Quantitative Analysis and Characterization of Biofunctionalized Fluorescent Silica Particles

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A strategy for the production and subsequent characterization of biofunctionalized silica particles is presented. The particles were engineered to produce a bifunctional material capable of both (a) the attachment of fluorescent dyes for particle encoding and (b) the sequential modification of the surface of the particles to couple oligonucleotide probes. A combination of microscopic and analytical methods is implemented to demonstrate that modification of the particles with 3-aminopropyl trimethoxysilane results in an even distribution of amine groups across the particle surface. Evidence is provided to indicate that there are negligible interactions between the bound fluorescent dyes and the attached biomolecules. A unique approach was adopted to provide direct quantification of the oligonucleotide probe loading on the particle surface through X-ray photoelectron spectroscopy, a technique which may have a major impact for current researchers and users of bead-based technologies. A simple hybridization assay showing high sequence specificity is included to demonstrate the applicability of these particles to DNA screening.

1. Introduction

Particle-based libraries are becoming increasingly important for sensitive, reproducible diagnostic applications.[1,2] Characterization and quantification of functional groups is extremely important to provide an accurate description of the final surface of the particle. Such a procedure is important both in research and in industry to instigate a reliable quality assurance process for the materials produced. The surface modification strategy must produce a particle library that (a) allows attachment of a specific number of biomolecules, (b) incorporates a separate method of encoding the particles so as to track the reaction history of the library elements, and (c) allows calculation of the number of probes bound to the particles.

The sensitivity of a DNA bioassay is largely dependent on the accessibility and concentration of the oligonucleotide probes.[3,4] Research underpinning the development of microarray platforms has shown that when biomolecules are separated from the support by means of a short alkyl chain, the sensitivity of the resulting bioassay is increased.[3,4] Shchepiniv and Southern found that the most important factor impacting the sensitivity of DNA detection was the separation distance from the array in preference to the chemistry of the linker.[5] This is most likely due to steric effects when the probes are positioned too close to the surface of the array. As the distance between the probes and the surface is increased, the accessibility of the probe to the target increases, and the kinetics approach those observed in liquid-phase hybridization reactions. Evidently, insufficient probe concentration will result in a poor signal, but very low hybridization efficiencies have been reported for high-density surfaces. Peterson et al.[3,4] suggest steric hindrance as a reason for this phenomena rather than kinetic effects, indicating that no matter how the hybridization conditions are changed, the surface chemistry must be altered to gain the correct probe density for maximum fluorescent signal. For this reason, it is vital to have absolute control over the surface chemistry of the solid support, regardless of the nature of that support (i.e., array or particle library).

Particle-based libraries developed for complex biological screening require that each particle (or set thereof) include both a biomolecule attachment protocol and a unique encoding strategy to identify that biomolecule. A number of encoding techniques have been developed for a variety of applications including particle-based systems,[2,6,7] electrochemically active metallic rods,[8] and high-density microarrays.[9] Fluorescent encoding is suited to high-throughput screening when combined with advanced analytical tools such as high-performance flow cytometry and high-speed fluorescence microscopy.[7] The incorporation of fluorophores into particles to make them optically distinguishable has been reviewed.[6] A number of materials have been used for such platforms, including polymeric,[10] silica,[11] and magnetic[12] particles. Silica particles are very attractive for these applications, as they are stable in most solvent environments. For this reason, encoded libraries of colloidal silica have been highlighted as a new generation of optical biosensor applications.[7,11,13−19] Ver-

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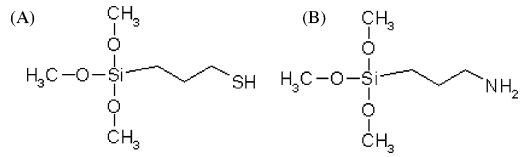

Figure 1. Molecular structures of (A) MPS and (B) APS.

haegh and van Blaaderen first produced fluorescent silica colloids for tracing experiments by coating Stöber particles in a dye-conjugated 3-aminopropyl trimethoxysilane (APS) layer.[20] A different class of particles were produced by Johnston,[21] which were microporous, enabling diffusion of dyes into the silica core and covalent attachment via functional thiol groups. These microporous particles exhibit a high degree of functional silanol loading, allowing a variety of biomolecule attachments.[21]

Micron-size silica particles were first synthesized by Stöber et al. in 1968 [22] via base-catalyzed hydrolysis and condensation of tetraethyl orthosilicate (TEOS). These "Stöber particles" have been thoroughly characterized, and extensive studies into the nucleation and growth mechanisms have been performed.[20,23,24] In a separate approach, microemulsion techniques have also been developed as sophisticated synthetic routes for the production of a wide array of hybrid "organosilica" particles. This is a versatile strategy due to the range of silanes, surfactants, and catalysts available.[25,26] Recently, investigations from our group[27] have produced micron-sized dispersions from acid-catalyzed oil-in-water microemulsions using 3-mercaptopropyl trimethoxysilane (MPS, Figure 1). The current study explores the suitability of the MPS particles as platforms for oligonucleotide attachment and simple biological screening.

