionalities and fluorophores onto an amino-terminated SAM on glass. The result is a number of complexing functionality–fluorophore combinations for sensing cations in a differential fashion. A large number of sensing surfaces can be generated without the need for designing and synthesizing a complex receptor, or target labeling and library deconvolution. Because of their binding properties these monolayers interact with an analyte and induce a change in the fluorescence emission intensity of the layer (Fig. 1).

Our original SAM sensing systems used TPEDA (*N*-[3-(trimethoxysilyl)propyl]ethylenediamine) to form an amino terminated monolayer on a glass surface[21,22] to which the fluorophore and complexing functionality were subsequently attached. However, it is known that direct attachment of amino terminated silanes onto glass results in monolayers that

† Electronic supplementary information (ESI) available: Fluorescence spectra, error analysis and stabilization studies. See http://www.rsc.org/suppdata/jm/b5/b502102b/

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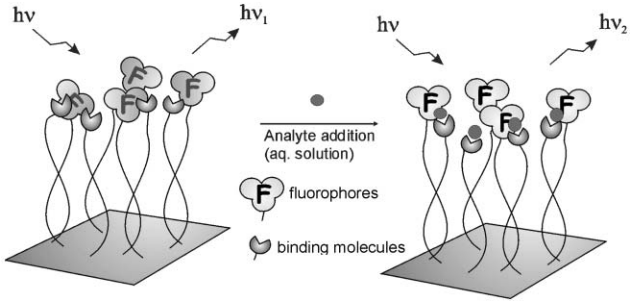

Fig. 1 Schematic representation of a fluorescent sensitive monolayer (SAM) on a glass surface. The sensitive fluorescent monolayer comprises a monolayer modified with fluorophores and the binding molecules. In presence of an analyte the fluorescence emission of the SAM changes due to interaction of the analyte with the layer.

are not highly ordered,[24] and it is likely that the lack of a well-ordered hydrophobic layer allows water to penetrate in the layer and hydrolyze the Si–O bonds, which will destroy the layer. In this paper, we show that by covalent attachment of a fluorophore to a SAM on glass, it is possible to monitor the stability of the layer in aqueous solution. By doing so, we were able to develop a new class of stable monolayers on glass for the fabrication of a parallel library of non-specific sensitive monolayers for cations in aqueous medium.

Experimental

Chemicals and procedures

All glassware used to prepare the monolayers was cleaned by sonicating for 15 min in a 2% v/v Hellmanex II solution in distilled water, rinsed four times with high purity (MilliQ, 18.2 MΩ cm) water, and dried in an oven at 150 °C. The substrates, quartz slides and silicon wafers were cleaned for 15 min in piranha solution (concentrated H_2SO_4 and 33% aqueous H_2O_2 in a 3 : 1 ratio. **Warning: Piranha solution should be handled with caution: it has been reported to detonate unexpectedly**). They were then rinsed several times with high purity (MilliQ) water, and dried in a nitrogen stream immediately prior to performing the formation of the monolayer.

Formation of the amino-terminated monolayers

Formation of the *N*-[3-(trimethoxysilyl)propyl]ethylenediamine self-assembled monolayer (SAM) (**1**, Fig. 2) was achieved in a glovebox under an atmosphere of dry nitrogen. The freshly cleaned substrate was immersed in a 5 mM solution of *N*-[3-(trimethoxysilyl)propyl]ethylenediamine, in dry toluene (freshly distilled over sodium) for 3.5 h. After the substrate was taken from the solution, it was rinsed with toluene twice (under nitrogen atmosphere) to remove excess silane and avoid polymerization. The substrates were then removed from the glovebox and rinsed thoroughly with toluene.

Millipore water (MilliQ, 18.2 MΩ cm) was used in all monolayer syntheses.

