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Novel one-pot synthesis and characterization of bioactive thiol-silicate nanoparticles for biocatalytic and biosensor applications

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Abstract

A novel one-pot neutral synthesis using bioinspired polymers to fabricate thiol-nanoparticles is presented. The thiol-particles may be directly tethered to metal surfaces such as gold, allowing the production of self-assembled nanostructured biocatalytic or biosensor surfaces. This one-pot method has also been used to entrap enzymes within the thiol-nanoparticles; it is apparent that once enzyme entrapment is carried out a bimodal distribution of particles is formed, with particles of one mode being very similar in size to thiol-nanoparticles without enzyme entrapped, and particles of the other mode being much larger in size. To this end, efforts have been made to separate the two modes of particles for the sample containing enzyme and it has been observed that the larger mode thiol-nanoparticles do indeed contain significant amounts of enzyme in comparison to the smaller mode ones. As the enzyme-containing thiol-nanoparticles can now be isolated, this means that there are many future possibilities for the use of thiol-particles containing enzyme, as they may be used in a wide range of processes and devices which require catalytic functionalized surfaces, such as biosensors and biocatalytic reactors.

S Supplementary data are available from stacks.iop.org/Nano/20/055612

1. Introduction

At present there is great interest in the production of biomimetic and functionalized nanomaterials for use in many areas of research from biology through to physics and engineering [1]. However, for most of the above research areas and industries, lengthy and complex processes using harsh chemicals and conditions are required to fabricate the desired product. Therefore, the future of the field of biomimetics and functionalized materials needs to be simplified in order to reduce production times whilst at the same time keeping optimum conditions for biological materials.

The method of silicate nanoparticle fabrication which we have used so far is one which is based on natural biosilication [1–8]. *In vitro* specialized proteins called silaffins can catalyze silication, which occurs at near neutral pH and ambient temperature and produces silicate nanoparticles and nanostructures down to around 50 nm in size [1, 2]. Recent work has demonstrated that polybasic peptide mimics such as polyethyleneimines (PEI) [3, 4] are also efficient at directing silicate deposition into nanoparticles in a similar manner to silaffin peptides [5, 6]. The silicate nanoparticles and nanostructures produced using PEI can be used to entrap enzymes for a number of applications in the fields of biosensors and biocatalysis. This silication method has great potential as it may be used by many industrial sectors and it has the major advantage that is does not use harsh chemical conditions or processing and may be used in manufacturing products from pilot to bulk scale [5, 7, 8].

There are several other strategies for the production of silicate nanoparticles. However, most are based on the Stöber process [9] in which alkoxy silanes are mixed with alcohol and polymerized in the presence of ammonia. Although this method has been used successfully for many years it involves

heating the particles to temperatures well above ambient and also the use of highly basic pH [9, 10]; both conditions are highly damaging to proteins and other biomolecules.

As well as the method of nanoparticle production, the way in which nanoparticles are functionalized is an important factor. Most nanoparticle functionality is carried out by surface modification of nanoparticles [11]. However, surface modifications have the disadvantage that they usually require complex processing steps and surface coverage is low, especially when using silica nanoparticles as substrates [11]. Recent work [11] describes a method of producing silicate particles which are modified with thiol groups. However, this method [11] requires many hours to progress and the size of the particles produced is substantially greater than those produced using the method presented in this paper. Moreover, their methodology does not control the pH of the system which is very important when considering immobilization or entrapment of biological molecules within nanoparticles, as the biological molecules may require more neutral and ambient conditions than might otherwise be used.

A method able of producing silicate particles which contain biological components, such as enzymes entrapped within them, is advantageous in a number of fields [5, 7, 8]. In addition, it is highly desirable if the particles could also be pre-functionalized with reactive groups such as free thiol groups and give similar results, independent of the enzyme type used, as it would enable an approach that could be more widely applied to other biosensor, biocatalytic and other biofunctionalized surfaces and materials. Furthermore, if such a methodology could also be produced with an improved fabrication method in terms of facileness and timescale than previously used it would mean a new innovative method had been produced which is advantageous over previous methods for the reasons mentioned above.

Here we present a novel one-pot method of fabrication and the subsequent characterization of silicate nanoparticles which are pre-functionalized with free thiol groups. The whole process has important advantages in that it is very rapid, when compared to other recently described methods [11] and, as well as being within the nanometre size range, the particles are pre-functionalized therefore requiring no further surface modification. Additionally, the method has the advantage that it is carried out under neutral pH conditions and at room temperature without the use of ammonia or alcohol which are often used to produce silicate nanoparticles. The thiol-silicate nanoparticles are highly useful in producing self-assembled and biomimetic systems as they can be easily tethered to metal surfaces or metal particles. Moreover, they have the possibility of incorporating enzymatic activity within them by immobilizing enzymes within the particles or by surface immobilization via available reactive groups. We have termed the silicate nanoparticles which contain thiol groups 'thiol-nanoparticles'. Here we focus mainly on the results of the production of thiol-particles and the characterization of thiol-particles containing β-galactosidase enzyme [8]. However, in addition, we have also used glucose oxidase [5, 12] and acetylcholinesterase [12] which are commonly used in the biosensor and biocatalysis fields. They have been successfully entrapped within thiol-particles using our new method and the characterization results are also presented (see section 3.3).

2. Materials and methods

2.1. Materials

Hydrochloric acid, 25 kDa polyethyleneimine, PIPES buffer, sodium phosphate, sodium chloride, potassium phosphate, potassium chloride, *N*-ethylmaleimide (NEM), glucose and sulfuric acid were purchased from Sigma-Aldrich. β-galactosidase (from *E.coli*), glucose oxidase and acetylcholinesterase (from electric eel) were also obtained from Sigma-Aldrich. The silane precursors that were used were 3-mercaptopropyltrimethoxysilane (3-mPTMOS), tetramethyl orthosilicate (TMOS), ethyltrimethoxysilane (ETMOS), propyltrimethoxy silane (PTMOS) and trimethoxymethylsilane (TMOMS) and they were also supplied by Sigma-Aldrich and used without any further purification.

Gold electrodes were obtained from the Tyndall Institute, Cork and comprised 200 nm gold onto a 50 nm chromium adhesion layer on SiO_2. The sensor working area was 0.785 mm.

