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Novel epoxy-silica nanoparticles to develop non-enzymatic colorimetric probe for analytical immuno/bioassays

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Abstract

We have developed a novel method to develop epoxy silica nanoparticles (EfSiNP) in a single pot. High surface coverage of epoxy functional groups between 150 and 57000 molecules per particles ($\sim 10^{13}$ – 10^{16} molecules/mL of 200 nm EfSiNPs) was achieved for different preparation conditions. We then created a red colored probe by conjugating Fuchsin dye to the epoxy functionalities of EfSiNPs. Anti-mouse IgG was co-immobilized with Fuchsin and their ratios were optimized for achieving optimum ratios by testing those in functional assays. Dye to antibody ratios were in good negative correlation with a coefficient of -1.00 measured at a confidence level of over 99%. We employed the developed non-enzymatic colorimetric immunonanoprobe for detecting mouse IgG in a direct immunoassay format. We achieved a sensitivity of 427 pg/mL with the assay.

Graphical Abstract

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Contributions

CKD conceived the idea. SB, JF and CKD developed and optimized the synthesis process. CKD, SB, and JM characterized the particles. CKD, JFR, and SLS analyzed and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.aca.2018.04.044>.

Keywords

Epoxy silane; Silica nanoparticles; Fuchsin; Tetraethylorthosilicate; Antibody; Conjugation; Crosslinking

1. Introduction

Physical properties, such as shape, size, charge, and functionalities of nanoparticles (NP) can easily be manipulated thus are widely used in analytical chemistry and biology [1–7]. This is attributed to our better understanding of NP formation and surface functionalization [8–12]. However, silica nanoparticles (SiNP) are still mostly favored for applications associated with surface functionalization [10], due to the availability of self-grafting polymer precursors, such as silanes, that exist in constructs containing variety of functional groups [1,7,13].

Bioconjugation onto nanoparticles serves an important role in various applications, such as immunoassays, drug delivery, and imaging [1,2,14–16]. SiNPs have been used for decades for these applications. Adding amine functionality on SiNP using 3-aminopropyltriethoxy silane [17] is by far the most common approach that allow for bioconjugation using several crosslinkers, such as amine to amine homobifunctional glutaraldehyde and carboxyl to amine heterobifunctional carbodiimide [1,18,19].

In this first ever report of creating epoxy-functionalized silica nanoparticles (EfSiNP) in a single pot process (Scheme 1), we have synthesized EfSiNPs by customized modification of ‘Bhakta method’, which is our published novel approach [20] and employed it for rapid bioconjugation and assay applications. Previously, Ishimura’s group synthesized one pot epoxy NPs by precipitating epoxy silane alone under different Stöber formulations [21]. They were able to synthesize NPs in only 0.5 mL batches with reaction times obscurely long extending between 1 and 3 days. There are several other reports grafting epoxy silanes on metal NPs, such as TiO₂ [22], Fe₃O₄ [23] etc. but only few reports were found pertaining to the epoxy functionalization of the pre-synthesized SiNPs [24–31]. The major disadvantage of grafting functionalities post synthesis is the poor surface coverage of the functionality-bearing chemical moieties. On the contrary several reports claim significant improvement of total number of functional groups and their distribution homogeneity on NP surface with co-condensation [6,32–34] with some reservations [35]. Therefore, we conceived the idea of functionalizing particles during their synthesis by adding epoxy silane into the backbone of the SiNP. This will also allow us to conjugate desired molecules on the surface of these particles, which we have demonstrated by conjugating fuchsin dye to the particle surface and created red colored silica nanoparticles.