Formation of the 11-aminoundecyltrichlorosilane self-assembled monolayer (**4**, Fig. 2) was achieved following a slightly modified protocol described previously by our group.[17] Substrates were submerged in a 0.1 vol.% solution of 1-cyano-11-trichlorosilylundecane in freshly distilled toluene previously cooled in an ice bath. After 35 min at 0 °C they were removed and rinsed copiously with toluene, then dried in an air stream. Reduction of the cyano group was achieved by submerging the substrates in a 10 vol.% solution of Red-Al in freshly distilled toluene under N_2 at 45 °C for 4 h. The slides were removed and sonicated in a 1 M HCl solution for 5 min, then sonicated with 0.5 M NaOH for 1 min, followed by copious rinsing with millipore water and drying in an air stream.

Immobilization of the fluorophore on the amino-terminated monolayer

Silicon and glass substrates functionalized with the amino-terminated monolayer were submerged in 50 mL of a 1 mM acetonitrile solution of fluorophore (dansyl chloride, 7-dimethylaminocoumarin-4-acetic acid succinimidyl ester, or TAMRA (5-(and 6-)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) *mixed isomers)) with 100 μL Et_3N for 4 h under N_2. After 4 h the substrates were removed from the fluorophore solution then they were rinsed sequentially with acetonitrile, ethanol and dichloromethane to remove physisorbed material and dried in an air stream.

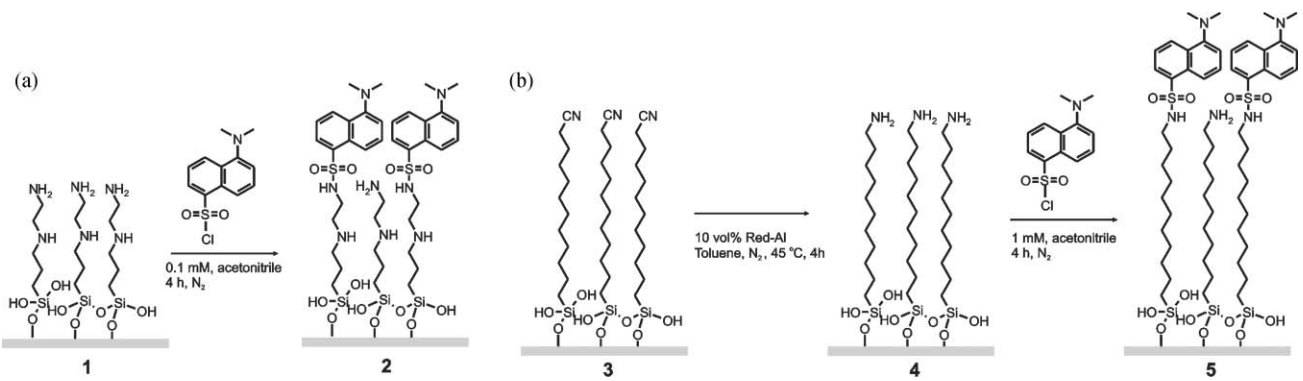

Fig. 2 Synthesis scheme for the preparation of the self-assembled monolayers (a) (dansyl-substituted (*N*-[3-(trimethoxysilyl)propyl]ethylenediamine) SAM (**2**), and (b) dansyl-substituted 1-amino-11-silylundecane SAM (**5**) on silicon and glass surfaces tested for water stability.

Immobilization of the ligands on the surface

Silicon and glass substrates with previously immobilized fluorescent SAM were submerged in 50 mL of a 50 mM acetonitrile solution of benzenesulfonyl chloride or 4-isopropylphenyl isocyanate. In the case of benzenesulfonyl chloride, 100 μL Et_3N was added. The reaction took place under N_2 for 16 h, at which time the substrates were then sequentially rinsed in acetonitrile, ethanol and dichloromethane to remove physisorbed material, and dried in an air stream.

