

Templated xerogels as platforms for biomolecule-less biomolecule sensors

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Abstract

We report on a new sensor strategy that we have termed protein imprinted xerogels with integrated emission sites (PIXIES). The PIXIES platform is completely self-contained, and it achieves analyte recognition without a biorecognition element (e.g., antibody). The PIXIES relies upon sol-gel-derived xerogels, molecular imprinting, and the selective installation of a luminescent reporter molecule directly within the molecularly imprint site. In operation the templated xerogel selectively recognizes the target analyte, the analyte binds to the template site, and binding causes a change in the physicochemical properties within the template site that are sensed and reported by the luminescent probe molecule. We report the PIXIES analytical figures of merit for and compare these results to a standard ELISA. For human interleukin-1 the PIXIES-based sensor elements exhibited the following analytical figures of merit: (i) ~2 pg/mL detection limits; (ii) <2 min response times; (iii) >85 selectivity; (iv) <6% R.S.D. long term drift over 16 weeks of ambient storage; (v) >95% reversibility after more than 25 cycles; and (vi) >85% recoveries on spiked samples. © 2006 Elsevier B.V. All rights reserved.

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1. Introduction

The goal to simultaneously measure “everything” within a sample is common in many disciplines [1]. This desire has helped drive the development of a wide variety of sensor array strategies [2–8], including artificial “noses” and “tongues” [9]. Interestingly, current array-based sensor platforms have much in common with biochips, DNA microarrays, and protein microarrays [10–12]. For example, each technology requires that one identify an appropriate recognition element (e.g., antibody, cell or phage lysate, recombinant protein or peptide, oligonucleotide/oligonucleoside) that can selectively recognize the target analyte (e.g., a protein, DNA fragment), one must implement a suitable detection/transduction method, and one must “immobilize” the recognition element [13–15]. In an ideal platform the recognition element must also remain stable over time, the target analyte must have access to the recognition element, and the tar-

get analyte-recognition element association/interaction should be, for a sensor or any other device designed for real-time monitoring, “reversible” or at least easily dissociated/reset following each measurement.

Several research teams have developed biosensor arrays [16–20] for simultaneous multi-analyte detection. Representative examples include the Tan group’s molecular beacon-based DNA biosensors [16], the electrostatically complexed monolayers deposited on photolithographically patterned gold microelectrodes described by Revzin et al. [17], the photopatternable enzyme membranes reported by Moser et al. [18], the Walt group’s high density fiber optic arrays that are based on intact bacteria and yeast cells [19], and McDevitt’s “electronic taste chips” based on immunochemistry [20]. A more recent development has been work from our laboratories coupling pin printing, protein-doped xerogels, and light emitting diodes to form novel (bio)sensor arrays [21].

Despite the progress on biosensor and protein microarrays based on proteinaceous biorecognition elements [2–20], there are well-documented limitations associated with this strategy [22–25]. For example, according to Swanson and co-workers

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[22], “. . . immuno-based assays are difficult to implement . . . owing to poor stabilities of antibodies and the need for unstable reagents.” Thus, although biologically based recognition elements are clearly being used to detect proteins, there are compelling reasons for developing inexpensive, robust, and reusable alternatives for these expensive and labile biorecognition elements. What if one could develop “biomolecule-less” sensors for the detection of biomolecules?

Over the past decade, the creation of specific binding domains within synthetic polymers by template-directed cross-linking of functional monomers has attracted considerable attention [23,24]. Molecular imprinting involves arranging polymerizable functional monomers around a suitable template molecule (e.g., the actual target analyte or a surrogate) followed by polymerization and template removal. Arrangement is typically achieved by non-covalent interactions (e.g., H-bonds, ion pairing) or reversible covalent interactions. After template removal, these molecularly imprinted polymers can recognize and bind the template (i.e., the target analyte). The advantages of molecularly imprinted polymer-based materials include [23,24]: (i) specificity comparable to a biomolecule (nM dissociation constants have been reported); (ii) robustness and stability under extreme chemical and physical conditions; and (iii) an ability to design recognition sites even for analytes that lack suitable biorecognition elements *or* for unknown analytes.

