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Charge-Neutral, Stable, Non-Cytotoxic, Near-Infrared SnS Aqueous Quantum Dots for High Signal-to-Noise-Ratio Biomedical Imaging

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Abstract

We have synthesized charge-neutral, stable, non-cytotoxic, bright, near-infrared (NIR) SnS quantum dots (QDs) by first making Cysteamine-capped SnS QDs in glycerol under acidic conditions followed by lengthening the capping molecule with peptide bonds by reacting the capping molecules with glycine and subsequent heat treatment at 200°C for 4 hours. The obtained stable SnS QDs exhibited a band gap of 1.5 eV and a strong, narrow NIR emission peak at 830 nm with a quantum yield of 4.6%. The suspension could be stable for more than 1 month without aggregation or emission decay. The positively charged SnS QDs were further neutralized by 3-mercaptopropionic acid (MPA) through electrostatic attraction. The MPA neutralized SnS QDs were shown to be non-cytotoxic at concentrations 6 times the typical QDs concentration for immunostaining. Low-noise, optimal NIR immunofluorescent imaging of vascular endothelial growth factor (VEGF) on 3T3 cells and Tn antigen on HT29 cells was achieved by using streptavidin (SA)-linked MPA-neutralized SnS QDs with a SA:QD molar ratio of 22:1 to bind to biotinylated secondary antibody bound on the primary antibody that was bound on the targeted antigen on the cell membrane with a high signal-to noise ratio (SNR) of 35.

Keywords

Bioimaging; Near-infrared; Non-cytotoxic; Quantum Dots; Tin Sulfide

1. Introduction

Near infrared (NIR) fluorescence plays an important role in deep tissue imaging because it can avoid the tissue autofluorescence in the visible range^[1] and the strong absorption of visible light by tissues.^[2] Among the popular fluorescence probes is the cyanine dyes and indocyanine green (ICG) is the only FDA approved probe for *in vivo* imaging. However, ICG has a low intrinsic quantum yield which decreases drastically in aqueous environment.

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Conflict of Interest

The authors declare no conflict of interest.

[3] Meanwhile, semiconductor quantum dots (QDs) have good quantum yield with narrow emission wavelengths tunable by particle size. So far, the potential of using QDs for *in vivo* imaging have been largely unfulfilled due in part to the fact that most QDs emit visible light. As such, NIR QDs have been investigated for *in vivo* imaging applications including visual guide for sentinel-lymph-node mapping^[4] and intra-operative imaging-guided surgery.^[5] However, so far NIR QDs such as CdTeSe/CdS, InAs/InP/ZnSe, PbS, and CdHgTe/ZnS^[6] all contain toxic and/or heavy-metal elements, making them unfit for *in vivo* imaging. It was reported the toxicity of CdSe QDs is due to deterioration of the particle surface over time and heavy metal release,^[7] which limits them for *in vivo* application. Also for NIR QDs, CdTe QDs and CdTe/CdS QDs showed cytotoxicity due to the release of Cd²⁺ ions from surface of QDs into the cytoplasm.^[8] Ag₂Se and Ag₂S QDs also exhibit photoluminescence in the NIR range.^[9] Ag₂Se QDs have been prepared in organic solvents^[10] which needs solvent exchange from organic to aqueous for *in vivo* applications. However, the PL intensity and stability of the QDs were compromised drastically.^[11] Hocaoglu *et al.* synthesized Ag S^[12] 2 under aqueous conditions with 2-mercaptopropionic acid as the capping molecule. However, the highly negatively charged surface may increase non-specific binding with cell surface.^[13] Therefore, NIR QDs that do not contain toxic heavy metal elements and are made in aqueous condition with charge-neutral surface is needed for *in vivo* NIR imaging.