Characterization of the layers

Ellipsometry measurements of the layers were made on silicon wafers, and were performed on a Plasmon ellipsometer (λ = 632.8 nm) assuming a refractive index of 1.5 for the monolayer over the silicon oxide layer (refractive index 1.46). Raster scans were taken of 25 points per silicon wafer (maximum size 7 mm^2), and their values averaged. Values for the layers averaged 1.44 ± 0.14 nm for cyano layers, 1.44 ± 0.16 nm for amino layers. Ellipsometry values for the fluorescent monolayers are summarized in Table 1.

All the values are in good agreement with a monolayer thickness modelled with the software WebLab Viewer v2.01. Comparing the calculated and experimental data small deviations of values have been found. Nevertheless, it is expected that the fluorophores and/or complexing functionalities are lying flat on the amino surface thus explaining the small deviations.

Contact angle measurements of the monolayers were performed on functionalized silicon wafers with MilliQ water. Measurements were performed on a Krüss pendant drop contact angle measurement system G10, a sessile drop system mounted with a CCD camera, using drop shape analysis 1.51 software. Drop fitting was done with DPA32 Tangent 1 and Tangent 2 analysis methods. Values of the angles averaged 65 ± 3° (advancing) and 50 ± 3° (receding) for the cyano monolayers and 60 ± 2° (advancing) and 32 ± 6° (receding) for the amino monolayers. Contact angle values for the fluorescent monolayers are summarized in Table 1.

Values for the cyano and amino terminated monolayers are in good agreement with previous reports in the literature;[17] the low hysteresis value (difference between advancing and receding value) indicates high order of the formed layer.

Fluorescent spectroscopy of the layers confirmed the introduction of the fluorophores, maximum emission peaks were found around 520 nm on the dansyl layers, 585 nm for the TAMRA layers and 440 nm for the coumarin layers.

Table 1 Ellipsometry and contact angle data of each fluorescent monolayer of the sensing library

	Ellipsometry	Contact angle/°	
Layer	Thickness/nm	Advancing	Receding
DA	1.86 ± 0.24	65 ± 3	50 ± 3
DS	2.50 ± 0.37	69 ± 1	30 ± 8
DU	1.95 ± 0.14	67 ± 4	32 ± 6
CA	2.22 ± 0.38	71 ± 5	28 ± 1
CS	2.23 ± 0.34	73 ± 5	32 ± 9
CU	1.89 ± 0.17	70 ± 4	28 ± 1
TMA	1.23 ± 0.11	65 ± 3	35 ± 4
TMS	1.33 ± 0.16	68 ± 2	33 ± 6
TMU	1.57 ± 0.16	66 ± 1	30 ± 4

Spectrofluorometric measurements set up

Fluorescence experiments were performed on an Edinburgh FS900 spectrofluorimeter with a 450 W xenon arc lamp as excitation source (λ_{ex} = 545 nm for TAMRA, 340 nm for dansyl and 330 nm for coumarin). M300 gratings with 1800 l mm^{-1} were used on both excitation and emission arms. Signals were detected at an angle of 90° with regard to the excitation source by a Peltier element cooled, red sensitive, Hamamatsu R928 photomultiplier system. For the fluorescence spectroscopy measurements a general procedure was followed: the quartz cuvettes (45 mm × 12.5 mm × 12.5 mm, volume 3.5 mL) were cleaned by placement for 15 min in a 60 °C 2% v/v Hellmanex II solution in demi-water and afterwards rinsed with ultrapure (MilliQ) water and dried in an air stream. Ultrapure (MilliQ) water at pH 7.0 (HEPES) was added, the functionalized quartz slide (H × W × D, 40 mm × 17 mm × 1 mm) was placed at an angle of 45° in the cuvette, and the cuvette placed in a holder on an externally tunable platform. Normally an angle between −10° and −20° was used for the measurements. For dansyl and coumarin a 375 nm filter was used, and for TAMRA a 550 nm filter was used. Excitation wavelengths were 340 nm for dansyl, 330 nm for coumarin and 535 nm for TAMRA. The analytes used were chloride salts of Hg^{2+}, Ca^{2+}, Cu^{2+}, and Co^{2+}. For each cation, two measurements were first taken in the absence of analyte to ensure layer stability. A solution of the cation was added so that the concentration of the analyte in the cuvette was 10^{-4} M, and a spectrum taken after 1 min. An additional spectrum was taken 2 min later to detect any additional changes. The slide was then removed and a spectrum of the solvent was measured. The individual fluorescence values given in the text are the average of between two and six measurements. For examples of fluorescence emission spectra and a list of errors in the measurements see supporting information.