Molecularly imprinted polymers have been developed for amino acid derivatives, nucleotide bases, pesticides, pharmaceuticals, polycyclic aromatic hydrocarbons, proteins, sugars and their derivatives, and vitamins. However, according to Lam and co-workers [25], “One of the major issues in the development of molecularly imprinted polymer-based biomimetic sensors is signal transduction.” There is literature on the use of amperometry/voltammetry [26], acoustic waves [27], conductometry [28], colorimetry/fluorimetry [29], and radio-labeled assays [30] to convert a molecularly imprinted polymer into a sensor; however, reports are scarce on luminescence-based molecularly imprinted polymer sensors for detecting non-luminescent analytes [25,31,32].

Sol–gel processing is an attractive method to sequester and exploit chemically active species [33,34]. Sol–gel-derived xerogels are also attractive because one can tailor a xerogel’s physicochemical properties by using different precursor(s) or changing the processing protocol [33,34]. Xerogel-based sensor platform can also exhibit remarkable stability over time [35]. Further, as demonstrated by, for example, Lam and co-workers [25] and Edmiston and co-workers [31] one can exploit molecularly imprinted xerogels to design luminescence-based chemical sensors for the detection of small molecules. In related research, the Chambers group [36] has used molecular imprinting to prepare molecularly imprinted xerogels that can recognize the protein Ricin. However, although Chambers and co-workers used the intrinsic fluorescence from the tryptophan residues within Ricin to investigate the Ricin–xerogel interactions, these authors did *not* actually report a sensing strategy.

High-throughput screening (HTS) methods are common in combinatorial chemistry and drug discovery [37–40]. For example, HTS methods have been used to identify promising H₂

producing catalytic materials [41], ceramics [42], flame retardants [43], dielectric thin films [44], and other materials [45–47]. We became attracted to HTS as a way to more efficiently prepare and screen biodegradable polymer and sol–gel-derived xerogel formulations for bioengineering and general sensor applications, respectively [48,49].

Despite the obvious attraction of luminescence-based detection, xerogels, and molecular imprinting, researchers have *not* developed a protein detection strategy that exploits the power of luminescence, the tunability of xerogels, and molecular imprinting. In this paper we report on a new strategy for fabricating protein-responsive chemical sensor elements based on sol–gel-derived molecularly imprinted xerogels. We term these new xerogel-based sensor elements protein imprinted xerogels with integrated emission sites (PIXIES). We outline the PIXIES production protocol, we describe a methodology for rapidly producing and screening a wide variety of sol–gel-derived xerogel-based formulations, and we compare the analytical figures of merit for our PIXIES to standard antibody-based assays.

2. Experimental

2.1. Reagents and supplies

The following reagents were used: human serum albumin, ovalbumin, tetramethoxysilane (TMOS) and urea (Sigma–Aldrich); human interleukin-1 alpha and beta (R&D Systems); tetraethoxysilane (TEOS) and methyltrimethoxysilane (C1-TMOS) (United Chemical Technologies); ethyltrimethoxysilane (C2-TMOS), *n*-propyltrimethoxysilane (C3-TMOS), *n*-butyltrimethoxysilane (C4-TMOS), *n*-hexyltrimethoxysilane (C6-TMOS), *n*-octyltrimethoxysilane (C8-TMOS), *n*-decyltriethoxysilane (C10-TEOS), bis(2-hydroxy-ethyl)aminopropyltriethoxysilane (HAPTS), 3-aminopropyltriethoxysilane (APTES), ureidopropyltriethoxysilane (U-TEOS), and 3,3,3-trifluoropropyltrimethoxysilane (TFP-TMOS) (Gelest, Inc.); succinimidyl ester of BODIPY FL (BODIPY-FL,SE) and 4-azido-2,3,5,6-tetrafluorobenzyl amine, hydrochloride (ATFA) (Invitrogen); HCl (Fisher Scientific); and EtOH (Quantum Chemical).