In recent times several enzyme-free immunoassay approaches (Supplementary Table 1) have been developed. Enzyme-mimics, such as metal oxides [36,37], metal complexes/hemin [38], or Palladium-Iridium nanoparticles [39], can catalyze colorimetric substrate reactions similar to that of horseradish peroxidase constituting indirect colorimetric methods. Dye-doped colored particles have also been demonstrated as probes for enzyme-free immunoassays. Most of these particles are either colored dye-doped polymeric [40–44], *viz.* latex/polystyrene, or doped nanoparticles [2,29,45–47]. Inherently colored nanoparticles,

such as gold nanostructures, are also routinely employed in immunosensing applications [48]. Several intuitive strategies, such as gold nanoparticle-catalyzed decolorization [49], have also been reported for enzyme-free colorimetric immune/bioassays. In the present manuscript we demonstrated the development of red colored EfSiNPs via surface conjugation of fuchsin dye and employed it as a signal probe in an immunoassay.

We present herein: (i) novel single pot synthesis of epoxy-functionalized silica nanoparticles, and (ii) an approach to develop Fuchsin-conjugated non-enzymatic color probe for performing immuno/bioassays. In order to achieve these goals, we developed a nanoprobe by single pot conjugation of Fuchsin dye and anti-mouse IgGs to the EfSiNPs and employed the conjugates for performing non-enzymatic colorimetric immunoassays.

2. Experimental

2.1. Synthesis of epoxy-silica nanoparticle

SiNP were synthesized according to our previously described novel 'Bhakta process' [20]. Briefly, TEOS (90 mM) was first hydrolyzed in a basic ethanole–water medium by adding 2 M NaOH at a final concentration of 18 mM with continuous stirring (600 rpm). (3-Glycidyloxypropyl)trimethoxysilane (GPTMS) was then added to the reaction mix at final concentrations of 25 mM, 50 mM, 100 mM, and 150 mM. Each TEOS:GPTMS concoction was sampled at 5, 10, 20, and 30 min duration. Later, the condensation process was further carried out for an additional 60 min and all the samples were thoroughly washed three times each with ethanol and water, respectively. SiNPs synthesized following Bhakta process were employed as a control without any functionality. Later, a part of bare SiNP was grafted with epoxy silane (GPTMS) following our previously described process for amine silanization [6–8].

2.2. Fuchsin and antibody conjugation and quantification

Fuchsin and Protein conjugation with surface epoxy groups of selected EfSiNP was performed in 10 mM PBS at pH 8.0. Various dye to antibody molar ratios (mM), viz. 1:0, 1:0.001, 1:0.01, 1:0.1, 1:1, 0:1, were prepared and the concoction was added to 4.25 mg/mL EfSiNP and reaction was allowed to proceed for 60 min under continuous stirring at 200 rpm. Later particles were centrifuge washed 3 times with water and 3 times with PBS. The amount of loaded dye was quantified as described in Supplementary Information (SI). For quantification of antibody content [50] bicinchoninic acid (BCA) assay was performed [51]. All the conjugates were also tested via a functional assay in order to characterize best dye to antibody ratio to be further employed for developing immunoassay (SI).

An immunoassay was then performed to assess the functional parameters of conjugates (SI). Briefly, mouse IgG (10 mM PBS, pH 8.0) at 100 μ L/well was adsorbed in 48 wells of a customized microtiter plate [52] in 8 different concentrations 10 μ g/mL, 1 μ g/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, 10 pg/mL, 0 pg/mL by incubating for 2 h at 37 °C. Each concentration was repeated six times on each plate with three repeats in total (n = 18). Later, HRP-antibody and EfSiNP-antibody-dye conjugates were employed to perform and analyze the technical parameters of the immunoassays.

2.3. Analytical considerations

Limit of detection (LOD/sensitivity), repeatability (%CV), and recoveries were calculated for each data set in order to characterize the performance of the probe against enzymatic labels, such as horseradish peroxidase (HRP). LOD was calculated according to conventional method where Absorbance (LOD) = Absorbance (blank) $\pm$ 3SD (standard deviation).