SnS is a good candidate material for such NIR QDs in that Sn is much more tolerant than other heavy metals to human. Also the band gap and emission peak of bulk SnS are both in the NIR range.^[14] Meanwhile the minimal risk level (MRL) of Sn to humans is 0.3 mg/kg/day,^[15] which is far larger than the typical amounts of Sn needed during imaging. A major challenge for making SnS QDs (i.e., nanoparticles) for biomedical applications is that SnS has high solubility in water and dissolves under neutral and basic pH conditions (see supporting information Figure S1). For SnS QDs to be viable for *in vivo* imaging, they must resist dissolution and remain stable in water at neutral pH. Several studies have examined the syntheses of SnS QDs in ethylene glycol^[16] or in water^[16b]. SnS thin films and SnS QDs have also been investigated for potential solar cells applications.^[17] However, these studies did not address the stability of SnS QDs in an aqueous environment, especially at neutral pH. Nor were there studies assessing the cytotoxicity of SnS QDs or the ease of conjugation of SnS QDs to receptors. These concerns are essential for *in vivo* imaging.

The goal of this study is to synthesize SnS QDs that are stable in an aqueous environment, non-cytotoxic, as well as easy to conjugate for potential *in vitro* and *in vivo* biomedical imaging applications. To make SnS QDs suspensions stable our strategy was to lengthen the capping molecules because long capping molecules could (1) provide sufficiently large inter-particle interaction energy barrier to prevent QD aggregation^[18] and (2) create diffusion barriers to minimize the dissolution of the QDs. Since SnS is known to precipitate only under acidic conditions, we will first synthesize SnS nanoparticles under acidic conditions in glycerol using cysteamine (Cys) as the capping molecule. The thiol group on one end of cysteamine will chelate to the metal cation,^[19] Sn²⁺. With further adjustment of the amount of cysteamine and Sn, bright photo-luminescent SnS was obtained. A schematic of the Cys-capped SnS QDs is shown in Scheme 1(a). The positive charge of the amine on the other end of cysteamine and the high viscosity of glycerol stabilize the SnS nanoparticles. We will call

thus synthesized nanoparticles SnS seed QDs because they tended to be small and not fully crystallized. Fully crystallized and stable SnS QDs with bright photoluminescence will be obtained by subjecting SnS seed QDs to repeated reaction with glycine at neutral pH to lengthen the capping molecule and further heat treatment. The formation of the peptide bond between the carboxyl group of the glycine and the amine group of the cysteamine on the QDs surface is schematically shown in Scheme 1(b). The same process is repeated to further lengthen the capping molecules as schematically shown in Scheme 1(c). The details of the synthesis and capping molecule lengthening are described in Experimental Section.

Finally, non-cytotoxic SnS QDs were achieved by neutralizing the positive charges of the amine groups on the QD surface with the negatively charged 3-mercaptopropionic acid (MPA). The obtained MPA-neutralized SnS QDs exhibited an NIR emission peak at 830 nm with a quantum yield of 4.6%, were non-cytotoxic under the immunostaining conditions that permitted NIR immunofluorescent imaging with an unprecedented high signal-to-noise ratio (SNR) of 35.