Results and discussion

To find a suitable fluorescent monolayer for aqueous solution measurements two different amino terminated monolayers were functionalized using dansyl chloride (Fig. 2). The first type of layer (**2**) was fabricated by direct functionalization of the glass substrate with a (*N*-[3-(trimethoxysilyl)propyl]ethylenediamine) SAM (**1**) and subsequent functionalization with the dansyl chloride. Fluorescence stability studies performed with **2** at pH 7.0 (see Experimental part) showed the monolayer to be unstable over time, with almost total loss of the fluorophore molecules into the solution after 15 min. Due to the instability of these layers, a stepwise chemical synthesis of a long chain amino terminated monolayer (**4**) was then performed (Fig. 2), because indirect synthesis of amino terminated monolayers yields a well-defined structure in the siloxane network increasing the stability of the monolayer.[25–29]

Dansyl-substituted 1-aminosilylundecane SAM (**5**, Fig. 2) was fabricated starting from 1-cyano-11-trichlorosilylundecane SAM (**3**). Reduction of the cyano groups resulted

in the amino-terminated monolayer (**4**). Reaction of the monolayer **4** with dansylchloride afforded **5**. The dansyl-substituted SAM (**5**) showed complete stability and integrity of the fluorescence signal in water at pH 7.0 (0.1 M HEPES) over at least 1 h (Fig. 3). Furthermore, no fluorescence signal was detected in the solution after removal of the functionalized slide from the spectrofluorometer cuvette ruling out the disassembly of the fluorescence material from the glass slide and the presence of physisorbed material. Consequently, monolayer **4** was used as starting material for the fabrication of the cation sensing library.

A series of two binding molecules (benzenesulfonyl chloride and 4-isopropylphenyl isocyanate) and three fluorophores (dansyl chloride, 7-dimethylaminocoumarin-4-acetic acid succinimidyl ester, and TAMRA (5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester as mixed isomers) were then sequentially reacted with the amino-terminated SAM **4** in different combinations, resulting in a library of sensing surfaces of nine distinct complexing functionality–fluorophore pairs (Fig. 4). Each sensing layer has one complexing functionality known to bind to cations (*i.e.*, amino (A), aryl-urea (U), aryl-sulfonamide (S)) and one fluorophore (*i.e.*, short excitation wavelength dansyl (D) or coumarin (C), or long excitation wavelength TAMRA (TM)). The resulting modified layers were characterized by contact angle and ellipsometry measurements, confirming the covalent linkage of the components.

Each layer was tested for signal stability prior to analyte addition. While dansyl layers (DA, DS and DU) were immediately stable, coumarin and TAMRA layers required sonication in water and in the 0.1 M HEPES solution to achieve stability. The most effective protocol to achieve stable coumarin and TAMRA layers involved 5 min sonication in a 0.1 M HEPES solution followed by 5 min sonication in water at 40 °C.[30] Following this methodology the stability of the silane monolayer attached to the glass slide and the absence of physisorbed material is assured.