Aqueous solutions were prepared from deionized water that was treated with a Barnstead NANOpure II system to a specific resistivity of >18 MΩ cm.

Glass microscope slides (Fisher Scientific Co.) were used as the substrates. The slides were cleaned by an EtOH rinse, 1–2 h soak in 1 M NaOH, 1–2 h soak in 1 M HCl, deionized water wash, and oven drying at 80 °C.

2.2. Formulation development and preliminary PIXIES screening

We set out to prepare, screen, and identify xerogel formulations that yielded PIXIES with good analytical figures of merit. Toward this end, we used a statistical design strategy [50] and prepared a series of sols based on several of the precursors that were listed in the previous section. As an example, a representative series of sols was prepared from TEOS, APTES,

C8-TMOS, and HAPTS, where the mole percentage of each precursor was varied from 0 to 100 mol%. Although other sols were also prepared and evaluated, our focus in this paper is on TEOS/APTES/C8-TMOS/HAPTS-based formulations only.

All sol solutions were prepared under ambient conditions. The *initial* sol solution contained 5 mmol of total silane, 20 mmol of H₂O, and 0.4×10^{-4} mmol of HCl [21,35].

We assessed each formulation by determining the relative response from the corresponding PIXIES (F_{analyte}/F_0), where F_0 is the luminescence signal from the PIXIES in absence any target analyte and F_{analyte} is the PIXIES luminescence signal in the presence of a fixed target analyte concentration.

2.3. Instrumentation

Fig. 1 illustrates the system that was used for producing the sol formulations, fabricating the sol-gel-derived xerogel-based sensor elements and sensor arrays, and performing the spectroscopic measurements on the xerogel formulations. The system consists of (i) a Gilson model Quad-Z 215 automated liquid handler for producing the sol formulations; (ii) a Cartesian Technologies model ProSys 5510 robotic pin printer system with TeleChem model CMP-3 quill pins for forming the xerogel-based sensor arrays [21]; (iii) a spectroscopy system that consists of a laser (He–Cd, 325/442 nm; argon-ion, 351/364/488 nm), an Ocean Optics model xyz-200 \times 150 \times 100 robotic 3D mapping table and Ocean Optics model USB 2000 spectrometer/CCD; and (iv) a CCD-based Olympus BX-FLA epi-fluorescence microscope system with a Princeton Instruments model TE/CCD-1317-K with model ST-138 controller or an Optronics model QuantiFire 4-megapixel CCD.

Thin films were produced with a Specialty Coating Systems model P6700 spin coater. Thin films were formed by spin casting

(2000 rpm, 30 s) an 80 μ L aliquot of a particular sol solution onto clean glass microscope slides.

The pin printing protocol typically used a pin velocity of 5–50 mm/s and pin-to-surface contact time of 5–50 ms. Although the PIXIES feature size (diameter) depended on the printing conditions, the PIXIES analytical figures of merit did not appear to be affected by the pin velocity or the pin-to-surface contact time.

Xerogel feature sizes and thicknesses were determined by using field emission scanning electron microscope (SEM, Hitachi model S-4000). The pin printed and spin cast xerogels are 1–2 μ m thick depending on the actual formulation composition.

2.4. PIXIES fabrication protocol

There are several key challenges that one must overcome to develop molecularly imprinted polymer-based optosensors for selective protein detection. First, one must form the protein selective molecularly imprinted polymer. Second, one must place a luminescent reporter group in close proximity to the template site so as to effectively transduce the protein-molecularly imprinted polymer binding event. Figs. 2 and 3 outline the new PIXIES production protocol.

We begin the PIXIES formation process by reacting ATFA with BODIPY FL, SE in THF in the dark to form a luminophore-tagged aryl azide (**1**) (Fig. 2). **1** is subsequently used (see below) to luminescently tag the protein-templated sites within a molecularly imprinted xerogel.