Silica particles possess a high silanol concentration that allows a wide variety of surface reactions to facilitate biomolecule attachment. The incorporation of both a short alkyl chain and an oligonucleotide onto the particle surface requires a simple organic synthesis strategy to be developed. Organosilane coatings involving APS produce a high concentration of primary amine groups,[28,29] enabling reactions with acid groups via carbodiimide chemistry. APS coatings have previously been developed for ceramic electrode coatings and building materials and in light-scattering research.[24,30] APS is a unique molecule in that it has been reported as having virtually limitless solubility in basic solutions;[24] however, it will precipitate onto fused or hydrated silica substrates, generating a high concentration of reactive primary amine groups.[31−34] The specific mechanism of APS surface modification on silica particles is a controversial topic in the literature; however, it is generally accepted that the process involves condensation of the acidic surface-silanols and the basic APS−silanol groups due to acid−base interactions.[35,36] In our study, APS was used to modify the surface of the MPS particles, thus generating a high yield of surface primary amines. The resultant APS modification is stable in both acidic and basic conditions.[34] It is vital to develop a reliable method for determining the APS, spacer, and oligonucleotide probe concentrations on the particle surface to establish quantitative bioassays. Such quantitative data assist both the development and the optimization of these assays and establish reproducibility and robust error analysis in biological screening studies. Current methods for analyzing the concentration of functional groups and DNA probes on particle surfaces involve indirect methods such as spectrophotometric[37] or fluorimetric[38] analysis of specific reporter molecules that are chemically lysed from the functional group of interest. However, data obtained from these techniques can be confounded by nonspecific adsorption of the lysed DNA, either on the particle or cuvette surface, and the extinction coefficients for calculation of Beer's law will vary greatly depending on the solvent environment. There are few elemental analysis techniques available that are suitable for particle surfaces. X-ray photoelectron spectroscopy (XPS) is a technique widely used to determine the surface structure of planar SiO_2 films;[31,33,34,39] however, its use for investigating spherical surfaces is limited. The silanol/siloxane ratio cannot be determined because of overlapping spectra,[39] but this method provides an extremely useful platform to investigate surface modifications of the silica particles. Structural and chemical characterization of various functional groups at the surface is important to ensure that the desired surface is produced after each modification. XPS provides useful data in two ways: (1) it provides qualitative information about the surface environment and chemical interactions, and (2) it provides semiquantitative data that provides relative atomic ratios of the chemical species involved at that surface. The technique has previously been used to determine the structure of self-assembled organosilane monolayers at hydrated and fused silica surfaces for applications ranging from ceramic electrode coatings to the stationary phase for chromatographic studies.[32] To our knowledge, this is the first investigation into the application of XPS to determine the DNA concentration on particles for quantitative hybridization assays.

The synthesis and structural characterization of the MPS particles used in this study have been published elsewhere,[21,27] where the particle morphology and mechanism of particle formation is described. To enable biomolecule attachment, subsequent chemical modification of the particles is required to attach oligonucleotide probes to the particle. This process comprises several steps, namely, (i) APS coating to provide a high concentration of primary amines, (ii) attachment of short alkyl chains to separate the oligonucleotide sequence from the particle surface (and provide a functional carboxylic acid group), and, finally, (iii) attachment of the specific oligonucleotide sequence. Furthermore, it has been demonstrated that the incorporation of fluorescent dyes into the particle does not affect the efficiency of the surface modifications described above, and vice versa. We describe here the characterization of the particles during surface modifications with the goal of quantifying the concentration of functional groups on the available surface. From

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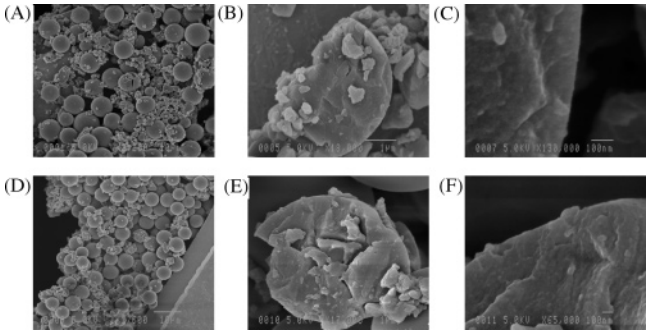

Figure 2. SEM images of MPS particles. (A−C) Uncoated particles. (D−F) APS-coated particles. Samples were partially fractured to show internal morphology.

these results, the applicability of the particles for biological assays will be discussed.