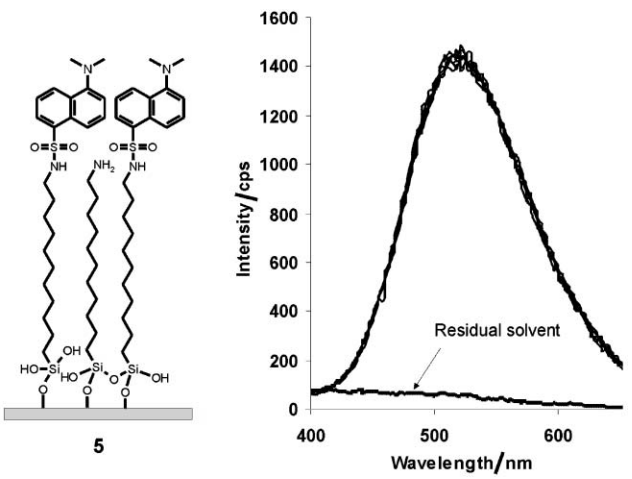

Fig. 3 Spectra of the dansyl-funtionalized 1-amino-11-silylundecane SAM (**5**) after 0, 3, 5, 10, 15, 30 and 60 min immersed in 0.1 M HEPES solution, and the spectrum of the residual solvent after removal of the functionalized glass slide from the spectrofluorometer cuvette.

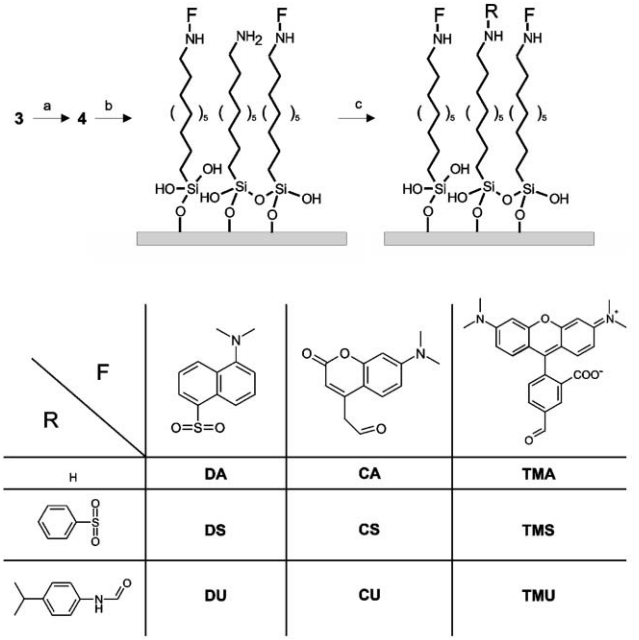

Fig. 4 Synthesis scheme for the preparation of the fluorescent SAMs (DA, CA, TMA, DS, CS, TMS, DU, CU and TMU) on glass and silicon surfaces. (a) Red Al, toluene, 40 °C, (b) Dansyl chloride, 7-dimethylaminocoumarin-4-acetic acid succinimidyl ester, or 5-(and 6-)-carboxytetramethylrhodamine, succinimidyl ester, acetonitrile, rt. (c) Benzenesulfonyl chloride or 4-isopropylphenyl isocyanate, acetonitrile, rt.

Table 2 Percentage[a] of the fluorescence intensity changes[b] of the layers (DA, DS, DU, CA, CS, CU, TMA, TMS, TMU) upon addition of 10^{-4} M (pH 7.0, 0.1 M HEPES) of chloride salts of Ca^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+}

	DA	DS	DU	CA	CS	CU	TMA	TMS	TMU
Ca^{2+}	−9	−12	−14	−4	−1	−1	−3	−1	0
Hg^{2+}	−25	−24	−18	−16	−6	−9	−5	7	6
Co^{2+}	−11	−14	−15	−1	−3	−17	−19	−7	0
Cu^{2+}	−50	−43	−39	−39	−30	−29	−55	−16	−26

[a] The response of the layer to the analyte was calculated as the percentage of the difference in fluorescence emission of the layer in absence and presence of the analytes. Positive values correspond to an enhancement in the fluorescence emission intensity of the layer while negative values represent a quenching of the fluorescence emission intensity of the layer. [b] Each response is the average of measurements taken several times of slides made in two separate batches (see supporting information for complete data).