2.2. Thiol-particle fabrication

The thiol-nanoparticles were produced by the condensation of 3-mercaptopropyltrimethoxysilane (3-mPTMOS) which had been prehydrolyzed for 15 min with 1 mM hydrochloric acid before mixing with 25 kDa polyethyleneimine (PEI), in the presence of sodium phosphate buffer (pH 7.5). The final concentrations of the substituents were 0.1 M 3-mPTMOS, 0.1 mM PEI and 29 mM phosphate buffer to give a phosphate concentration to PEI repeat unit ratio of 0.5. The PEI was added to the phosphate buffer and the silane was added last at which point the solution was mixed by vortex mixing. The whole mixture was left to incubate for 15 min period. After this time, the precipitate was washed twice by centrifugation at 16 873g, 2 min, before resuspending in water. All particles were fabricated at room temperature ($\sim$22 °C).

2.3. Thiol-particle fabrication with enzyme entrapped

In this case the PEI and phosphate buffer were incubated together at the same concentrations as for producing thiol-particles without enzyme entrapped. Concurrently a mixture of hydrolysed 3-mPTMOS (final concentration 0.1 M), β-galactosidase enzyme (0.5 mg ml^{-1} final concentration) and PIPES buffer (29 mM final concentration) was separately incubated together. Both mixtures were incubated for 15 min before adding the two solutions together, vortex mixing and incubating for a further 15 min at which time a cloudy solution formed. After this time, the precipitate was washed twice by centrifugation at 16 873g, 2 min, before resuspending in water. All particles were fabricated at room temperature ($\sim$22 °C). The same procedure was used for glucose oxidase and acetylcholinesterase, but here the final concentrations of enzyme used were 10 mg ml^{-1} and 5 units ml^{-1} respectively.

2.4. Thiol-particle characterization—thiol group and enzyme activity

After synthesis of the thiol-particles with or without β-galactosidase enzyme entrapped, the particles were tested in two different ways in order to ensure that the thiol-particles contained free thiol groups and were β-galactosidase active [8]. The first test used Ellman's reagent [13] (5,5′-dithio-bis(2-nitrobenzoic acid)) which reacts with free thiol groups to produce yellow thio-nitrophenol. The presence of a yellow colour shows that there are reactive free thiol groups present on the surface of the particles which could be used for attachment of the particles to metal surfaces. Secondly β-galactosidase activity was measured using ortho-nitrophenyl β-D-galactopyranoside (ONPG) which is converted to yellow ortho-nitrophenol and galactose by the β-galactosidase enzyme.

2.5. Thiol-particle characterization—particle size distribution

In order to determine the particle size distribution, the particles in suspension were sized by analysis of Brownian motion using video microscopy [14]: the Nanosight system used employs laser-light scattering to enable particle motion tracking [15]. The particles were also sized using the Malvern Zetasizer NS [16] which uses dynamic light scattering for particle size determination. In dynamic light scattering, in order to determine the speed at which the particles are diffusing due to Brownian motion, the rate at which the intensity of the scattered light fluctuates is measured. The hydrodynamic diameter of the particles can then be calculated by the software using the Stokes–Einstein equation [15, 16].

2.6. Thiol-particle characterization—FEGSEM

The thiol-particles were characterized by field emission gun scanning electron microscopy (FEGSEM). The thiol-particles were either analysed directly from the particle suspension where a drop of a particle suspension was placed onto an aluminium stub and left to dry before sputter-coating with platinum/palladium or the thiol-particles were incubated with gold electrodes to see if they would bind to gold surfaces. In this case, solutions of thiol-particles were incubated for 1 h with gold electrodes that had been thoroughly cleaned using fresh piranha solution comprising 30% (v/v) hydrogen peroxide and 70% (v/v) sulfuric acid. The electrodes were then gently rinsed in deionized water before drying using a stream of nitrogen. The electrodes were mounted on to FEGSEM aluminium stubs for imaging.

2.7. Thiol-particle characterization—specific binding to gold surfaces

Experiments were carried out to determine if the gold-thiol-particle binding was specific to the thiol-particles. This was carried out by blocking the free thiol groups using *N*-ethylmaleimide (NEM) which alkylates thiol groups. The use of NEM for blocking free thiol groups for enzyme inhibition assays has long been used [17, 18] with NEM covalently binding to thiol groups at pH 6.5–7.5 [19].

The experiments were carried out by making a 20 mM solution of NEM in 0.1 M phosphate buffer at pH 6.5. The use of this pH prolongs the half-life of the NEM which means that the solution and reaction will be stable for a longer time period than the length of time the experiment takes to perform. Thiol-particles with and without enzyme were diluted with 20 mM NEM to give a final NEM concentration of 10 mM. Before incubation of the NEM-thiol-particles with the gold electrodes samples of the particles were tested using the Ellman's test as mentioned previously [13] to confirm the reactive thiol groups had been blocked.

During the experiment it was possible to see that the particles contained free thiol groups before NEM treatment as a yellow colour was produced from the thio-nitrophenol ion. After NEM treatment for 1h the particles were tested and no yellow colour was detected, suggesting that the thiol groups had been successfully blocked. After the NEM-treated thiol-particles had been tested for free thiol groups samples of NEM-thiol-particles and NEM-thiol-particles with β-galactosidase were incubated with freshly cleaned gold electrodes for 1 h. The electrodes were then gently washed and dried as was the case for all electrodes studied.

2.8. Thiol-particle characterization—activity of particles on a gold electrode surface

Gold electrodes were cleaned in piranha solution and subsequently incubated with thiol-particles with entrapped glucose oxidase for 1 h before rinsing in water. Chronoamperometry was then carried out. The classical three-electrode system was employed with the particle/gold electrode as the working electrode plus a platinum counter electrode and a Ag/AgCl reference electrode. Measurements were performed using an μAutolab-III FRA12 and the general purpose electrochemical software operating system GPES4 from EcoChemie (Ultrecht, Netherlands) in 10 mM pH 7 phosphate buffered saline. Activity of the immobilized enzyme was observed by the release of hydrogen peroxide in response to the addition of the glucose substrate. This was achieved by the addition of aliquots of glucose corresponding to increases in substrate concentration of 1 mM. The oxidation of hydrogen peroxide was monitored at +600 mV as carried out previously with a similar system using carbon electrodes [12]. Intensive stirring of the solution was carried out during the experiment and the baseline current was allowed to settle before injections of substrate were commenced.

