Biomolecular screening with novel organosilica microspheres

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Organosilica microspheres synthesised *via* a novel surfactant-free emulsion-based method show applicability towards optical encoding, solid-phase synthesis and high-throughput screening of bound oligonucleotide and peptide sequences.

With the completion of the first draft of the human genome sequence, there has been an exponential increase in the amount of biological sequence information. This has led to a high demand for biomolecular screening strategies in genomics, proteomics and drug discovery. Important biomolecular interactions include those between ligands and receptors, enzymes and substrates, and oligonucleotides and their complementary sequences.

A recently developed biomolecular screening strategy utilises microspheres encoded with a unique combination of fluorescent dyes, which can be decoded using flow cytometry.[1] Immobilising specific probe molecules on the surface of the microspheres, and fluorescently-labelling either the probe or its interacting partner, allows their interaction to be measured as a change in fluorescence.[2]

There are several key criteria that such microspheres need to satisfy in order to be suitable for the proposed strategy. Monodispersity of size and uniform structure are important for reducing variability in bioassays and facilitating the use of simple statistics to quantify target loading. Covalent attachment of fluorescent dyes is preferred over entrapment to ensure that dyes cannot leach out of the particles during synthesis or assaying of biomolecules on the surfaces of the microspheres. Utilising two chemically distinguishable functional groups, *e.g.* thiols and amines, provides separate pathways for dye or biomolecule attachment.

Different methods for incorporating functional groups into silica microspheres have been developed. One approach first produces a monodisperse population of non-functionalised silica microspheres using the method of Stöber *et al.*, followed by synthesis of a functionalised shell.[3] Another approach involves the hydrolysis and condensation of organosilica precursors to form polydisperse droplets, which are then cross-linked by exposure to ammonia.[4]

In this letter we report a method for the production of thiol-functionalised organosilica microspheres with a narrow size distribution. We show the suitability of these microspheres for optical encoding with fluorescent dyes and their use in biomolecular screening applications. We further compare the stability of dyed organosilica microspheres and polystyrene–divinylbenzene microspheres towards phosphoramidite oligonucleotide synthesis reagents.

Thiol-functionalised organosilica microspheres were synthesised *via* a two-step process involving acid-catalysed hydrolysis and condensation of 3-mercaptopropyltrimethoxysilane (MPS) in aqueous solution, followed by base-catalysed condensation.[5,6] Fig. 1 shows microscopy images of the MPS solution before and after addition of a base catalyst, triethylamine (TEA). Under basic conditions, the linear oligomers produced during the acid-catalysed condensation are rapidly cross-linked and precipitate to form micron-sized emulsion droplets (*cf.* dispersion polymerisation of organic polymers). Due to the brief nucleation period and negative surface charge under basic conditions ($\zeta_{\mathrm{pH\ 9}} = -65$ mV),[5] the emulsion droplets have a narrow size distribution.

The emulsion droplets continue to condense to form solid uniform microspheres of a similar size distribution (Fig. 2a). The size distribution is multi-modal due to the presence of larger microspheres resulting from the coalescence of two or more emulsion droplets at an earlier stage. Average size of the microspheres formed is dependent on the initial concentration of MPS, the time period of acid-catalysed condensation and the choice of base catalyst. The microspheres are sufficiently solid (Fig. 2b), with solid-state ^{29}Si NMR spectroscopy showing 83% of the maximum possible siloxane bonds have formed.[5]

To demonstrate the applicability of the microspheres for optical encoding, the thiol-reactive dye Alexa Fluor 546 succinimidyl ester was added to a population of microspheres. After removal of unreacted dye, confocal fluorescence microscopy shows the dye molecules have covalently bound to free thiol groups distributed throughout the interior of the microsphere (Fig. 2c). A range of other thiol-reactive dyes, *e.g.* isothiocyanates, succinimidyl esters and maleimides, have been similarly incorporated. This indicates that the core of the silica particles is highly accessible. Conversely, the synthesis of an amine-functionalised shell using 3-amino-propyltriethoxysilane (APS) makes the particles more impermeable to the dye molecules, resulting in the dye being localised within a surface layer of the particles (Fig. 2d).

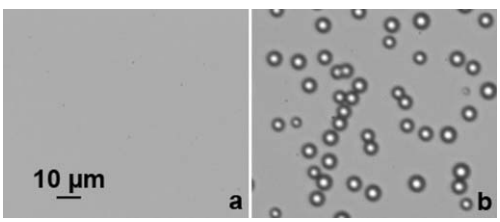

Fig. 1 *Formation of emulsion droplets.* a) Aqueous MPS solution after acid-catalysed hydrolysis and condensation. b) Micron-sized emulsion droplets are rapidly formed upon addition of a base catalyst, TEA.