The protein-templated xerogel is produced in a multi-step process (Fig. 3). We begin by preparing a *initial* sol solution (vide supra). We then mix the *initial* sol (1:1, v/v) with buffer (10 mM pH 7.0 phosphate). We then introduce the target protein

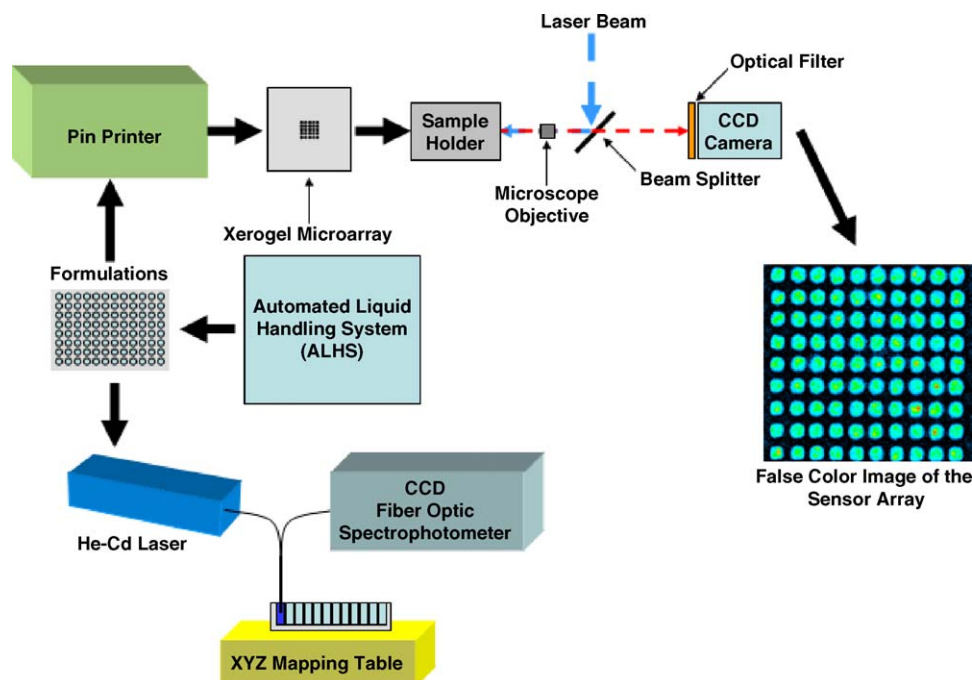


Fig. 1. Simplified schematic of the robotic sol production and xerogel screening system.

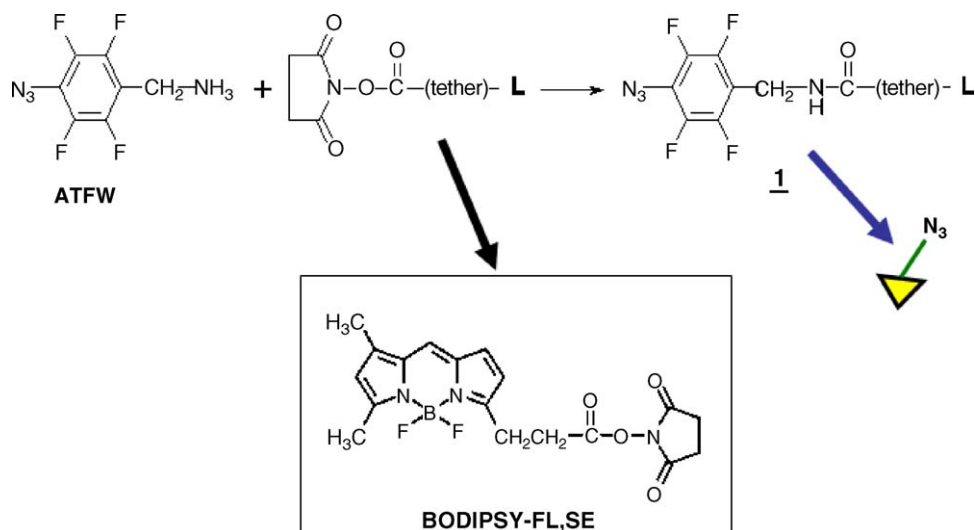


Fig. 2. Creation of the photoactivatable luminophore (L).