3. Results and discussion

3.1. Thiol-particle characterization

Before characterization by FEGSEM, the thiol-particles were tested for the presence of reactive free thiol groups on the surface of the particles which could be used for attachment of the particles to metal surfaces. A yellow colour was produced when Ellman's reagent was used to test the thiol-particles, confirming the presence of thiol groups (figure 1S(a), supplementary data available on stacks.iop.org/Nano/20/055612).

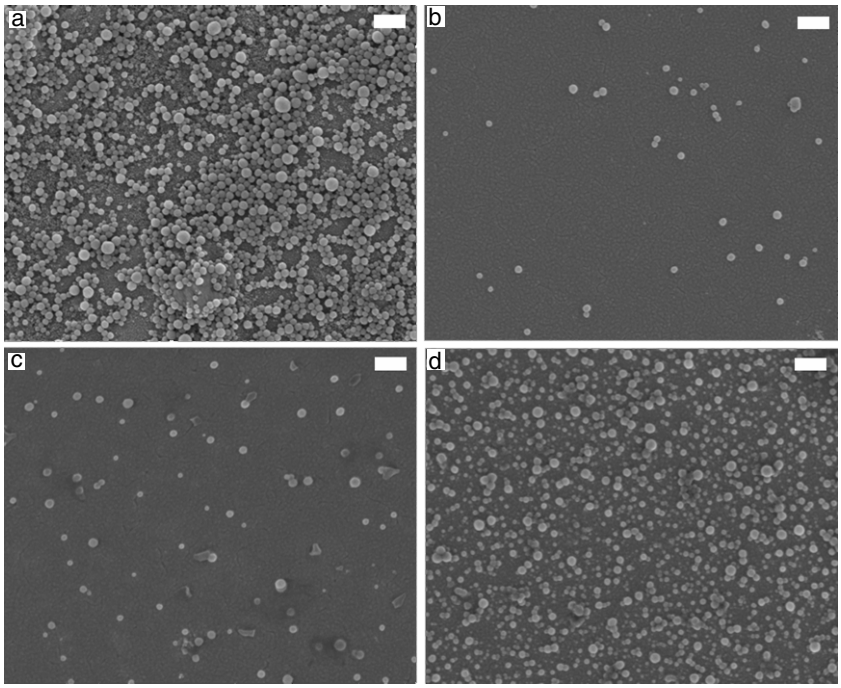

Figure 1. Effect of concentration of thiol-particle suspension on surface loading of gold electrodes and comparison with particles directly dried from a water suspension without any binding or washing steps. Particles were imaged using a LEO1530 Gemini FEGSEM. (a) Freshly prepared thiol-particles from water suspension were imaged using FEGSEM; the particles were dried directly on to aluminium stubs and the relative concentration of the suspension was ∼1 mg ml^{-1}. Different concentrations of thiol-particle suspension were incubated with a clean gold surface which was then rinsed and dried before FEGSEM imaging. The concentrations used were: (b) ∼1 mg ml^{-1}; (c) ∼2 mg ml^{-1}; (d) ∼10 mg ml^{-1}. The scale bar is 500 nm.

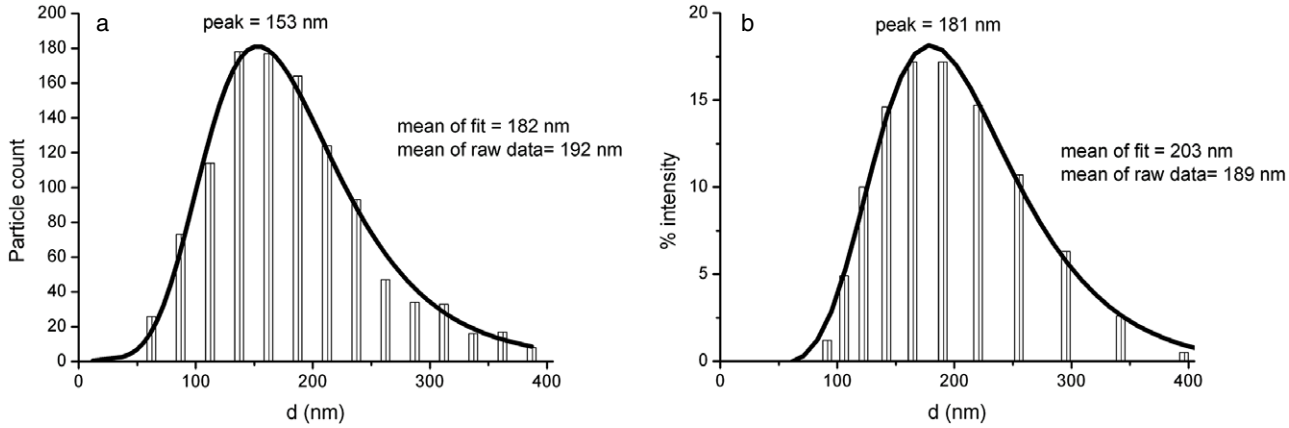

Figure 2. Particle size distribution of thiol-particles using (a) Nanosight nanoparticle tracking analysis technology and (b) dynamic light scattering. Particle sizing was performed using (a) a Nanosight LM10 with data processing carried out using the manufacturer's software (version 1.5) and (b) a Malvern Zetasizer NS and Dispersion Technology Software (version 5.02). An extreme value distribution was fitted to the data in order to best fit the distribution of the data. The peak value of the fit is written in the figure. The confidence level was 95% and correlation coefficient (r) values for the fit in (a) and (b) were 0.996 and 0.997 respectively.

A FEGSEM image of the thiol-nanoparticles (figure 1(a)) shows that there was little aggregation of the particles which are freely dispersed in solution. It can be seen that the average particle diameter as determined from the FEGSEM image is around 100–200 nm. A visual comparison with the scale bar (500 nm) reveals that there is a small amount of particles which are greater in size than 250 nm (these results are later corroborated in figure 2). The particles are spherical or quasi-spherical in shape and are more regularly shaped than particles which are larger due to the presence of enzyme (see later, figure 5).

When the same solution of thiol-silicate particles as imaged directly (figure 1(a)) was incubated with a gold electrode the resulting FEGSEM image (figure 1(b)) shows that the particles are attached to the gold surface and that there are individual particles with very little aggregation occurring. This is a property of nanoparticles which is highly desirable in the colloids and sensors fields of research. The image

shown in figure 1(b) shows particles which are fairly dispersed with around 0.5–1.0 μm between particles. If these particles are to be used in functionalized surfaces such as particle attachment to electrodes for sensor applications it is important to have as high a load as possible to increase any signal which is produced. Therefore, experiments were carried out to determine if the surface could be loaded with different amounts of particles (figures 1(b)–(d)).