2. Results

2.1. Particle Morphology. To enable calculation of the surface concentration of functional groups, the accessible surface area was established. Scanning electron microscopy (SEM) images revealed relatively smooth, continuous outer surfaces with low surface roughness. To check for porosity, particles were fractured and investigated under high-resolution SEM, but the internal surfaces also revealed a solid, continuous morphology. (Figure 2A−C). Note that there were small particles visible under SEM, which were present only upon fracturing the larger MPS particles, and are assumed to be a product of the fracture process. Therefore the total surface area available for modification was calculated assuming a hard-sphere model and excluding surface roughness and internal porosity:

$$\mathrm{TSA} = \pi N_p d_p^{\,2} \qquad (1)$$

where TSA = total surface area, d_p = particle diameter, and N_p = particle concentration. The size distribution of the MPS particles, determined via a Coulter counter, can be described by a unimodal distribution (Figure 3) with mean size $\mu = 6.3\ \mu$m and standard deviation $\sigma = 1.8\ \mu$m. Similarly, the particle concentration, determined by hemocytometer counting, can be described by a Gaussian distribution with quadratic variance, $N(\mu, \mu + 0.012\mu^2)$,[40] with $\mu = 1.29 \times 10^8$/mL.

2.2. Impact of Encoding Dyes on Surface Properties. For two populations of fluorescently dyed particles, the impact of dye inclusion on the net surface charge of the particles was monitored by measuring the ζ potential. The structures of these dyes are illustrated in Figure 4. A change in surface functionality would be evident as a shift in ζ potential from that of the untreated control particles. The ζ potential values for dyed and nondyed particles (shown in Table 1) indicated that the presence or absence of dyes made only a small, or no difference in the overall surface charge of -45 ± 5 mV in MilliQ water. It was concluded that incorporation of the dyes did not detrimentally affect particle stability or APS surface modification.

The concentration of primary amine groups on the particle surface as a result of APS modification was determined by the ninhydrin test. The test has had widespread use in solid-phase peptide chemistry and is a fast, simple, colorimetric method for quantifying primary amines in particle suspensions.[41] Positive

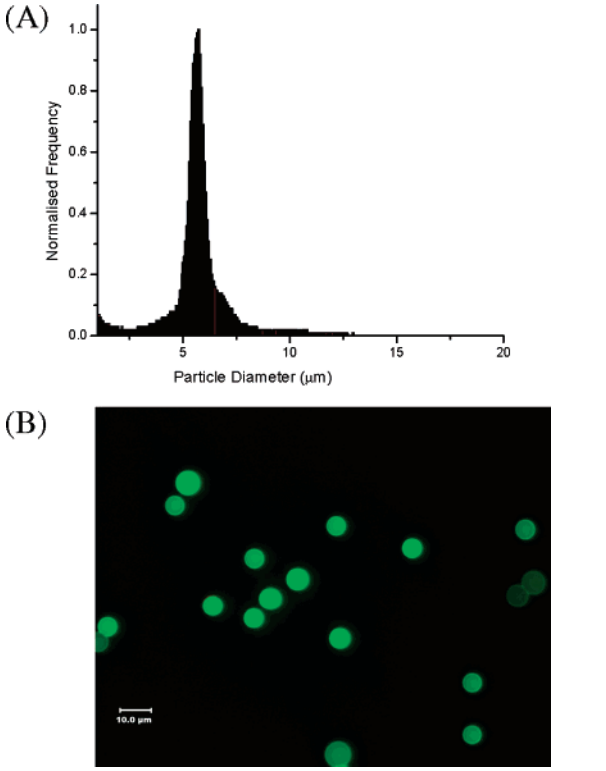

Figure 3. MPS particle characteristics. (A) Frequency histogram showing size distribution as measured by the Coulter method. (B) Fluorescence micrograph of A546-dyed MPS particles.

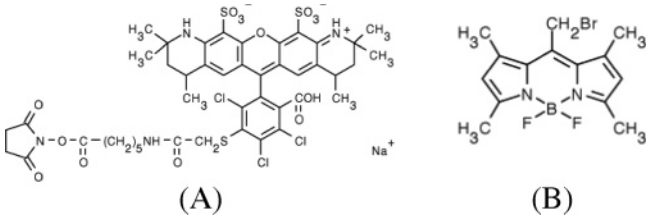

Figure 4. Structure of fluorescent dyes used for ζ potential and staining investigations: (A) A546; (B) B493.

Table 1. ζ Potential Measurements for Particles with and without Fluorescent Dyes Incorporated

sample description	ζ potential (mV)
untreated particles	-46.4 ± 4.9
particles + A546	-40.4 ± 2.3
particles + B493	-47.8 ± 5.2

controls are vital in determining the validity of the final data, so standard concentrations of ethanolamine were used to establish reproducibility and to determine the molar absorption coefficient for Beer's law calculations. The primary amine concentration on the surface of the particles was established to be independent of dye presence. This confirms that dyes do not impact APS coating within the reported error of the techniques (Figure 5).

2.3. APS Surface Coating. Ninhydrin test data alone do not provide any insight into the morphology or uniformity of the APS coating. SEM revealed spherical particles with smooth surfaces (Figure 2). The particles were again fractured and subjected to high-resolution SEM to discern whether the APS coat had introduced a morphological change. No surface artifacts or extra layers were evident in the APS-modified particles when compared to the unmodified particles. If indeed there is a coating present, it is continuous around the particle surface, ensuring an even surface coverage and constant APS surface density.