3. Result and discussion

3.1. Epoxy silica nanoparticle synthesis

EfSiNPs were synthesized via co-condensation to create homogeneous coating of epoxy functionality on SiNP. The presence of epoxy groups on the EfSiNPs was confirmed with FTIR along with other biophysical approaches. Epoxy functionalization was successfully achieved as reported in previous several studies. However, the functionality was grafted on pre-synthesized NPs [24,26–31]; this method of functionalization, as now realized by researchers, creates a non-homogeneous coverage on the particle surface [5,32–34]. Ishimura has published the only report to address this bottleneck by synthesizing epoxy NPs directly using an epoxy silane precursor (2-(3,4-epoxycyclohexyl)ethyltrimethoxysilane) without employing TEOS [21]. In the absence of an appropriate precursor their method was constrained by obscurely longer duration of synthesis, *viz.* 1–3 days for 0.5 mL batches and also created inconsistent structures. Plausible reason to this finding may be that an epoxy silane contains only three (3) hydrolysable ‘OR’ groups in comparison to silicate (TEOS) that has a fourth ‘OR’ group. The lack of this fourth ‘OR’ group on epoxy silane may create enough structural hindrance for the condensation reaction to occur in all the directions that may result in a significant increase in reaction times due to slow kinetics. While compared against Ishimura’s one-pot synthesis where they employed condensation of only 2-(3,4-epoxycyclohexyl)ethyltrimethoxysilane in the presence of ammonium hydroxide, we synthesized EfSiNPs via condensation of TEOS and co-precipitation of epoxysilane [(3-Glycidyloxypropyl) trimethoxysilane] in water:ethanol medium with NaOH using our previously described ‘Bhakta method’ [8,20]. Unlike Ishimura’s approach, we can ably control the extent of functionalization as well as the sizes of the nanoparticles via modulating the time of addition of (3-Glycidyloxypropyl)trimethoxysilane in synthesis process and duration of the reaction. Also, NaOH, the catalyst in our method, is more potent than NH₄OH and has been explained in detail in one of our previous reports [8]. The most important aspect of our synthesis approach is that we were able to synthesize batches of 500 mL volumes within 2 h (1000 times bigger batch size and 12 times smaller synthesis time compared to Nakamura’s process). Therefore, our approach of synthesizing EfSiNP through co-precipitation of GPTMS with TEOS in water:ethanol medium have significantly addressed the issue of long synthesis times. We synthesized 500 mL batch in less than 2 h. The synthesized nanoparticles were consistent in their shape and size, as expected (Fig. 1). The presence of characteristic FTIR band for epoxy group at 1150 cm⁻¹ can be clearly distinguished, which was not observed in the bare SiNPs. In addition, characteristic bands for stretching C-H bending at 2950 cm⁻¹ and bending vibrational bands near 1400 cm⁻¹ also confirmed the presence of epoxy groups on the surface (Fig. 1g) [53,54].

In order to optimize the synthesis process, we worked out several GPTMS (epoxy silane) to TEOS compositions such that for each composition GPTMS was added after 5, 10, 20, and 30 min after initiating the nucleation process. This has helped us in understanding the effect of GPTMS on nucleation behavior (Supplementary Table 2). From the size analysis, with respect to SiNPs synthesized without GPTMS, the sizes of all EfSiNPs were significantly smaller for similar times of synthesis. With increasing concentration of GPTMS the sizes of EfSiNP decreased for all the GPTMS:TEOS ratios in the order of 0:90 > 2.5:9 > 5:9 > 10:9 > 15:9. In addition, we have consistently recorded that if GPTMS was added closer to the start of the nucleation process the EfSiNP sizes were smaller which increased in the order of the addition of GPTMS at 5 < 10 < 20 < 30 min. From our understanding of nucleation of precursors in SiNP synthesis [8] we can make an informed assumption that GPTMS is causing a disruption of the densification of TEOS on the silica seed. The reason may be that with only '3' hydrolysable groups on epoxy silane reaction is restricted for the number of reaction centers as against '4' groups on TEOS thus, cease to react with other silicate/silane molecules in the fourth direction, as discussed before, restricting the particle growth, as evident in our timescale studies (Fig. 1; Supplementary Table 2). Our findings of disruptive nucleation with the addition of GPTMS are consistent and clearly supported with those reported by Nakamura [21], where they synthesized materials by precipitating only epoxy silane that took between 1 and 3 days for their single-pot synthesis while co-condensation with TEOS allowed us to synthesize EfSiNP particles within 2 h.