When the concentration of the thiol-particle solution was increased from 1 to 2, to 10 mg ml^{-1}, the surface loading increased accordingly (figures 1(b)–(d) respectively) with a highly covered surface at the 10 mg ml^{-1} concentration. It should be noted that the particles are attached to the aluminium surface (figure 1(a)) purely through direct drying on to the surface and that if the suspension is incubated on the aluminium stub and then rinsed as is the case for the gold electrode there is no binding of the particles to the surface.

Figure 2 shows the particle size distribution of the thiol-particles. The data presented are obtained with the Nanosight nanoparticle tracking analysis (NTA) (figure 2(a)) and the Malvern Zetasizer NS particle size analysis (figure 2(b)) systems. The data from the Nanosight NTA (figure 2(a)) and dynamic light scattering (figure 2(b)) show the peak particle diameters from the extreme distribution fitted to the data to be 153 and 181 nm respectively. However, the Nanosight system measures data from individual particles and the Malvern gives an overall analysis which depends on whether the percentage number, volume or intensity is chosen. Here the dynamic light scattering data are presented as the percentage intensity, although all the results from the dynamic light scattering were of a similar range with a maximum peak particle diameter of the extreme distribution of 191 nm even when using percentage volume data.

The mean values of the fit to the data and of the raw data are also presented in figure 2. The mean values of the fitted extreme distribution are similar to those of the raw data which are 192 and 189 nm for the data for figures 2(a) and (b) respectively. An extreme distribution was used instead of a Gaussian distribution as the correlation coefficients are greater for the extreme distribution and also because the particle size distribution is not symmetrical and therefore it is not amenable to use a Gaussian distribution for fitting the data. Particle size analysis processed a much larger data set than FEGSEM can and therefore the mean size may be slightly different. However, the mean values of the extreme distribution of the thiol-particles from both particle sizing methods are similar both between each other and also when compared to the average particle diameter obtained with FEGSEM.

The method to produce thiol-particles presented here is very simple, quick and easy to carry out and so far we have shown that there are free thiol groups present in the particles and that the particles attach to gold surfaces (figure 1). However, to confirm that the particles were specifically binding to the gold surface and not just attaching to the surface by absorption/drying two complementary experiments were carried out. Firstly, tetramethyl orthosilicate (TMOS) particles were tested in the same way as the thiol-particles. TMOS was used as it does not contain any thiol groups and a typical

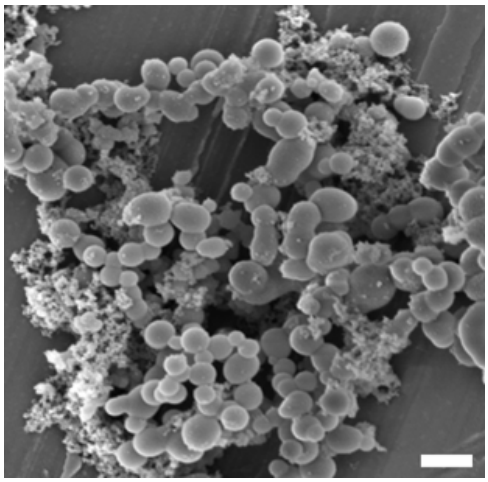

Figure 3. TMOS particles were imaged using a LEO1530 Gemini FEGSEM. Freshly prepared TMOS particles were imaged using FEGSEM. The scale bar is 500 nm.

image of TMOS particles can be seen in figure 3. Unlike the thiol-particles formed using 3-mPTMOS (figure 1), TMOS nanoparticles often showed an irregular silicate precipitate with uncondensed silicate matter present and the particles themselves often aggregated. Stirring partly abrogated this effect, but affected the morphology of the particles.

The TMOS particles were incubated with clean gold electrodes for 1h and after rinsing in water and drying were imaged using the FEGSEM where it was clear that the non-thiol containing particles did not adhere to the gold surface (figure 2(S)(b), supplementary data available on stacks.iop.org/Nano/20/055612). A sample fabricated with TMOS and enzyme was also tested in the same way as the TMOS particles; again, no particles adhered to the surface.

Secondly, *N*-ethylmaleimide (NEM) was used to alkylate the thiol groups in the thiol-particles. The use of NEM for blocking free thiol groups for enzyme inhibition assays has long been used [17, 18] with NEM covalently binding to thiol groups. The NEM-thiol-particle samples without and with enzyme respectively were also incubated with gold electrodes for 1 h before rinsing and the experimental data (figure 2S (c), supplementary data available on stacks.iop.org/Nano/20/055612) showed that NEM successfully blocked the thiol groups and this prevented any NEM-thiol-particles binding to the gold electrodes. As a control, TMOS particles/silica samples with and without enzyme were also tested with NEM. The results were the same as for the TMOS particles alone, in that there was no binding to the gold electrodes.

Another property of thiol-particles was also investigated as it has been noted that TMOS particles generally aggregate and that thiol-particles do not. Therefore, a series of experiments were carried out to determine the reason for this difference. A series of particles were fabricated in exactly the same way as TMOS particles and thiol-particles, but using different silane precursors with a similar structure to 3-mPTMOS which is used to make thiol-particles. The silane precursors that were

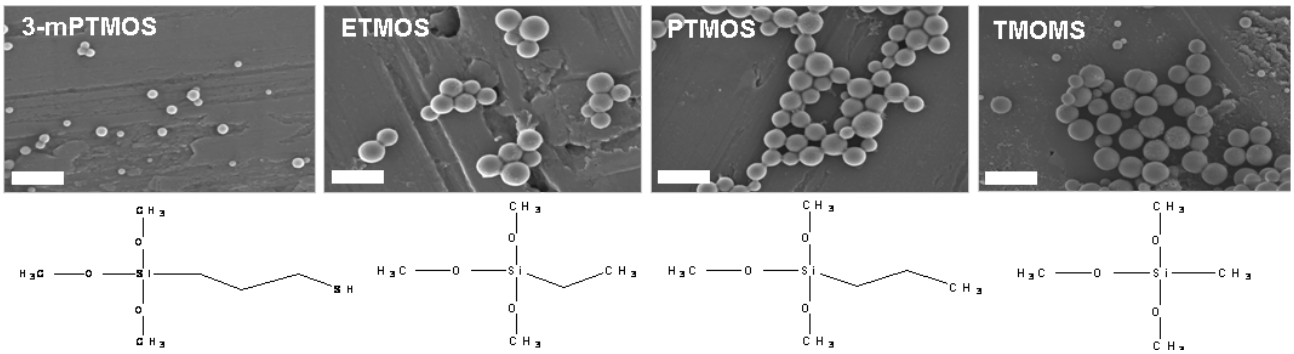

Figure 4. FEGSEM images of silicate particles produced using different silane precursors which contain alkyl chains. The chemical structure of the respective silane precursor is shown below the corresponding FEGSEM image. The scale bar is 1 μm.