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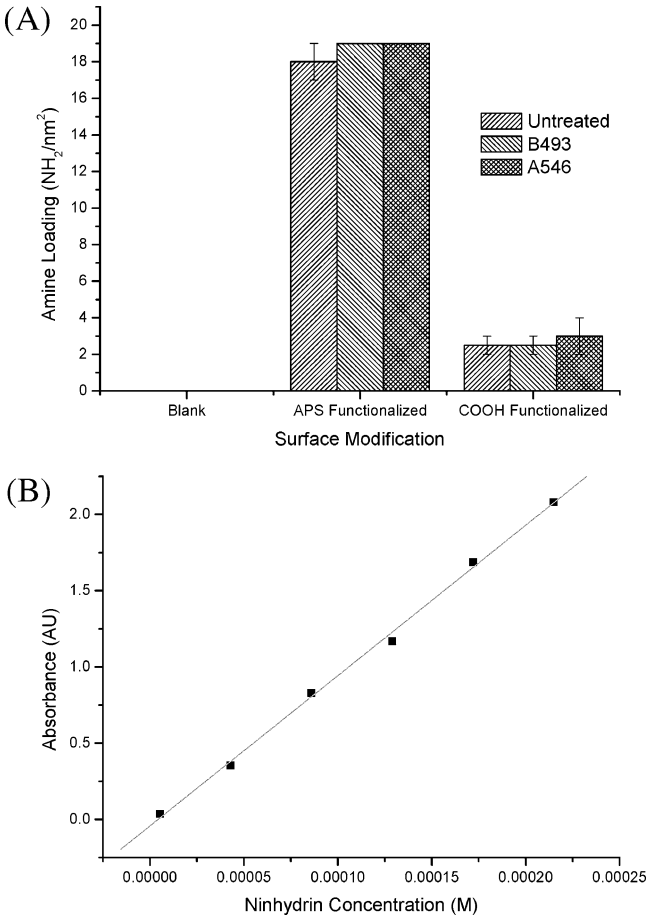

Figure 5. Results from the ninhydrin test. (A) Amine loading on particles as a function of incorporated fluorescent dyes. (B) Ninhydrin test calibration using ethanolamine standards.

The MPS particles were exposed to a fluorophore (A546) both in the absence and in the presence of an APS coat to visualize the extent and localization of the APS coat. The succinimidyl esters on the dye molecules react much faster with primary amines than with primary thiols,[42] so that, under the right conditions, the dyes can react exclusively to the amines. This produced a fluorescent shell around the particles, and it is proposed that this fluorescent shell is indicative of the localization and extent of APS coating. Figure 6 shows the comparison between APS-coated and uncoated particles, showing (a) a complete coat around the particle and (b) dye molecules localized on the particle surface as a result of attachment to the APS shell.

2.4. Grafting of Adipic Acid Linker onto Particle Surface. Adipic acid is a six-carbon (6-hexanedioic acid) linker used to separate the oligonucleotide probes from the surface (Figure 7). Carbodiimide chemistry was used to covalently attach the carboxylic acid group of the diacid to the amine functionality on the particles, thus reducing the free primary amine group. The primary amine concentration was also measured after adipic acid modification by the ninhydrin test as an indirect measure of adipic acid concentration. The comparative loss of amine groups after adipic acid modification suggested an ~80% conversion of amine functional groups to carboxylic acid functional groups.

2.5. XPS Analysis of Surface Structures. The XPS analysis of planar MPS surfaces has been reported previously,[43] and the

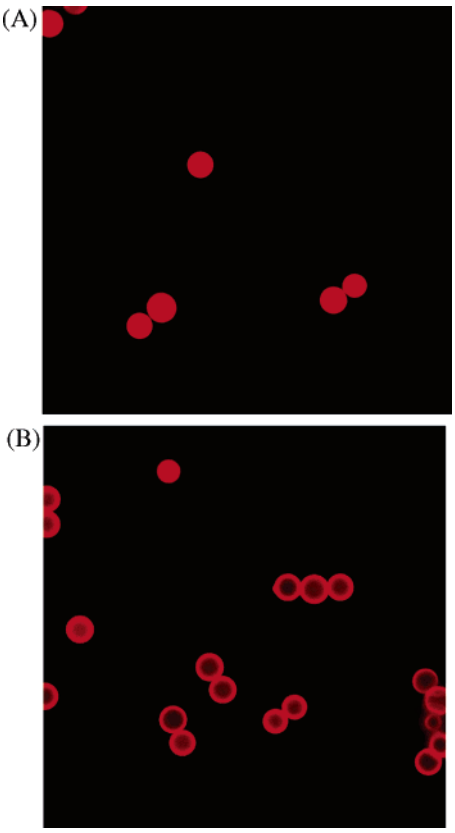

Figure 6. Confocal microscopy images of (A) A546 dyed particles and (B) APS-coated particles dyed with A546.