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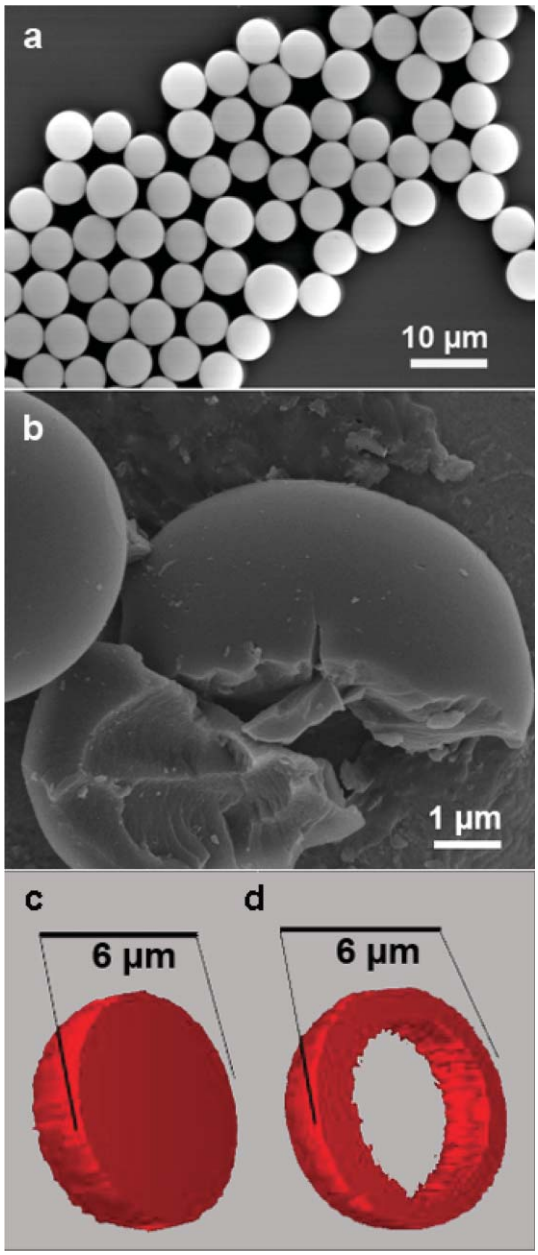

Fig. 2 *Organosilica microspheres formed by condensation of emulsion droplets*. Scanning electron micrographs of a) a representative population of microspheres, and b) a high-resolution image of a crushed microsphere, suggest a solid uniform structure. Z-stacks of confocal fluorescence microscopy images clearly show thiol-bound Alexa Fluor 546 dye c) evenly distributed throughout the interior of non-functionalised microspheres, or d) localised within a surface layer in APS-modified microspheres.

To further improve the stability of covalently-bound dye, ATTO 550 maleimide was coupled to free thiol groups of the organosilica microspheres. The thioether bonds thus formed possess greater chemical stability than thioesters or dithiocarbamates formed from succinimidyl esters or isothiocyanates, respectively. The ATTO 550-labelled organosilica microspheres displayed excellent retention of fluorescence intensity when individually subjected to each of the reagent steps involved in phosphoramidite DNA synthesis (Fig. 3).[7] In contrast, commercially available polystyrene–divinylbenzene microspheres,

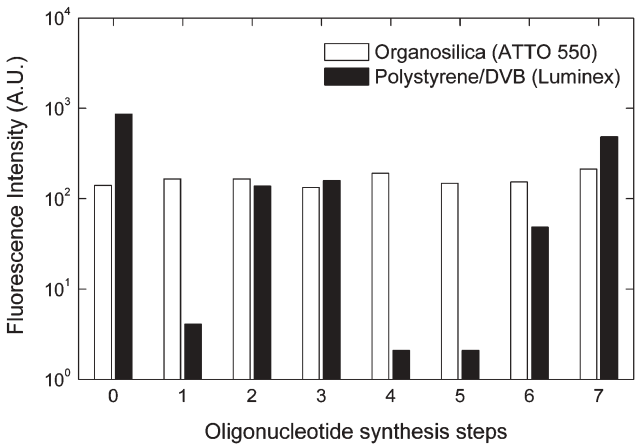

Fig. 3 *Stability of optical encoding towards oligonucleotide reagents*. Organosilica microspheres covalently labelled with ATTO 550 dye are stable towards each of the reagents used in phosphoramidite oligonucleotide synthesis.[7] In contrast, optically-encoded polystyrene–divinylbenzene (DVB) beads are unstable in most steps, in particular those involving dichloromethane and tetrahydrofuran.

which contained entrapped non-covalently-bound fluorophores, lost significant fluorescence intensity under most conditions.