(ovalbumin was used as our initial protein target) from an aqueous buffer solution into this sol solution. The protein-to-Si molar ratio is typically between 1:250 and 1:1000. After allowing the sol solution to hydrolyze within a sealed vial for 1 h, we form the xerogel. For the screening experiments we pin print [21] the PIXIES sol solutions onto clean glass microscope slides and create an array of formulations. For analytical measurements we use the lead formulations from the screening experiments and we form spin cast PIXIES films onto glass microscope slides. We remove [36] the ovalbumin after the xerogels have formed (48 h in the dark at ambient temperatures) by using 5 M aqueous urea *or* dilute phosphoric acid.

To luminescently tag the ovalbumin-templated sites within the molecularly imprinted xerogels we prepare a 1:1 mixture of ovalbumin (micromolar protein) and **1**. Under these conditions, steady-state fluorescence anisotropy measurements show that more than 98% of **1** is ovalbumin bound. (*Note*: The association

between ovalbumin with **1** is not unique; there is a substantial literature on the binding of a wide variety of fluorescent “ligands” to proteins [51,52].) Thus, we are essentially using the protein target (ovalbumin in this specific example) to selectively deliver the luminophore (**1**) into the template site. We then immerse the ovalbumin-templated xerogel films in the ovalbumin–**1** solution, filling all accessible ovalbumin-templated xerogel sites. After 15 min, we removed the films from the ovalbumin–**1** solutions and illuminate the films with the filtered output ($\lambda < 360$ nm) from a 450–1000 W Xe arc lamp. (*Note*: A few PIXIES were fabricated by using the 325 nm output from a He–Cd laser for this task.) Photoillumination creates the aryl nitrene [51] which undergoes high efficiency C–H insertion into the xerogel template site. After being illuminated for 10 min, we then rinse the ovalbumin-templated xerogel films with aqueous buffer [36], 5 M aqueous urea *or* dilute phosphoric acid to liberate any ovalbumin and unreacted **1**. This washing step also removes any ovalbumin to which **1** may have reacted.

3. Results and discussion

Fig. 4 presents a partial set of screening results for 16 TEOS/APTES/C8-TMOS/HAPTS-based PIXIES. The actual composition for each formulation (labeled F1–F16) is listed in Table 1. Fig. 4A illustrates the numbering scheme for each formulation and PIXIES. Fig. 4B presents the false color CCD image from an array of PIXIES formulations in buffer ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} > 500$ nm). Fig. 4C is the same array in Fig. 4B when it is challenged with 50 μM ovalbumin in buffer. Fig. 4D is the F_{analyte}/F_0 results from each of the 16 formulations. Of the formulations shown here, no. 12 appears to be the most analytical useful. (*Note*: The donut shape of the pin printed features results from an un optimized printing conditions (e.g., humidity, temperature, printing solution viscosity, surface tension and density, pin geometry, wettability and surface chemistry, substrate wettability and surface chemistry, and pin velocity and pin-to-surface contact time).)

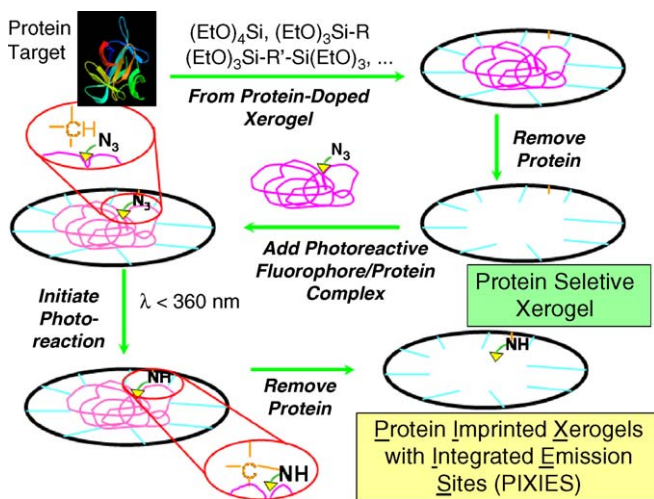


Fig. 3. The PIXIES fabrication process. This is *not* an exhaustive listing of R and R' groups that one could use. Examples of alkoxides that have been tested are listed in the text.