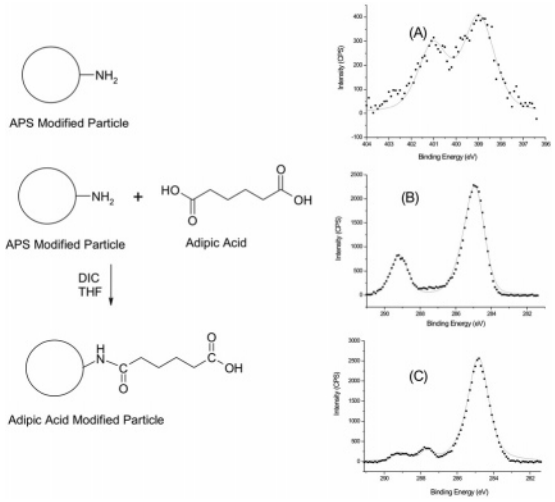

Figure 7. Reaction scheme for adipic acid grafting to produce a carboxylated surface. Insets are N1s spectra for (A) APS-modified particles (399/401 eV; see text for details); and C1s spectra for (B) pure adipic acid and (C) particle surface functionalized with adipic acid, illustrating the amide carbon (287.2 eV) and the carbonyl carbon (289.1 eV).

data presented here (Table 2) supports these observations. A characteristic double peak is apparent (399/401 eV) in the N1s spectra as a result of APS treatment. This peak has been attributed to a mixture of primary amines and protonated amines because of the basic conditions of the APS modification reaction.[31,33,34,44] XPS characterization of both the adipic acid precursor and the adipic acid-modified particles support the proposed surface structure illustrated in Figure 7. In both samples, the large peak

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Table 2. Atomic Ratios of the Elements Sulfur (S2p), Silicon (Si2p), Oxygen (O1s), Nitrogen (N1s), and Carbon (C1s) Determined through XPS Analysis

	MPS uncoated		APS modified		adipic acid modified		DNA modified	
	binding energy (eV)	RAP[a] (%)	binding energy (eV)	RAP (%)	binding energy (eV)	RAP (%)	binding energy (eV)	RAP (%)
S2p	163.2	10.8	163.2	6.4	163.2	4.5	163.2	4.3
Si2p	102	12.6	102	11	102	7.8	102	7.4
O1s	532	26.1	532	22.8	532	23.5	532	21.6
N1s			399/401	3.2	399	3.3	399	6.6
C1s	285	50.4	285	56.5	285	60.9	285	60.2

[a] Relative atomic percentage.

Table 3. Key Parameters for Calculation of DNA Loading

parameter	APS modified	DNA modified
Si/N[a]	3.4:1	1.1:1
concentration (N/nm^2)[b]	17	52.5
concentration (species/particle)[c]	2.3×10^9	4.7×10^7

[a] Ratio of silicon to nitrogen in terms of RAP. [b] Nitrogen atoms per square nanometer. [c] Species refers to either APS or DNA; as explained in the text, the DNA concentration is normalized for the number of nitrogen atoms in one molecule of the oligonucleotide.

at 285.0 eV corresponds to a combination of hydrocarbon contamination[43] and the C−C bonds from the alkyl chain. The free carboxylic acid groups are visible at 289.1 eV on both samples, but the modified sample shows both the carboxyl and the amide functionalities in the same ratio, with the latter peak at 287.2 eV. A small peak was also visible at 286 eV, corresponding to another source of contamination, most likely C−O bonds left as residue from the tetrahydrofuran (THF) solvent.[43] As further evidence, the N1s spectra for the modified particles narrowed into a single peak, which may be attributed to amide bonds formed by the adipic acid modification, which did not occur in the purified sample. Table 2 summarizes the key XPS results for each stage of particle functionalization.

2.6. DNA Loading and Biological Activity. An amine-modified oligonucleotide (csf1r) was coupled to the adipic acid groups on the surface of the particles. DNA loading was calculated by combining the data from XPS and the ninhydrin tests. It is proposed that increases in the atomic percentage of nitrogen after APS modification are directly attributable to the covalent coupling of the oligonucleotides. Therefore, using the quantitative data obtained from only APS-modified particles, the subsequent increase in nitrogen loading due to DNA can also be expressed in terms of the quantitative surface loading of DNA. Considering that there are 149 nitrogen atoms in the csf1r oligonucleotide, the DNA loading for the csf1r-modified particles was calculated as 4.7×10^7 species per bead (Table 3).

After the DNA loading was established, fluorescently labeled target sequences were hybridized to the csf1r probes attached to the silica particles. The raw data recorded are presented in the form of a histogram (Figure 8), with log fluorescence intensity on the *x*-axis and frequency on the *y*-axis. The number of fluorescent targets hybridized to the probes was analyzed by flow cytometry. Particles on which the target was bound were observed in the FL1 (510−550 nm band-pass filter), and the range of detection was determined as approximately two log decades in terms of fluorescence intensity. To investigate the specificity of the DNA hybridizations, the experiments were repeated using a csf1r probe containing a single-base substitution (or single-nucleotide polymorphism, SNP). A reproducible decrease of ∼20% in fluorescence intensity was observed for the single mismatch duplex when compared to that of the complementary duplex.