Two separate biomolecular screening assays were performed with APS-modified organosilica microspheres: a hybridisation assay between oligonucleotide probe-coupled microspheres and a fluorescently-labelled target sequence (Fig. 4a),[8] and a protease screening assay on microspheres containing a peptide sequence with a terminal fluorescent label (Fig. 4b).[9] Both assays were analysed using flow cytometry;[10] in the case of the hybridisation assay, an increase in on-bead fluorescence represented bound target sequence, whereas for the protease screen, a decrease in on-bead fluorescence represented cleavage of the recognition site (and hence the label) by the enzyme.

The hybridisation assay demonstrated that the probe-coupled microspheres can detect a DNA target sequence with high specificity. The complementary duplex showed greater than a 100 : 1 ratio of fluorescence intensity in comparison to the completely mismatched duplex (Fig. 4c). Furthermore, the fluorescent intensity for the single base mismatch (denoted by **A/T** in the sequence[8]) was reproducibly 20% lower than that of the complementary duplex – a vital requirement for single nucleotide polymorphism (SNP) applications.

Results from the protease screening assay demonstrated that peptide sequences containing lysine (AKAAKA) are recognised and cleaved by the lysine/arginine-specific trypsin, resulting in a 67% loss of peptide-bound fluorophore compared with the control samples (Fig. 4d). As expected, microspheres displaying the serine-containing peptide (*i.e.* ASAASA) show a negligible decrease in on-bead fluorescence, indicating little or no proteolysis has taken place. These results suggest the organosilica microspheres can be used as solid supports for the production and screening of peptides synthesised by the Fmoc peptide synthesis strategy.

Organosilica microspheres suitable for optical encoding have been synthesised *via* a novel emulsion-based method. Unlike optically-encoded polystyrene microspheres, the covalent attachment of thiol-reactive fluorophores *via* formation of a

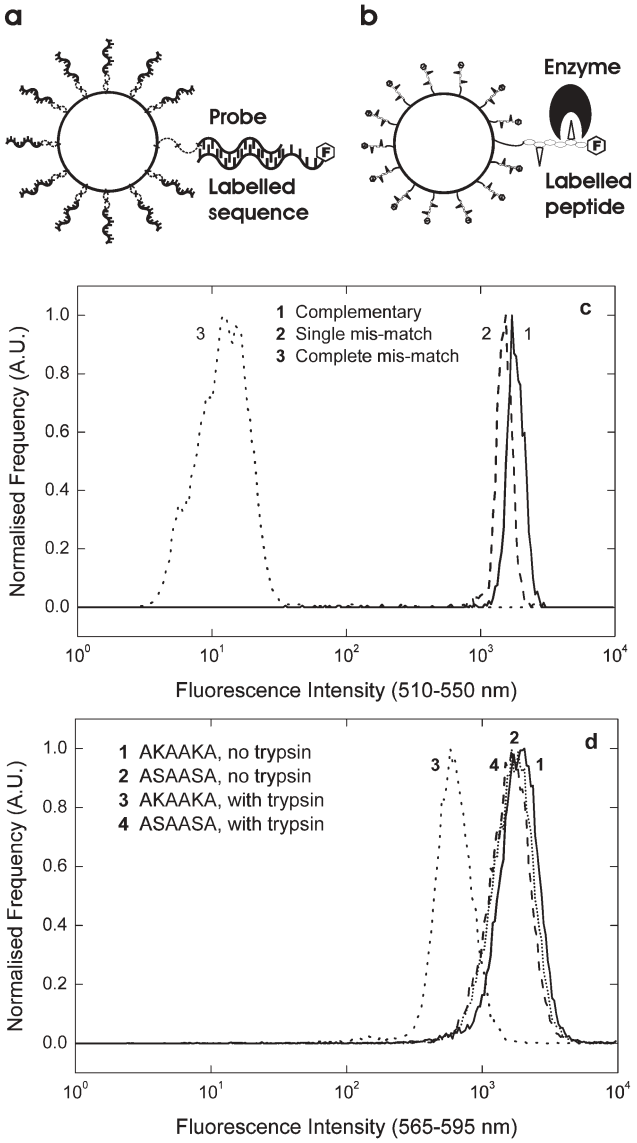

Fig. 4 *Biomolecular screening*. Biomolecular interactions can be detected by a) hybridisation of fluorescently-labelled target sequences to bound probe molecules, or b) cleavage of bound fluorescently-labelled peptide sequences. Using organosilica microspheres with bound probe molecules, c) complementary duplexes are distinguishable from single mismatch duplexes, and easily separable from complete mismatch duplexes, while d) lysine-containing peptide sequences (AKAAKA) are cleaved by trypsin, resulting in a 67% decrease in on-bead fluorescence, compared to negative (ASAASA) and control samples.

thioether bond proved to be stable towards phosphoramidite oligonucleotide synthesis reagents and common organic solvents. These organosilica microspheres have also been successfully used as solid supports in bioassays involving oligonucleotide probes and peptide sequences.