We have quantified the amount of pendent epoxy groups for all samples in order to optimize GPTMS loading efficiency as a function of time of addition (Supplementary Table 1). By calculating recoveries of the conjugated Fuchsin, the number of epoxy groups was estimated. Fuchsin ranging between 10^{13} – 10^{16} molecules/mL EfSiNP solutions (Supplementary Table 2) indicates 150 to 57000 molecules per EfSiNP. Theoretically, a SiNP with effective diameter of 200 nm can accommodate ~59700 epoxy molecules while the obtained values are 300-fold–1.03-fold less than expected. Due to lower epoxy grafting with 25 mM GPTMS-based recipe we chose to further investigate samples with 50 mM GPTMS since for this recipe the particle size and epoxy-loading efficiency were in good balance.

We employed 50 mM GPTMS-based strategy for further investigating the effect of times of addition on sizes and particle charges and employed pre-synthesized SiNPs grafted with epoxy silane prepared according to our previous protocol (Dixit et al., 2016; Roy et al., 2012b, 2010) as a control. By delaying GPTMS addition the particle sizes increased and zeta potential became more positive in the order of 5 < 10 < 15 < 20 > 30 min (Table 1). The zeta potential shift for 5 and 10 min EfSiNPs with respect to unfunctionalized SiNPs was similar to that reported for grafting-based approaches [55,56]; however, EfSiNP batches with GPTMS addition between 15 and 30 min have significantly higher positive shifts than grafting-based methods. This indicates that delaying the GPTMS addition by 15 min or more allowed for a better seeding of the TEOS creating a stable core nucleus, which apparently improved the holding capacity of GPTMS as a function of particle growth. Although a stable seeding may be required for effective GPTMS co-condensation, the epoxy loading efficiency of our particles, as quantified (Fig. 2), were at least 100-fold higher than grafting GPTMS on pre-synthesized SiNPs. Fuchsin recoveries were in a range of 10^{15} – 10^{16}

molecules/mL EfSiNP solution (Fig. 2) indicating 7000 to 57000 molecules per EfSiNP at an approximate surface density between 0.06 and 0.45 epoxy groups per nm², respectively. For grafting-based epoxylated SiNP we recovered 10¹³ Fuchsin molecules/mL of SiNP solution. At least 100- to 1000- fold excess of Fuchsin was conjugated on EfSiNP with respect to the latter indicating that co-condensation is a better approach to functionalize NPs.

3.2. Antibody conjugation and immunoassay performance

Linker-free single-step conjugation of dyes is crucial for developing enzyme-free colorimetric probes. Epoxy functional groups can facilitate this direct conjugation [57]. For creating an effective immunonanoprobe anti-mouse IgG should be co-immobilized with Fuchsin on EfSiNPs. Therefore, we optimized the ratios of Fuchsin to the antibody in order to achieve best assay functionality. Fuchsin and antibody were conjugated to epoxy groups of EfSiNPs (4.25 mg/mL; as employed for epoxy group quantification) in various ratios, *viz.* 1:0, 1:0.001, 1:0.01, 1:0.1, 1:1, 0:1, respectively.