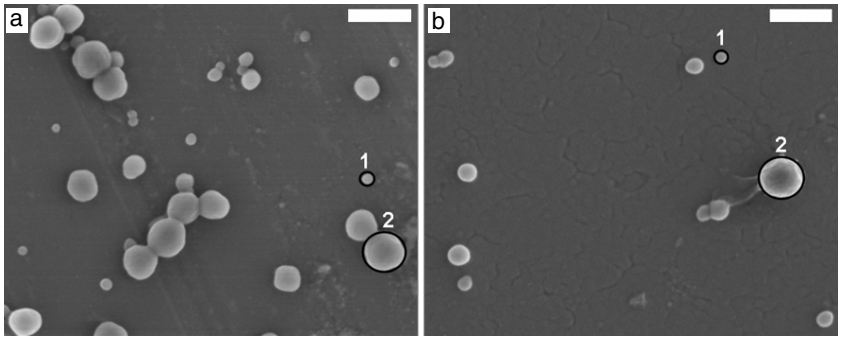

Figure 5. Enzyme loaded thiol-particles. Particles were prepared as described as to incorporate β-galactosidase. (a) Typical smaller particle (1) and typical larger particle (2). (b) Enzyme loaded thiol-particles were incubated with a clean gold surface which was then rinsed before FEGSEM image. Typical smaller particle (1) and typical larger particle (2). The scale bar is 500 nm.

used were ethyltrimethoxy silane (ETMOS), propyltrimethoxy silane (PTMOS) and trimethoxymethyl silane (TMOMS). The structures of these precursors can be seen in the lower part of figure 4. FEGSEM images of the particles made with these silane precursors are shown in the top part of figure 4.

The FEGSEM images show some similarities and differences between the different particles made with 3-mPTMOS, ETMOS, PTMOS and TMOMS. When comparing the particles made with ETMOS, PTMOS and TMOMS, indeed there are very few differences that can be observed. However, when comparing the particles formed by these three silane precursors with those made with 3-mPTMOS it is obvious that they are much larger in size than the thiol-particles as the thiol-particles have an mean size of around 100–200 nm and the other particles have an approximate average particle diameter of around 500 nm.

The main similarity between thiol-particles (formed using 3-mPTMOS) and the other three particle samples is that all of the particles are not aggregated which is not the case for particles made with TMOS as the silane precursor (figure 3). This is thought to be due to the presence of the alkyl chain in the silane precursor which most likely introduces some kind of repulsive forces between the particles. Since there is no alkyl chain present in TMOS, it is most likely that the particles are more hydrophilic and there is less repulsion between them allowing them to easily aggregate.

3.2. Characterization of enzyme-containing thiol-particles

The particle suspensions were tested for enzyme activity. The results of the test on a particle suspension of thiol-particles containing β-galactosidase showed a yellow colour after the reaction of ONPG with the β-galactosidase containing thiol-particles (figure 1S (b), supplementary data available on stacks.iop.org/Nano/20/055612), confirming that the particles contain active enzyme as well as free thiol groups.

When β-galactosidase enzyme was introduced into the silicate particle fabrication process the resultant particles showed a bimodal particle distribution with average particle sizes of 100–200 and 300–400 nm for the two types of particles (figures 5(a) and 6(b), described later). Since the thiol-particles without enzyme observed with FEGSEM directly from a water suspension (figure 1(a)) have a particle diameter of 100–200 nm, we propose that the larger particles present in the β-galactosidase thiol-particle sample are those that contain the enzyme. This is shown in figure 5(a) where one smaller nanoparticle ($\sim$120 nm (1)) and one larger nanoparticle ($\sim$340 nm (2)) have been highlighted. The smaller particles are spherical or quasi-spherical in shape although the sphericity of the larger particles and their surface appearance is more amorphous, most likely due to the presence of the relatively disordered structure of the enzyme present in the sample.

Figures 6(a), (b) show the comparison of the particle size distribution of thiol-particles without and with enzyme entrapment respectively carried out using the Nanosight system. The

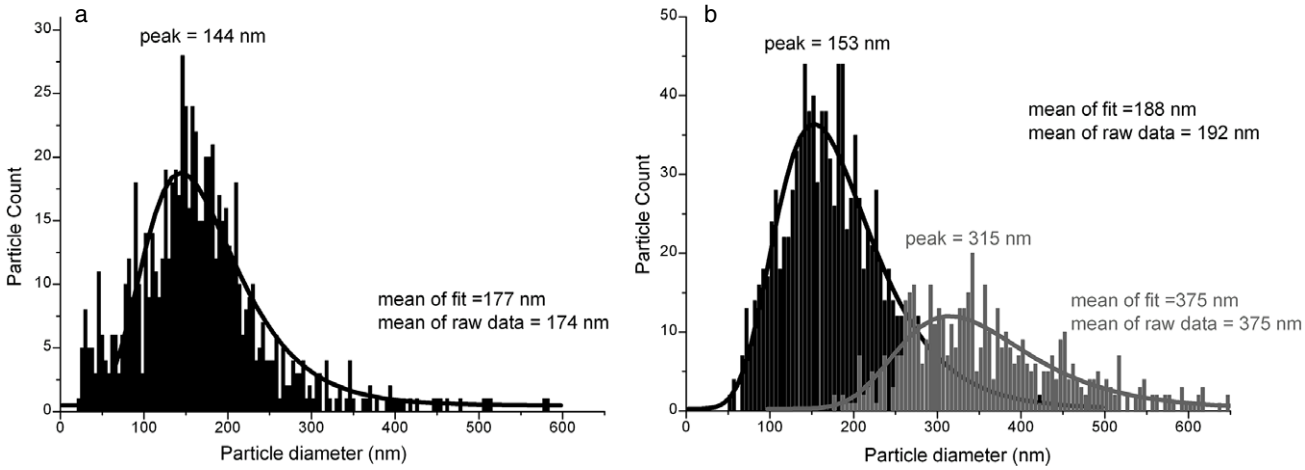

Figure 6. Nanosight NTA particle size distribution of thiol-particles in (a) the absence and (b) the presence of β-galactosidase enzyme, where black bars and line are data for smaller thiol-particles with β-galactosidase and grey bars and line are for larger thiol-particles with β-galactosidase. Particle sizing was performed using a Nanosight LM10 with data processing carried out using the manufacturer's software (version 1.5). An extreme value distribution was fitted to the data in order to best fit the distribution of the data. The peak value of the fit is written in the figure. The confidence level was 95 %. The correlation coefficient (r) value for (a) was 0.911 and for the case of (b) were and 0.954 (small) and 0.888 (large) respectively.