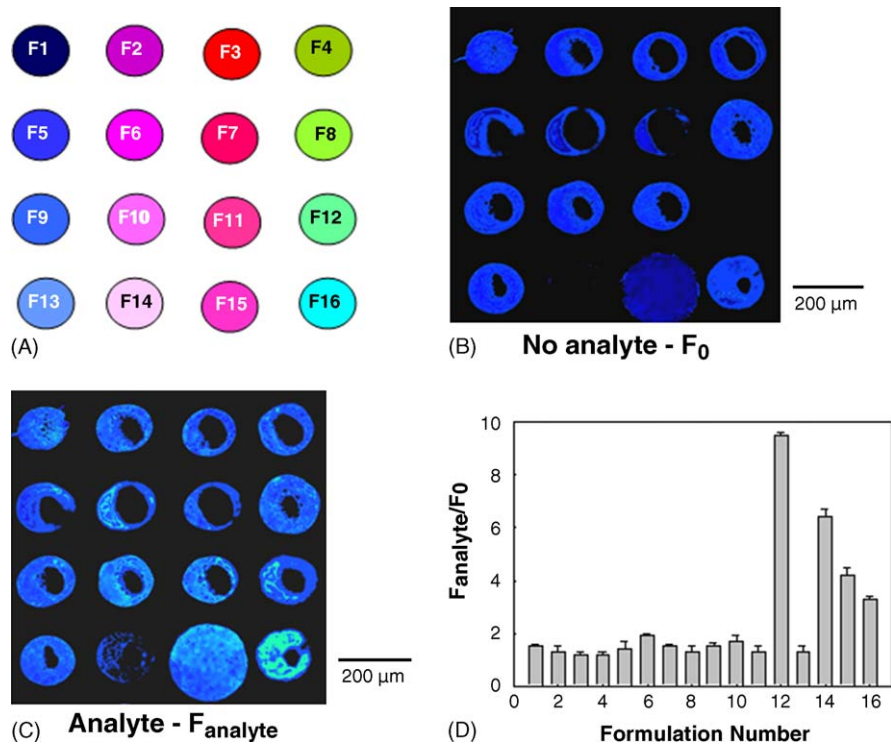


Fig. 4. Screening results from 16 TEOS/APTES/C8-TMOS/HAPTS-based PIXIES. The actual composition of formulation F1-F16 is given in Table 1. (A) Formulation numbering scheme. (B) False color CCD image from an array of PIXIES formulations in buffer ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} > 500 \text{ nm}$). (C) Same as (B) when challenged with $50 \mu\text{M}$ ovalbumin. (D) F_{analyte}/F_0 from (B) and (C). Formulation no. 12 appears to be the most analytical useful.

Fig. 5 presents a typical response profile from an ovalbumin-templated PIXIES film. The actual composition of this particular PIXIES-based sensor element is 55 mol% TEOS, 2% mol% APTES, 3 mol% C8-TMOS, and 40 mol% HAPTS. The molar ratio of ovalbumin to alkoxide Si was 1:750 and BODIPY FL was used as the luminescent reporter molecule. When we challenged these ovalbumin-templated PIXIES with ovalbumin we see that the luminescence increases. As an initial ovalbumin-templated PIXIES selectivity test we took a solution of ovalbumin, reacted it with a 15-fold molar excess of phenyl-SO₂Cl to block all the

accessible primary amines on ovalbumin, and we re-determine the PIXIES response to phenylsulfonamide of ovalbumin; there was no observable response over the concentration range tested.