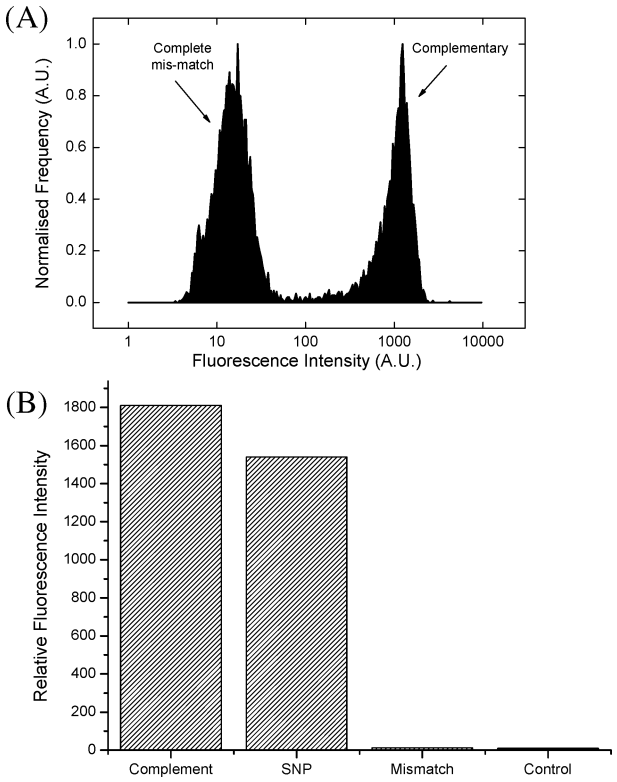

Figure 8. Flow cytometry histograms showing results of biological screens. (A) Fluorescence histogram showing range of detection between complementary hybrids and complete mismatch sequences. (B) Column chart showing the fluorescence emission from a complementary duplex, a single-base mismatched duplex, a complete mismatch duplex, and a set of unfunctionalized particles as the control.

3. Discussion

This study represents a systematic approach to the design and characterization of surface modifications required to produce particle libraries suitable for DNA screening applications. Due to the increased interest in particle supports for biological and chemical screening studies, such methods are necessary to determine the final concentration of the molecules of interest.

The particles were characterized based on the assumption of a "hard-sphere" model. This assumes that the particles are spherical, continuous, and solid with negligible porosity. While high-resolution SEM supports the use of this model, the diffusion of fluorescent dyes into the particle interior suggests that there is some accessibility. It therefore appears that the particles do in fact allow "molecular diffusion" of very small molecules, and these channels are below the detection limit of the high-resolution SEM technique. A possible explanation for this behavior is that the particles may swell in different solvents. Miller et al. showed that the ratio of cross-linked silica to linear silica chains was approximately 1:1 by nuclear magnetic resonance[27] (Si NMR), which easily allows swelling to occur in different solvent environments. When the particles were analyzed under SEM conditions, the evacuated chamber would cause any pores to close, removing any evidence of porosity. However, the hard-sphere assumption is still valid for calculating functional group loading, as the APS layer appears to be localized to the surface (discussed further below). Therefore, any calculations to determine the concentration of functional groups for biomolecule attachment are made under the assumption that these groups appear only at the surface.

While it has been proven that APS is a useful molecule for functionalizing silica surfaces, the reaction mechanism is still largely unknown. Many studies have been published outlining the formation of self-assembled monolayers (SAMs)[45] on metal and oxide coatings, but, in those systems, chemical bonding occurs through the propylamine functionality of APS.[43,46,47] We have proven in this work that free amine groups are available for reaction on the surface of the particles, suggesting that it is in fact the APS−silanol groups (obtained upon hydrolysis of the methoxy groups in basic conditions) that chemically bond to the MPS particle surface. Two variations on this basic mechanism have been proposed in other studies: (1) direct nucleophilic attack of the APS−silanol on the MPS surface silanols, and (2) reaction of the terminal silanol of an APS oligomer with the MPS surface in the same way. Acid−base interactions are obviously very important in the mechanism, as is hydrogen bonding in the aqueous solution.[31,34] Further investigations using NMR are underway to elucidate the exact mechanism so that a broader set of applications can be explored.

It is proposed that the APS functionalization produces a silane coating localized to the particle surface. It appears that small molecules cannot penetrate through to the interior of the particles after APS treatment, as evidenced by the confocal microscopy experiments performed (Figure 6). This suggests that larger, highly charged species such as oligonucleotides will also align themselves at the surface to facilitate biological reactions. We propose that this behavior is based largely on the hydrophobic nature of the MPS particles. The freshly prepared particles have been observed to aggregate in pure water, suggesting a hydrophobic nature imparted by the organic component of the MPS monomer. Hydrophilic molecules will prefer to align themselves on the particle surface rather than bury into the hydrophobic matrix. For this reason, hydrophilic molecules should remain at the surface, available for further reactions.