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Notes and references

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6 Particle synthesis: 10 ml of 3-mercaptopropyltrimethoxysilane (MPS) (95%, Lancaster) was added to a stirred solution of 1.6 ml of 0.1 M HCl and 80 ml of MilliQ water. After 18 hours, the aqueous phase was separated using centrifugation. 100 µl of triethylamine (TEA) (99%, Sigma) was then rapidly injected into the stirred aqueous solution. After 30 minutes, the microspheres thus formed were washed multiple times *via* centrifugation and resuspension in ethanol using ultrasonication. Amine-functionalised microspheres were obtained by further reaction with 10 µl of 3-aminopropyl triethoxysilane (APS) (97%, Aldrich), 50 µl of 28% ammonia, and 940 µl of ethanol for 90 minutes.
7 Optical encoding stability: organosilica microspheres (5.7 µm) were covalently labelled with ATTO 550 maleimide dye (ATTO-TEC) in tetrahydrofuran (THF). Optically-encoded polystyrene–divinylbenzene microspheres (5.6 µm) were obtained from Luminex Corporation (Austin, TX). Both types of microspheres were washed three times in acetonitrile (ACN) and individually subjected (*i.e.* not consecutively) to DNA synthesis reagents (Beckman) for 16 hours at room temperature: Step 0: as received; Step 1: 5% dichloroacetic acid in dichloromethane; Step 2: 0.5 g adenosine phosphoramidite monomer in 10 ml ACN; Step 3: 4% tetrazole in ACN; Step 4: 10% acetic anhydride, 10% 2,6-lutidine, 80% THF; Step 5: 17% *N*-methylimidazole, 83% THF; Step 6: 1–3% iodine, 1–3% water, 5–25% pyridine, 70–90% THF; Step 7: 25% ammonia. Samples were prepared for flow cytometric analysis[10] by washing three times with ACN, once with ethanol, and three times with 0.2% sodium dodecyl sulfate (SDS).
8 Hybridisation assay: APS-modified microspheres were transferred into THF, and reacted with 5 mg of adipic acid (99%, Aldrich) and 4 µl of diisopropyl carbodiimide (DIC) (99%, Aldrich) for 2 hours. The coated microspheres were transferred into ACN and split into three batches. Each batch was reacted with 4 µl DIC and 5 µl of a 50 µM solution of one of three oligonucleotide probes (5′-NH_2-TACTCATTA-TTTTGTGTGTG**A/T**TGGCTGGATTATTGGAAC-3′, 5′-NH_2-TACTCATTATTTTGTGTGTG**T**TGGCTGGATTATTGGAAC-3′ or 5′-NH_2-ATGAGTAATAAAACACACACTACCGACCTAA-TAACCTTG -3′) (Geneworks, SA) for 2 hours. 5 µl of probe-coupled microspheres were combined with 1 µl of a 1 µM fluorescently-labelled target sequence (3′-ATGAGTAATAAAACACACACTACCGAC-CTAATAACCTTG-FITC-5′) in a buffer solution (70 µl of 600 mmol sodium chloride, 60 mmol sodium citrate and 1% SDS). Each sample was incubated in the dark at 53 °C and shaken for 60 minutes. Samples were left to stand at room temperature for 10 minutes before flow cytometric analysis[10].
9 Protease screen: non-functionalised microspheres were stabilised by treatment with concentrated aqueous ammonia, and then APS-modified as above. A bi-functional polyethylene glycol (PEG) spacer (*O*-(*N*-Fmoc-2-aminoethyl)-*O*′-(2-carboxyethyl)-undecaethylene glycol) and peptides with the amino acid sequences AKAAKA or ASAASA were then synthesised on the microspheres using Fmoc chemistry. Amino acids, PEG spacer and activating reagents were obtained from Novabiochem. The fluorescent dye, TAMRA (Molecular Probes), was attached to the amino terminus of the peptides. After deprotection by trifluoroacetic acid, samples were washed with 50 mM ammonium carbonate/0.02% Igepal buffer solution. 125 µl of a 20 µg/ml trypsin solution was added to each sample and incubated at 37 °C for 1 hour. Control samples were incubated in a trypsin-free buffer solution. Each sample was washed once in 1% SDS and stored at 4 °C until flow cytometric analysis[10].
10 Flow cytometry: all samples were analysed by a multi-laser flow cytometer (MoFlo, DakoCytomation Inc.); 100 mW 488 nm argon ion laser: FITC (530/40 nm), TAMRA (580/30 nm), ATTO 550 (580/30 nm); 12.5 mW 633 nm diode laser: Luminex (670/30 nm).