In a second experiment we tested the ovalbumin-templated PIXIES selectivity by using human serum albumin (HSA) as an interfering species. The results show that an ovalbumin-templated PIXIES is selective for ovalbumin over HSA and the phenylsulfonamide of ovalbumin. We estimate that these specific PIXIES exhibits a selectivity factor (i.e., response to target analyte divided by response to an interferant at an equivalent concentration) greater than 200. When we challenged an ovalbumin-templated PIXIES with a ternary protein mixture

Table 1
Composition (in mol%) of the TEOS/APTES/C8-TMOS/HAPTS-based PIXIES formulations shown in Fig. 4

Formulation no.	TEOS	APTES	C8-TMOS	HAPTS
1	100	0	0	0
2	0	100	0	0
3	0	0	100	0
4	0	0	0	100
5	50	50	0	0
6	0	50	50	0
7	0	0	50	50
8	50	0	0	50
9	50	0	50	0
10	25	25	25	25
11	91	3	3	3
12	55	5	5	35
13	30	30	20	20
14	60	0	0	40
15	50	10	10	30
16	30	20	20	30

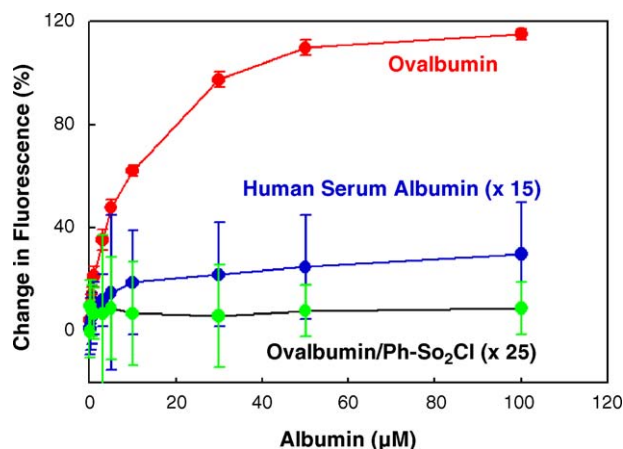


Fig. 5. Calibration curves for an ovalbumin-selective PIXIES. $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} > 510 \text{ nm}$.

that contained ovalbumin, the phenylsulfonamide of ovalbumin, and HSA, we observed responses that were equivalent only to the ovalbumin content of the samples ($\pm 5\%$). Together, these results illustrate our new PIXIES strategy as a means to develop luminescence-based sensors for protein detection and quantification.

We also carried out continuous flow experiments with an ovalbumin-templated PIXIES sensor by injecting plugs of ovalbumin followed by pure buffer. The response time (time to reach 90% of the maximum signal change) for 1.2 μm thick PIXIES-derived films was on the order of 70 s and the response is reversible ($\pm 4\%$) after more than 25 cycles. (*Note:* A cycle consists of flowing 0.01 M phosphate buffered saline past the sensor, injecting an aqueous protein containing sample in a stopped flow manner, waiting 1–2 min and recording the signal, and dissociating the protein-PIXIES association with 5 M aqueous urea or dilute phosphoric acid.) We also performed a Scatchard analysis on a series of PIXIES that we prepared by using different xerogel compositions. The results of these experiments are summarized in Table 2. These results clearly show that we can use the xerogel composition to tune the PIXIES response and selectivity.

We next questioned if we could use the PIXIES strategy to develop sensors for discriminating between two structural similar proteins. Toward this end we targeted human interleukin-1 alpha and beta ((h)IL-1 α and (h)IL-1 β) in human plasma. We also compared the PIXIES performance to results from standard ELISA kits for these same proteins (Amersham RPN 2750 for (h)IL-1 α and RPN 2751 for (h)IL-1 β). The results of these experiments are reported in Table 3. (*Note:* The PIXIES that were used in these experiments were identified after an abbreviated materials screening campaign [48,49] (256 xerogel formulations).) The composition of the (h)IL-1 α responsive PIXIES is 2 mol% TEOS, 22 mol% APTES, 27 mol% C8-TMOS, and 49 mol% HAPTS. The composition of the (h)IL-1 β responsive PIXIES is 15 mol% TEOS, 6 mol% APTES, 14 mol% C8-TMOS, and 65 mol% HAPTS. The results from these *partially* optimized PIXIES-based sensor elements are very promising with detection limits and selectivity factors within a factor of 2–3 of an ELISA kit and response times at least two orders of magnitude better in comparison to the corresponding ELISA.