We have employed BCA assay for antibodies (SI Fig. 1a) and Fuchsin standard curve analysis by microplate-based assays (SI Fig. 1b). In all the concoctions, Fuchsin concentration was kept constant at 1 mM while obtained recoveries (SI Table 1) indicate that only 95.9 nM Fuchsin was bound to the surface, which is ~10⁴-folds less than the concentration used in this study. Therefore, we offset the total bound Fuchsin to 95.9 nM that corresponds to 50 mM GPTMS EfSiNPs at 20 min addition time as 100% (Fig. 2a). Similarly, antibody recoveries from these samples were calculated with respect to the amount added for each respective concentration. We recovered Fuchsin between 85 and 103% (81.5–98.7 nM) while antibody recoveries were in a range of 90–115% (Fig. 3a); these recoveries were within the range recommended by the US FDA for bioanalytical methods. There was a strong negative correlation between Fuchsin and antibody recoveries (Fig. 3b) indicating a decrease in the amount of conjugated dye with respect to increasing antibody concentration. This suggests that antibody is binding significantly as a function of increasing concentration. This behavior may be attributed to the presence of ~84 primary amines [58] against only 2 reactive amines on the Fuchsin molecule. Based on these findings we employed 1000:1 and 100:1 ratios of dye to antibody for functional testing via a direct immunoassay using 10 mg/mL particle concentration for each. Assays were linear for both the ratios; however, higher sensitivity was observed with 1000:1 dye to antibody ratio, as expected (Supplementary Fig. 2). In addition, the recoveries for each coated concentration of antibody were within 10% deviation, *viz.* for 10 µg/mL surface coating concentration of antibody we recovered between 9 and 11 µg/mL from functionality testing. According to the US FDA guidelines, recoveries of analytes in a sample using optical methods should be within 80–120% of the original concentration and we were well within that range. Based on these experiments we employed 1000:1 dye to antibody ratio.

Optimized EfSiNP-Fuchsin-antimouse IgG nanoprobe was then employed for detecting various concentrations of mouse IgG adsorbed on the microtiter plate as previously reported [59–61]. Six replicates of each concentration was tested per run on three different days (n = 18). For validation, an assay was performed in parallel using antimouse IgG-HRP conjugate towards colorimetric detection; standard curves for both these assays are in SI (SI Fig. 3).

With the developed biosensor we obtained a sensitivity of 427 pg/mL, which was ~15 fold less sensitive than enzyme-based colorimetric assay that has a sensitivity of 28 pg/mL (Fig. 4). Both the non-enzymatic and enzymatic colorimetric immunoassays were in strong linear correlation up to higher picogram concentrations of mouse IgG confirming adequacy of the developed probe in detecting low analyte concentrations significantly lower than the concentrations of most biomarkers of clinical relevance that are in nanogram ranges [62]. Although at a lower sensitivity the developed nanoprobe can effectively be employed for creating immune/bioassays. Our findings also corroborate results from previous studies pertaining to colorimetric assays [44,63,64].

In several other non-enzymatic immunoassays, which were mostly based on chromogenic substrates and enzyme-less heavy metal catalysis (SI Table 1), the assay sensitivities were within a range of lower nanograms to lower picograms. Sensitivities recorded in current study are within the range as reported by previous studies [29,43,47,48]. For any type of nanoparticle-based optical probes, color or fluorescence dyes were entrapped within the particles during synthesis [6]. One big challenge with these particles is customization according to the need. If the entrapped dye is to be changed then the whole synthesis process needs to be optimized again. We have addressed this lacuna with our epoxy silica nanoparticles. Dyes can be conjugated on the surface of these particles as demonstrated with the Fuchsin dye thus obviating the need of optimization of the whole process. In addition, various indicator dyes, such as in our case with Fuchsin, can be conjugated to EfSiNPs reported here and employed for monitoring physicochemical properties like solution pH [65,66]. Nanoparticle conjugated dyes can also be employed for developing lateral flow assays [66]. However, dyes with poor stability that need to avoid direct contact with the microenvironments cannot be used in our system and are better off entrapped within the particles [7].