data presented are those of thiol-particles and thiol-particles containing β-galactosidase (bimodal distribution of small and large particles as mentioned above). Particle size analysis processes a much larger data set than FEGSEM can and therefore the average size may be slightly different. However, the mean particle sizes of the fit to the data of the thiol-particles with and without enzyme are similar in value to those found with FEGSEM. The data (figure 6) clearly show that the particles which do not contain enzyme (figure 6(a)) do not have a bimodal distribution, whereas there is a bimodal distribution produced in the thiol-particle solution which contains enzyme (figure 6(b), small particles, black and larger particles, grey). Moreover, the size of the smaller particles (from sample containing enzyme) is almost identical to the size of the thiol-particles which do not contain enzyme, further supporting our hypothesis. It is worth noting than in this case the peak and mean values of the extreme distribution for the thiol-particles (figure 6(a)) are slightly lower than the value seen in figure 2(a) since a new batch of particles was used in order to compare particles with and without enzyme made on the same day. Nevertheless, the difference in the peak values of the fit of the data of the two batches of particles without enzyme is less than 7%, indicating the method is fairly reproducible.

A similar experiment to the one shown in figures 1(b)–(d) was carried out to look at the surface loading of the thiol-particles sample containing β-galactosidase enzyme (figures 7(b)–(d)). The FEGSEM images clearly show the bimodal distribution of particle size of the particles attached to the gold surface and that the surface loading increases with concentration as in the case of thiol-particles without enzyme (figures 1(b)–(d)). However, the surface coverage is significantly lower in the case of thiol-particles with β-galactosidase and so if this method is to be used as a method of biosensor or biocatalytic surface fabrication, the method of attachment of the particles to the surface would have to be greatly improved. Figure 7(a) is an FEGSEM image of thiol-particles containing β-galactosidase which are directly attached to the aluminium surface purely through direct drying on to the surface. The presence of two distinct modes of particles can be clearly seen.

Due to the fact that there are two modes of particles produced when enzyme is introduced to the system and that the loading of the particles with enzyme shows there is a mixture of the smaller and larger particles, it was investigated to see if it was possible to fractionate the thiol-particles into two distinct modes. The fractionation was carried out in order to identify if the small particles from the enzyme-containing thiol-particle sample do not contain enzyme, or at least contain a significantly smaller amount of enzyme and if the larger particles contain the majority of the enzyme. If this would be possible it would be beneficial for two main reasons. The first reason would be that if the particles which contain a higher level of enzyme could be separated out they could be used for biosensors and biocatalysis with a more concentrated sample of bioactive particles which are also functionalized with thiol groups. The second advantage would be the fact that it would show that the thiol-particles form in distinct ways depending on whether enzyme is entrapped and that the presence of enzyme is necessary for larger particles to be formed.

The thiol-particles were fractionated by using a sucrose gradient to separate the particles on the basis of size. A sucrose gradient from 0.5 to 2.5 M was used and the thiol-particles (with or without enzyme) were layered on to the top of the gradient before centrifugation at 2110g, 10 min. After centrifugation of the thiol-particles containing enzyme there were two main fractions present: a fraction nearer to the top of the gradient (T) and a fraction at the bottom of the tube (B). Thiol-particles (no enzyme) were also fractionated using the sucrose gradient as a control and two samples were also taken from the sucrose gradient after centrifugation although only one band of particles was visible near the top of the gradient (T).

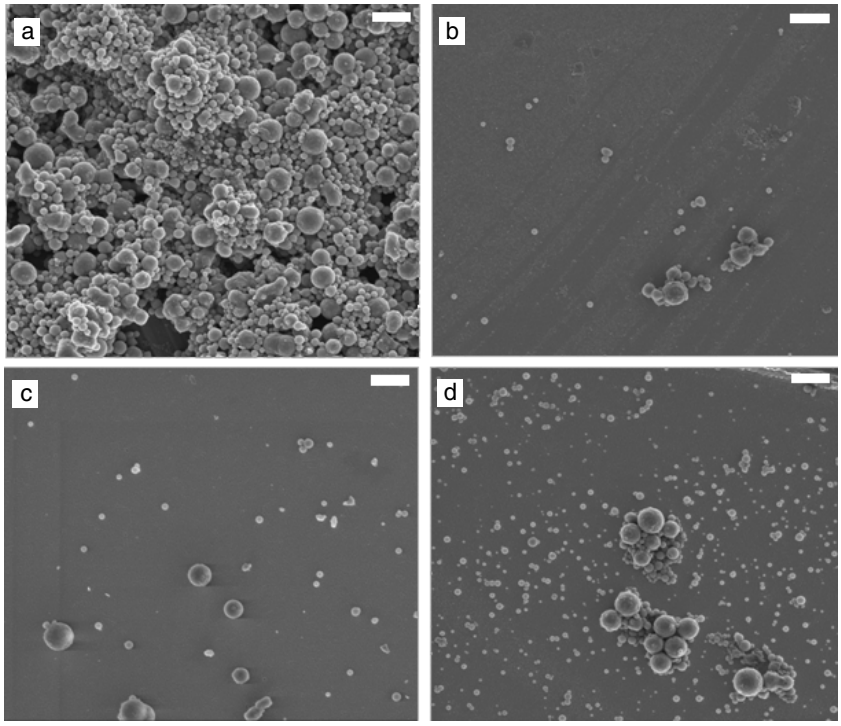

Figure 7. Effect of concentration of thiol-particle suspension on surface loading of gold electrodes and comparison with particles directly dried from a water suspension without any binding or washing steps. Particles were imaged using a LEO1530 Gemini FEGSEM. (a) Freshly prepared thiol-particles + β-galactosidase from water suspension were imaged using FEGSEM: the particles were dried directly on to aluminium stubs and the relative concentration of the suspension was $\sim$1 mg ml^{-1}. Different concentrations of thiol-particle + β-galactosidase ((b)–(d)) were incubated with a clean gold surface which was then rinsed before FEGSEM imaging. The concentrations used were: (b), $\sim$1 mg ml^{-1}; (c) $\sim$2 mg ml^{-1}; (d) $\sim$10 mg ml^{-1}. The scale bar is 1 μm.