We also performed recovery experiments on spiked (10 μM of (h)IL-1 α) human plasma samples. The recovery was 92%

Table 2
Recovered dissociation constants (K_d) for three ovalbumin (O)-templated PIXIES based on different precursor compositions

PIXIES composition ^a	K_d (nM) ^b			
	O	SO ^c		HSA
A	8	35	–	3000
B	2	19	79	352
C	18	125	56	650

^a [A] Same composition used for Fig. 5. [B] 15 mol% TEOS, 5 mol% APTES, 5 mol% C8-TMOS, and 75 mol% HAPTS. [C] 8 mol% TEOS, 14 mol% APTES, 7 mol% C8-TMOS, and 71 mol% HAPTS.

^b Where there are two entries this reflects two recovered dissociation constants.

^c Phenylsulfonamide of ovalbumin.

Table 3
Comparison of Amersham ELISAs and two PIXIES for the detection of (human)IL-1 α and (human)IL-1 β in human plasma

Template/analyte	Detection limits (pM)		Selectivity factor ^a	
	ELISA ^b	PIXIES ^c	ELISA	PIXIES
(human)IL-1 α	0.6	1.4	78:1 ^d	147:1 ^d
(human)IL-1 β	0.6	1.7	194:1 ^e	136:1 ^e

^a Signal of the target protein at five times the detection limit divided by the signal for an equivalent concentration of the interfering protein.

^b Assay time = 4 h.

^c Measurement time = 70 s.

^d (human)IL-1 β is the interfering protein.

^e (human)IL-1 α is the interfering protein.

Table 4
Comparison of antibody- and PIXIES-based cross reactivities^a

Target analyte	Antibody ^b		PIXIES ^b	
	Porcine	Human	Porcine	Human
(h)IL-1 α	0.13 \pm 0.03	1	0.22 \pm 0.04	1
(p)IL-1 α	1	0.18 \pm 0.04	1	0.15 \pm 0.04

^a In a cross reactivity experiment each system was challenged with 50 $\mu\text{g}/\text{mL}$ of (human)IL-1 α or (porcine)IL-1 α .

^b Observed response/response of intended target. A ratio of 1 means the target was tested. A smaller ratio implies less cross reactivity (e.g., a value of 0.18 means 18% cross reactivity).

for the Amersham RPN 2750 assay and 87% for the (h)IL-1 α -templated PIXIES. The recovered PIXIES analytical figures of merit are as follows: (i) ~ 2 $\mu\text{g}/\text{mL}$ detection limits; (ii) < 2 min response times; (iii) > 85 selectivity factor; (iv) $< 6\%$ R.S.D. long term drift of the PIXIES-based sensor elements when stored for up to 16 weeks under ambient conditions in the dark; and (v) $> 95\%$ reversibility after 25 use/reuse cycles.

Finally, we questioned if we could use our PIXIES strategy to develop sensors to discriminate between a particular protein (IL-1 α) from different animal species (human and porcine, homology = 66%). Preliminary results are presented in Table 4. These results demonstrate that the PIXIES-based platform provides a means to discriminate between the same proteins from different animal species.

4. Conclusions

We report a new biomolecule-less sensor platform for the selective detection and quantification of proteins. We have termed these new sensor elements protein imprinted xerogels with integrated emission sites. The PIXIES platform is based on tailored xerogels, molecular imprinting, and site-selective installation of a luminescent reporter molecule within the template site to form highly selective protein-responsive sensor elements without the use of a biorecognition element within the xerogel. Robotic liquid handlers, pin printing, and imaging systems are used to produce a wide variety of xerogel-based formulations and screen these formulations to identify those with the most desirable analytical figures of merit. The PIXIES-based sensors exhibit analytical figures of merit akin to an antibody-based

assay, they are faster in comparison to an antibody-based assay, and the sensor elements are substantially more robust compared to an antibody assay.

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