After formation of the stable monolayers their cation sensing properties were studied (Table 2). The chloride salts of Hg^{2+}, Ca^{2+}, Co^{2+} and Cu^{2+} were used as analytes. Each of the layers of the sensing library (DA, CA, TMA, DS, CS, TMS, DU, CU, TMU) (Fig. 4) was placed in a spectrofluorometer cuvette filled with 0.1 M aqueous solution (pH 7.0) of HEPES buffer and the fluorescence spectrum was measured. A solution of the corresponding cation was added so that the concentration of the analyte in the cuvette was 10^{-4} M and the fluorescence spectrum was measured again.

Looking at the library response as a whole, a few overall trends emerge. For different analytes the largest fluorescence response of all layers was to Cu^{2+} followed by Hg^{2+} and Ca^{2+}.

When we compare the influence of the different fluorophores (dansyl, coumarin and TAMRA) on the monolayer response, the dansyl monolayers exhibit more fluorescence quenching than the monolayers with the other fluorophores. The quenching of dansyl layers of the three ligands in DA, DS, and DU, respectively, in response to Ca^{2+} and Hg^{2+} were 9%, 12% and 14% for Ca^{2+}, and 25%, 24% and 18% for Hg^{2+} for layers. For TAMRA and coumarin bearing layers the response for Ca^{2+} was in all cases lower than 4% fluorescence quenching. In the case of Hg^{2+} only the layer CA showed a fluorescence quenching of 16%, comparable with the response of dansyl monolayers to Hg^{2+}. Within each fluorophore series, the effect of the individual complexing groups on the fluorescence response can evaluated. In the dansyl series, all three ligands gave similar responses to each particular analyte, which indicates that the dansyl group has a predominant influence on the analyte response and not the complexing functionality. Notice that when the sulfonyl chloride of the dansyl reacts with the amino terminated surface a sulfonamide bond is formed. This sulfonamide functionality is a good binding group for metal ions and may very well be responsible of the higher affinity of the metal ions for the dansyl layers. In the coumarin and TAMRA series, the more varied responses illustrate cases where the presence of ligands influences the sensing (Table 2).

There are few overall trends when we compare the amino, sulfonamide, and urea functionalities and monitor the effect of the fluorophores on the response. For example, the amino layers consistently give a high response for all three fluorophores for Cu^{2+}, whereas not all of the sulfonamide and urea layers respond as strongly. Analysis of both the sulfonamide (DS, CS, TMS) and urea (DU, CU, TMU) series reveals that some analytes exhibit different responses for different fluorophores. For example the fluorescence of the layers DS is quenched 24% in presence of Hg^{2+} while the layer CS shows a quenching of only 6% for the same cation. In the same way the layer CU displayed a 17% quenching for Co^{2+} while layer TMU does not respond to the presence of Co^{2+}.[31]

Thus, the nature of the functionalities of the fluorophore and the other ligating functionalities influences the sensing ability of the layer resulting in a range of responses to different analytes. Yet whether one component will be predominant in determining the fluorescence response or whether they will work together to form a unique sensing system is unpredictable. This is because the binding ability of each functionality–fluorophore pair is determined by a number of factors, *e.g.* where the analyte is binding relative to the two surface components, as well as possible steric constraints or additional surface interactions between the monolayer substituents (monolayer packing, van der Waals forces, cation–π or π–π interactions). The unpredictability of which components will constitute a successful sensing layer underlines the power of utilizing a 2D combinatorial parallel approach to the discovery of successful sensing systems. The library response towards metal cations can be used to search for either a unique response (individual ''hit'') or a whole ''fingerprint'' of responses (Fig. 5). Here, the ''fingerprint'' is the collection of the individual responses of each sensing layer to one cation. Rapid inspection of the library ''fingerprint'' (Fig. 5) provides a unique response for each cation.