Fluorescent dyes were incorporated into the particles to provide a means of uniquely identifying the particles and tracking the reaction history of the biomolecules subsequently attached to the particle surface. These dyes possess a terminal succinimidyl ester group that enabled direct conjugation to alkylthiol groups throughout the particles.[13,21] It was anticipated that incorporating the "bulky" dye molecules into the matrix of each particle could have potentially affected the subsequent attachment of the APS layer due to steric shielding of the reactive silanol groups. This could have resulted in a change in surface charge, leading to changes in the stability of the particle dispersion in different solvents and/or pH-buffered solutions. However it seems that, in aqueous solutions similar to those used for bioassays, the surface charge of the particles (Figure 5) and the amine loading (Table 1) is not affected by the incorporation of encoding dyes. These results establish the bifunctionality of the organosilica particles.

Measurement of DNA loading is regarded as an extremely desirable factor in developing diagnostic materials. To determine the specific concentration of DNA, the atomic ratio of nitrogen was monitored and compared to the absolute concentration of primary amines as measured by the ninhydrin test. The calibration procedure for the ninhydrin test highlighted a variation in the molar absorption coefficient for the ninhydrin reagent, suggesting that the calibration should be performed often to check variability within one batch and across multiple batches. This variation has been noted before[41] and can be included in calculations as a quantifiable error. The final DNA loading was determined as $\sim 4.7 \times 10^7$ per particle (based on the mean size), which is similar to results from other investigations[3,4,37,38] assuming a constant surface density. From the above discussions regarding the localization of the APS coating, it appears that these oligonucleotides are available at the particle surface for reaction. This means that there will be little or no detection of DNA hybridization events below the particle surface, negating any difficulties in fluorescence emission from within the particle interior.

The applicability of particle-based assays for DNA screening have been established on the basis of the range of detection between csf1r mismatches and complementary duplexes. However, a simple error analysis highlights an important issue yet to be overcome. The results from the hybridization reactions involving the csf1r sequences reveal a significant dispersion in fluorescence intensities for particles in the same reaction. The ability to determine the concentration of fluorescently labeled targets attached to the particles could establish particle-based platforms as a standard method for biomolecule quantitation. The random error for fluorescence detection can be at least estimated at a standard deviation of 1−2% of the mean, based on the alignment particles used for laser and stream alignment. However, the size distribution of the MPS particles as quoted above suggests ∼29% standard deviation, and this figure is doubled for surface area measurements. The systematic errors, therefore, lie with the particle size distribution, which directly correlates with the number of DNA probes attached per bead, assuming a consistent DNA surface density. While error analysis and reduction is an important issue for the development of particle-based assays, the impact of this error is rarely considered.[37,38]

4. Conclusion

Particle-based assays present a novel method for high-throughput DNA screening by recording the presence of a bound target molecule by flow cytometry. This paper has outlined a methodology for developing a bioactive surface for MPS particles and associated methods for quantifying the concentration of surface-active groups. A unique method for measuring biomolecule concentration on the particle surfaces has been developed based on the use of XPS. The distribution of particle sizes is an issue that needs to be improved, either through chemical optimization or through data analysis and normalization. A suite of analytical techniques has been presented to analyze and crosscheck the key material parameters for biological screening.

5. Materials and Methods

5.1. Fluorescent Dye Incorporation. Fluorescent dye (Alexa Fluor 546 and Bodipy 493, Molecular Probes) crystals were added to 500 μL of THF (EMD) to form a saturated dye solution. A 50 μL portion of the supernatant was added to a centrifuged pellet of silica particles ($\sim 2 \times 10^7$ particles) and shaken at 1400 rpm for 60 min on an Eppendorf thermomixer. Unreacted dye was removed by multiple washing steps using THF, followed by a transfer into 1% sodium dodecyl sulfate (SDS) to ensure complete removal of unbound dye molecules.

5.2. APS Coating. Particle suspensions were washed three times in AR-grade ethanol in preparation for the APS (Aldrich, U.S.A.) reaction (based on the method of Van Blaaderen[24]). A 940 μL portion of ethanol and 50 μL of a 25% ammonia solution (Ajax FineChem, Auckland, New Zealand) were added to the particle pellet, and the suspension was sonicated for 30 s. A 10 μL portion of APS was added, and the particles were again sonicated briefly before being left to rotate for 90 min in the dark. After the APS reaction, the particles were washed thoroughly in ethanol to remove ammonia and excess APS.

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The amine concentration on the surface of the particles was determined through application of the ninhydrin test. (Fluka, Switzerland) The APS-coated particles were transferred into dry hexane (EMD), and the solvent evaporated in an oven at 80 °C in a nitrogen environment. The test was carried out according to the manufacturer's standard instructions, and the resulting solutions were analyzed by UV/Vis spectrophotometry (at 570 nm). A dilution series of ethanolamine in methanol was prepared to calibrate the ninhydrin test. A 75 μL portion of an ethanolamine/methanol solution replaced the particles for this calibration, and the resulting Beer−Lambert plot was used to fix the molar absorption coefficient for the ninhydrin reagent.