4. Conclusions

Results above demonstrate successful synthesis of EfSiNPs in single pot with high density pendent epoxy groups on the NP surface. We used the particles for linker-free conjugation of dyes and proteins, *viz.* antibody, either alone or in combination. In addition, we established that EfSiNPs can be used for analytical applications. Successful development of a non-enzymatic colorimetric immunoassay using our novel Fuchsin-conjugated EfSiNPs validates these claims. EfSiNPs thus holds significant potential in various other applications with a broader coverage including point-of-care diagnostic analyses. Also, our novel EfSiNP-dye/antibody conjugates could host cheap alternative detection probes for resource-limited settings as indicated in the preparatory cost analysis (SI Table 3). HRP (20 mg) alone costs about USD 85.0 while crosslinkers and other reagents are additional. For 400 mg EfSiNP the total cost is about USD 50.0. Easy single step conjugation of amine containing chemical moieties, such as Rhodamine, amino acids, and proteins, also allows for customization of the probe and can be potentially used for various applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- First report of very fast synthesis of epoxy functionalized silica nanoparticles in a scalable single pot approach.
- Epoxy functionalized silica nanoparticles can be used for single step immobilization of variety of dyes like fuchsin and biomolecules.
- Fuchsin conjugated nanoparticles can be employed as enzyme/chemical reaction-free colorimetric probe for faster assay to result turnaround time.

Fig. 1.

TEM images of epoxy-silica nanoparticles synthesized by adding 3-Glycidyloxypropyltrimethoxysilane at different time; (a) 5 min, (b) 10 min, (c) 15 min, (d) 20 min, (e) 30 min, and (f) no-epoxy. As expected, the sizes increase with time of addition of the epoxysilane due to less cross-condensation process. (g) FTIR Spectra of 'i' bare silica nanoparticles and 'ii' epoxy-silica nanoparticles, where characteristic epoxy group peak at 1150 cm^{-1} along with stretching C-H bending at 2950 cm^{-1} and bending vibrational bands at 1400 cm^{-1} are clearly distinguishable compared to g.i.

Fig. 2. Surface epoxy functional group quantification, as depicted in 'a', by conjugating Fuchsin dye followed by recovering the amount of attached dye against its standard curve (b). Assumption is number of bound Fuchsin is equal to free pendent epoxy groups.

Fig. 3. Fuchsin dye and antimouse IgG antibody quantification after conjugation with EfSiNPs. (a) Dye and antibody recoveries were estimated using their respective standard curves. All the recoveries were within the US FDA stipulated range of 80–120% for bioanalytical measurements. (b) A perfect negative correlation (with coefficient -1.0) was established indicating that antibodies at higher concentration tends to decrease Fuchsin binding significantly due to higher number of competing amines per antibody (~ 84).

Fig. 4.

(a) Performance of non-enzymatic colorimetric assay against conventional ELISA. In immunoassay readouts using both methods were plotted indicating better sensitivities of enzymatic method against non-enzymatic colorimetry. (b) A positive correlation (with coefficient 1.0) was established between both approaches indicating usefulness of non-enzymatic colorimetric analysis in resource-limited settings.

Scheme 1.

Schematic presentation of epoxy-silica nanoparticle synthesis. Step 1 represents the starting of condensation process of tetraethylorthosilica (TEOS) in water-ethanol medium with NaOH as base catalyst. Step 2 describes the formation of epoxy-silica nanoparticle after addition of (3-Glycidyloxypropyl)trimethoxysilane at different time. The time of epoxysilane addition controls the size and surface medication of these epoxy-silica nanoparticles. Step 3 demonstrates the application of these nanoparticles for easy and faster conjugation of amine-containing biomolecules; top is Fuchsin conjugation that imparted the red color while lower panel is Fuchsin and antibody co-conjugation.

Table 1

Sizes and zeta-potentials of different silica nanoparticles in 1:1 water-ethanol medium ^a.

GPTMS addition time (min)	Hydrodynamic size (nm)	TEM size (nm)	Zeta Potential (mV)
5	198	105	-55.2 (±5)
10	226	140	-46.1 (±3)
15	201	162	-39.7 (±1)
20	314	235	-30.5 (±1)
30	332	280	-35.3 (±2)
SiNP Bhakta method	450	385	-62.5 (±3)
SiNP Bhakta method – GPTMS grafted	461	386	-56.3 (±2)

^a Tetraethylorthosilicate concentration was 90 mM and GPMS concentration was 50 mM.