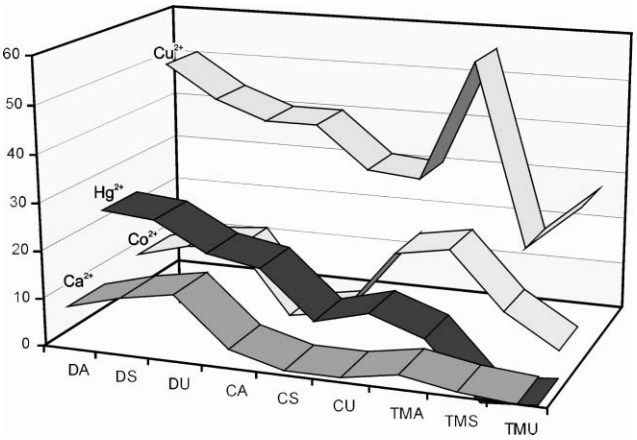

Fig. 5 Graphical representation of the data showed in Table 2. Changes in fluorescence emission intensity of the layers in presence of 10^{-4} M Ca^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+} chloride salts in 0.1 M HEPES (pH 7.0). Every line on the graph constitutes a unique ''fingerprint'' of each analyte. The *y*-axis represents percentage of quenching of the initial fluorescence of the layer upon addition of the analyte. The initial fluorescence of the layers before analyte addition has been normalized.

The detection limit and reusability of the layers functionalized with dansyl (DA, DS and DU) and TAMRA (TMA, TMS, TMU) fluorophores were also studied. The layers exhibited responses to Cu^{2+} down to 10^{-6} M.[32] Furthermore, the sensitive surfaces are fully regenerated by rinsing the analyte away with 0.1 M HCl (aq.). The layers were reused at least four times without losing their characteristic response.

In conclusion we have demonstrated that commercially available fluorophores and simple, small, off-the-shelf molecules can be used to fabricate a cation responsive library of SAMs on glass in aqueous solution. This is the first example of ion sensing in water by fluorescence on a SAM on a glass surface and shows that our original concept is fully transferable to an aqueous medium. The sensing library described is a highly sensitive probe for cation sensing in water, since it is able to produce a different fingerprint response for different analytes.

Additionally we have shown that the stability of the monolayer in aqueous solution can be easily monitored by the covalent attachment of a fluorophore to a SAM on glass. This methodology could also be applied to assess the stability, in principle, of other SAM used in biological and chemical sensing systems where knowledge of the stability of the monolayer or quantification of the molecular recognition event is required.

The extension of the sensing methodology to the microscale by both patterning of the monolayer surface and microchannel technologies for future development towards incorporation of the systems into a true microarray is underway in our group.

Acknowledgements

The research of M. C.-C. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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30 The sonication protocol may change depending on the layer composition. After sonication the fluorescence signal of the glass slides and the residual signal in the solvent are measured to check the absence of physisorbed material. The layers were cleaned until stabilization occurred and only minimal signal was seen in the residual solvent.
31 The analysis within the current library layers containing the TAMRA fluorophore TMA (amino functionality) and TMU (urea functionality) in the sensing of Ca^{2+}, Co^{2+} and Cu^{2+} offer some degree of comparison with the responses of their counterparts in acetonitrile from the previous library.[21,22] Although direct correlations cannot be made due to differences in the length of the amino SAM upon which the sensing components are placed, as well as differences in counterion (chloride *versus* perchlorate), it can be noted that the overall responses were not as large as in the acetonitrile system, which is understandable due to the more polar environment as well as the presence of a high concentration of other charged species (the HEPES and NaOH).
32 Due to the relatively low magnitude of fluorescence response across the library for Co^{2+}, Hg^{2+} and Ca^{2+} at 10^{-4} M, measurements with these cations were not taken at lower concentrations.