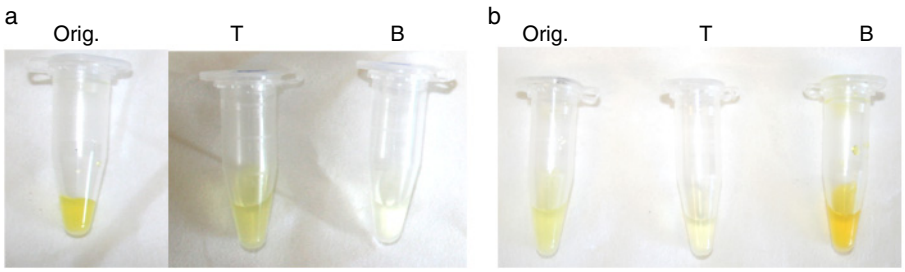

Figure 8. Effect of separation of thiol-particles with and without β-galactosidase using a sucrose gradient. (a) Thiol-particles and (b) thiol-particles containing β-galactosidase. Orig., refers to the original particle solution fabricated, T, to the top fraction (containing smaller particles), and B, to the bottom fraction (containing larger particles).

(This figure is in colour only in the electronic version)

Particles from the top and bottom fractions were then analysed using colorimetric assays for the presence of thiol groups or the presence of enzyme (figure 8). Figure 8 shows images of the tubes tested for thiol or enzyme activity, with the original sample and the fractions produced from the sucrose gradient. The thiol-particle (no enzyme) sample (figure 8(a)) shows that there were active thiol groups present in the sample and that this activity remained in the fraction collected from the top of the sucrose gradient. The finding that the top fraction contained particles with active thiol groups was expected since the thiol-particles are relatively small and so it would be more likely that they would remain at the top of the sucrose gradient than to progress down it.

Figure 8(b) shows the results of testing the sample of thiol-particles with β-galactosidase enzyme using ortho-nitrophenyl β-D-galactopyranoside, before and after separation on the sucrose gradient. Enzyme activity can be observed in the original sample, but it is significantly higher in the bottom fraction after separation on the sucrose gradient. This strongly suggests that there is a much higher content of enzyme in the larger particles which have gone to the bottom of the sucrose gradient due to their increased density and size in comparison with the particles in the sample that do not contain enzyme, or that contain much less enzyme. This corroborates our hypothesis that the larger particles of the bimodal distribution of thiol-particles

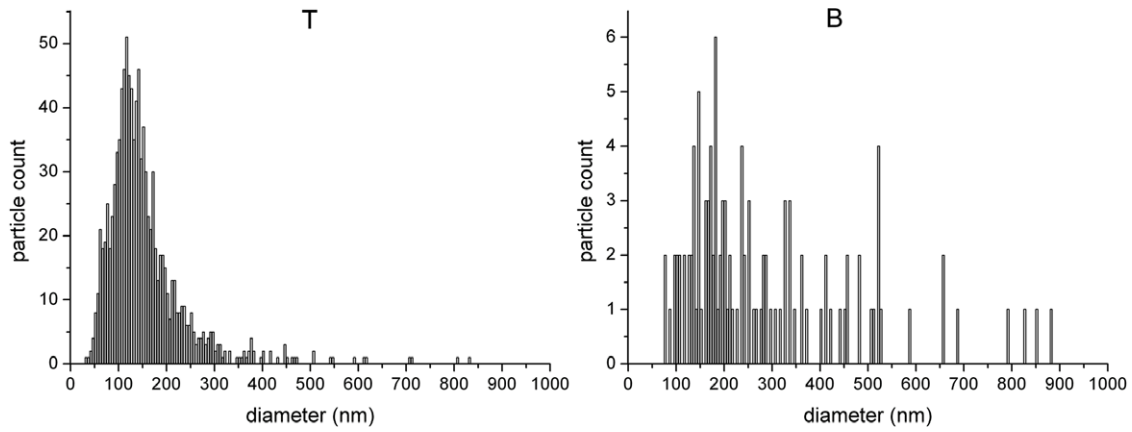

Figure 9. Particle size distribution of thiol-particles containing glucose oxidase from the top (T) and bottom (B) fractions after separation on a sucrose gradient. Particle sizing was performed using a Nanosight LM10 with data processing carried out using the manufacturer's software (version 1.5).

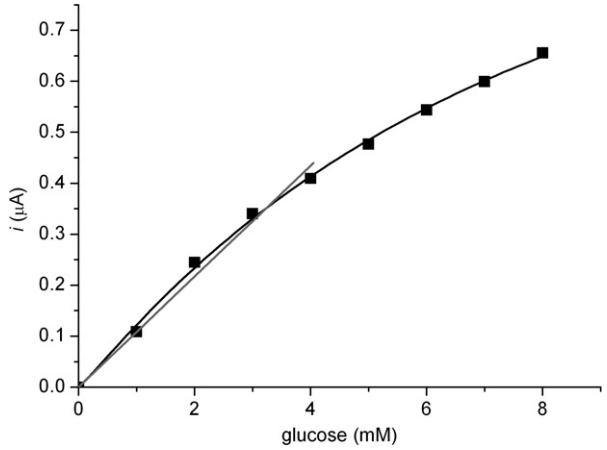

Figure 10. Amperometric glucose response of thiol-particle containing glucose oxidase modified gold electrodes. Amperometric responses were measured in 10 mM phosphate buffered saline, pH 7.4 at +600 mV with stirring.

with β-galactosidase are formed due to the presence of enzyme.

Nanosight nanoparticle tracking analysis was also carried out on the thiol-particles containing enzyme which were separated using a sucrose gradient. The results (figure 9) clearly show that there are small particles present in the top fraction (figure 9 (T)) with a relatively narrow particle size distribution and an average size of around 100–200 nm as mentioned previously. However, when the bottom fraction was analysed much larger particles were present (figure 9 (B)).