5.3. Modification of Particles to Enable Oligonucleotide Attachment. APS-coated particles were transferred into THF before adipic acid coupling to convert amine functionalities to carboxyl moieties. A 250 μL portion of a 20 mg/mL solution of adipic acid in THF was added to the particle pellet along with 5 μL of diisopropyl carbodiimide (DIC; Aldrich, MO). Suspensions were agitated using an Eppendorf thermomixer block for 12 h at 1400 rpm. Similarly, 5 μL of a 50 μM DNA ("csf1r": 5′-NH_2−C6-TTGCTGGCACAGGAGGTGACAGTGGTTGAGGGCCAGGAAG 3′; or csf1r single-base mismatch: 5′-NH_2−C6-TTGCTGGCACAGGAGGT*C*ACAGTGGTT GAGGGCCAGGAAG 3′; Geneworks, South Australia, Australia) was coupled to the particles by reacting the 5′-hexamethyl amino spacer on the oligonucleotide to the carboxylic acid group on the particles in a 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma) reaction mixture (50 mg/mL EDC, 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer). A 40 μL portion of the EDC reaction mixture was added to the particle suspension every 20 min for 1 h.

5.4. Particle Sizing and Counting. Particles were sized after APS coating by the Coulter method on a Beckman Coulter multisizer. For particle counts, samples were loaded onto a hemocytometer and analyzed in triplicate by light microscopy in accordance with the procedure published by Nielsen.[40]

5.5. DNA Screening Experiments. Oligonucleotides coupled to encoded silica particles were incubated in a hybridization buffer [60 mM NaCl (Chem-Supply, South Australia, Australia); 600 mM sodium citrate (Aldrich, U.S.A.); 1% SDS (ICN Biomedical, Ohio)] with 1 pmol of complementary Cy3-modified target oligonucleotide (5′-Cy3-CTTCCTGGCCCTCAACCACTGTCACCTCCTGTGC-CAGAAC-3′; Geneworks, South Australia, Australia). The hybridizations were all performed for 60 min at 53 °C unless otherwise indicated. Particles were maintained in suspension by agitation in an Eppendorf thermomixer. Following the reaction, the hybridized particles were analyzed using a Moflo flow cytometer (DakoCytomation, U.S.A.) where at least 5000 particles were used to confirm the basic statistical descriptors of the mean, median, and coefficient of variation. The Cy3-modified target was excited by a 488 nm argon-ion water-cooled laser (I90C, Coherent Scientific, Australia) at 100 mW. Emission data was recorded in log mode with the detector gain set on 650 V using a 580/30 band-pass filter.

5.6. XPS Analysis. Each sample was rinsed thoroughly with THF and then dried under nitrogen before being degassed overnight. Data were acquired using a Kratos Axis ULTRA X-ray photoelectron spectrometer incorporating a 165 mm hemispherical electron energy analyzer. The incident radiation was monochromatic Al X-rays (1486.6 eV) at 300 W (15 kV, 20 ma). Survey (wide) scans were taken at an analyzer pass energy of 160 eV, and multiplex (narrow) high-resolution scans were taken at 20 eV. Survey scans were carried out over a 1200−0 eV binding energy range, with 1.0 eV steps and a dwell time of 100 ms. Narrow high-resolution scans were run with 0.1 eV steps and 250 ms dwell time. Base pressure in the analysis chamber was 1.0×10^{-9} Torr, and, during sample analysis, it was 1.0×10^{-8} Torr.

All sample binding energies were calibrated to 284.8 eV for C−C/H/Si in the C1s spectrum.[43] To determine the structure of the MPS surface (and functionalized surfaces), the atomic percentages of each species were compared theoretically, and elemental analysis was performed to establish the C, H, and N levels (data not shown).

5.7. Confocal Laser-Scanning Microscopy. Particles were functionalized with fluorescent dyes and/or APS, as described above, and then mounted on a glass slide for examination by confocal microscopy. Dilute particle suspensions in THF were spotted onto a slide, and glycerol was added once the solvent had evaporated. A cover slip was placed over the glycerol, and all air was removed from the sample space. The instrument used was a confocal laser-scanning microscope (Bio-Rad MRC-600) mounted on a Zeiss Axioskop. The *xy* and *xz* planar sections were reconstructed in a *z*-axis using Bio-Rad MRC-600 CoMOS software to create a three-dimensional profile of the coated structure.

5.8. SEM Analysis. For SEM analysis, particles were transferred into MilliQ water and crushed using a mortar and pestle to expose the internal structures. Holey carbon-coated copper transmission electron microscopy (TEM) grids were used as the mounting medium, and samples were sputter-coated with platinum under vacuum to provide an electron-conductive layer. A high-resolution JEOL 890 microscope was used to provide maximum resolution images of both the external and fractured particle surfaces. Samples were analyzed using a 5 kV and 2×10^{-12} A current.

5.9. ζ Potential Analysis. Encoded particles were suspended in MilliQ water at a low enough dilution to prevent aggregation. ζ potentials were measured using an aqueous dip cell (3000HS ZetaSizer, Malvern, U.S.A.). The Smoluchowsky equation was used, and the Huckel approximation was used ($k_a = 1$) for particle suspensions in low ionic strength (in this case, MilliQ water). Five measurements were taken for each sample, and sample replicates were analyzed to check for variability.

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