Here we have studied the fabrication and characterization of thiol-particles and thiol-particles containing enzyme. As has been shown earlier by colorimetric tests (figure 1S (b), supplementary data available on stacks.iop.org/Nano/20/055612 and figure 8), the particles themselves are enzymatically active, which means they could easily be used in biocatalytic reactors and recovered by centrifugation after the desired product had been formed. These particles could also be used directly in biosensor applications as there is increasing use of particles as sensors where substrate molecules attach directly to modified particles, such as antigens attaching to antibody modified gold particles [1]. However, for these particles to be useful in traditional biosensor applications they need to be able to be attached to conducting surfaces and still be active once this has been carried out.

Glucose oxidase is an enzyme which is widely used in biosensors [5, 7, 12]. Figure 10 shows the amperometric glucose response when glucose oxidase was entrapped within thiol-particles which were then incubated with gold electrodes. The activity of the immobilized enzyme was observed by the release of hydrogen peroxide in response to the addition of the glucose substrate. This was achieved by the addition of aliquots of glucose corresponding to increases in substrate concentration of 1 mM. The linear range of the response was up to 4 mM glucose (grey line, figure 10) and the sensitivity within this range was 109 nA mM^{-1}. These values are in good agreement with previously gained data [12] and future work will optimize the attachment of particles to the gold surface and study a range of enzymes.

3.3. Generality of the protocol using different enzymes

So far in this paper we have focused on the results of the production of thiol-particles and the characterization of thiol-particles containing β-galactosidase enzyme. However, some results have also been presented for glucose oxidase and currently we have data on three different enzymes with varying properties (table 1) which are commonly used in the biosensor and biocatalysis fields that have been successfully entrapped within thiol-particles using this novel method.

Table 1 shows the properties of the enzymes which have been studied so far for the entrapment of enzymes within thiol-particles. These enzymes are commonly used for biosensor applications [5, 7, 8]. Although the molecular weight, estimated hydrodynamic diameter [20, 21] and isoelectric point (pI) of the enzymes are different, the size of the particles produced is very similar, suggesting that the method presented in this paper can be generally used for different types of

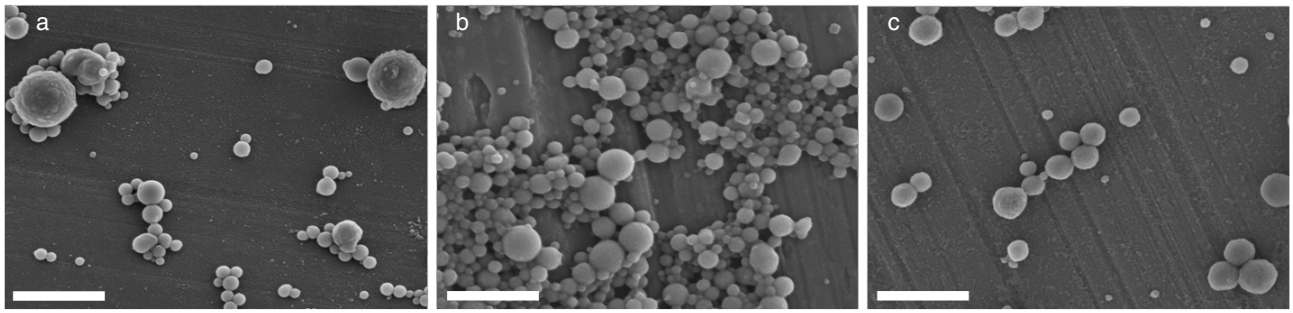

Figure 11. Freshly prepared enzyme loaded thiol-particles from a water suspension were imaged using a LEO1530 Gemini FEGSEM. Particles were prepared entrapping (a) β-galactosidase, (b) glucose oxidase and (c) acetylcholinesterase. The scale bar is 1 μm.

Table 1. Properties of three enzymes and thiol-particle systems studied to date.

Enzyme	Molecular weight (kDa)	pI	Estimated hydrodynamic diameter (nm)	Particle size (nm)	
				Smaller	Larger
β-galactosidase	465	4.6	16	100–200	300–500
Glucose oxidase	160	4.2	10	50–220	350–450
Acetylcholinesterase	280	5.5	12	50–170	350–450

enzymes and this means the method is potentially applicable to many applications in the biosensors or biocatalysis fields.

Figure 11 shows a comparison of FEGSEM images of samples of thiol-particles which contain either β-galactosidase, glucose oxidase or acetylcholinesterase. In all cases a bimodal distribution of particles can be seen and the particles look similar to each other (table 1).

4. Conclusions

In conclusion, we propose a novel methodology for the one-pot fabrication of silicate nanoparticles which contain active free thiol groups without the need for an additional surface modification step. As well as being a one-pot synthesis this novel method has important advantages such as that the production time of thiol-silicate nanoparticles is significantly reduced when compared to other recently described methods (from several hours to 30 min). These thiol-silicate nanoparticles formed with 3-mercaptopropyltrimethoxy silane do not aggregate as would be desirable for their use in biosensor and biocatalysis applications. This is not the case when using other silane sources such as tetramethyl orthosilicate which produces particles that aggregate easily. Furthermore, the method is able to produce particles within the nanometre size range under neutral pH conditions and at room temperature without the use of ammonia or alcohol, which are often needed to produce silicate nanoparticles. Additionally, the lack of ammonia or alcohol in the fabrication process allows the possibility of entrapment of labile biological molecules, such as enzymes, within the particles.

A further advantage of the method we have presented here is that enzymes can be introduced to the thiol-silication reaction and produce biofunctionalized silicate nanoparticles which have available thiol groups which would allow them to be tethered to metal surfaces such as gold, which is a very common substrate used in the biosensors field, allowing the self-assembly of nanostructured biocatalytic or biosensor surfaces. We have also shown that the thiol-particles containing enzyme form in two distinct distributions. The bimodal distribution of particles has been separated into two distinct groups of different size ranges and it has been shown that the larger particles contain a considerably higher level of enzyme than the smaller particles, providing an insight into the mechanism of enzyme entrapment with the silication method. This is of great significance as it means that this method of entrapment may be used for a wide range of processes and devices which use silica-entrapped enzymes, such as in biosensors and biocatalytic reactors.

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