2. Results and discussions

Figure 1(a) shows that particle size distributions of SnS QDs in glycerol after capping molecule lengthening with various Gly/Cys ratios and heat treatment at 200°C for 4 hours by Zetasizer. As can be seen, without lengthening (Gly/Cys=0), the size of the SnS QDs after heat-treatment was 16.7 ± 3.2 nm. With lengthening of the capping molecules by peptide bond formation, the size of the SnS QDs in glycerol after heat treatment increased from 16.7 ± 3.2 nm to 18.4 ± 3.6 nm, 19.9 ± 3.5 nm, 21.1 ± 4.0 nm and 22.4 ± 4.1 nm, corresponding to an increase of the size by 1.7 nm, 3.2 nm, 4.4 nm, and 5.7 nm for Gly/Cys=10, 20, 30, and 40, respectively. A peptide bond consists of one carbon-carbon bond and two carbon-nitrogen bonds and is about 0.4 nm in length. Thus adding 1 peptide bond would lengthen the capping molecules by 0.4 nm and increase the hydrodynamic size of the SnS QDs by 0.8 nm. Therefore, the increase of hydrodynamic size of the QDs by 1.7 nm, 3.2 nm, 4.4 nm, and 5.7 nm with Gly/Cys =10, 20, 30, and 40 corresponds to lengthening the capping molecules by 2.1, 4, 5.5, and 7.1 peptide bonds, respectively. The average size versus time of SnS QDs with capping molecules modified with various Gly/Cys ratios and after the heat treatment is shown Figure 1(b). As can be seen, the SnS QDs without glycine lengthening (Gly/Cys=0) aggregated even in a viscous medium such as glycerol resulting in an increasing size over time. The aggregation slowed with an increasing Gly/Cys ratios, and with Gly/Cys=30 and 40, the average size of the SnS QDs did not change for more than a month. Furthermore, the PL intensity of the SnS QDs with Gly/Cys=40 showed no decrease even after one month (see supporting information Figure S5). As longer capping molecules are known to be able^[18d] to maintain the stability of nanoparticles, the observed increased particle size with an increasing Gly/Cys ratio shown in Figure 1(a) is consistent with the results in Figure 1(b) that the stability of the SnS QDs improved with an increasing Gly/Cys ratio. Since SnS QDs modified with Gly/Cys =40 were most stable, all the following conjugation, immunostaining, and cytotoxicity studies were based on the SnS QDs modified with a Gly/Cys=40 and heat treated at 200°C for 4 hours unless otherwise specified. Higher Gly/Cys ratios were not examined in the current study and would be considered in future study.

Figure 2 shows the FT-IR spectra of glycine (Gly), SnS QDs before lengthening (Gly/Cys=0) and SnS QDs after lengthening with Gly/Cys=10 and with Gly/Cys=40. Most notably, a new peak at 1645 cm^{-1} emerged after lengthening with glycine that corresponds to the Amide I peak related to the stretching of the carbonyl (C=O) of the amide bond^[20]. The amide I peak was unique for the peptide bond formation because no such peak existed before mixing the glycine and the cysteamine-capped QDs. Furthermore, the peak intensity of the amide I peak increased with an increasing Gly/Cys ratio indicating that more peptide bonds were formed when more glycine was added during the lengthening procedure.

The photoluminescence spectrum and the UV absorbance spectrum of the SnS QD in glycerol obtained with Gly/Cys=40 and treated at $200\text{ }^{\circ}\text{C}$ for 4 h are shown in Figure 3. The photoluminescence spectrum obtained with an excitation wavelength of 690 nm exhibited a narrow NIR emission peak at around 830 nm. The UV-vis absorbance spectrum showed that the SnS QDs absorbed light above wavelength of 700 nm into the NIR range. The absorption spectrum was replotted according to the Tauc equation^[21] as shown in the insert. The band gap of the SnS QDs was estimated to be 1.5 eV (827 nm) as the x-intercept in the Tauc plot. This estimated band gap was consistent with an emission peak at around 830 nm and was also consistent with the band gap of SnS nanoflowers^[22].

To measure the quantum yield (QY) of the SnS QDs, Rhodamine 101 (RD) (Fisher Scientific, Fairlawn, NJ) which has a QY of 100% was used as the reference. For QY study, the absorbance and emission spectra of diluted RD solutions and diluted SnS QD suspensions were measured with USB4000 and PTI, respectively. The integrated emission intensity was plotted versus the absorbance for both the RD solutions and SnS QD suspensions as shown in Figure 4. Since RD has QY of 100%, the QY of the SnS QD was simply taken as the ratio of the slope of the SnS QDs to that of RD with the QY of RD. The thus obtained QY of the SnS QDs was $4.6 \pm 0.6\%$. In recent reviews on NIR quantum dots, the quantum yields are in the order of 20%–30 %.^[23] However, those quantum dots all contain heavy metals such as Cd, Te, etc. which are not friendly to human and the environment.

In Figure 5(a) we show the XRD pattern of the SnS QDs in the powder form as precipitated from the suspension by adding acetone. The XRD pattern shows that the SnS QDs had a cubic zincblende structure consistent with that found in SnS films.^[24] The crystallite size of the SnS QDs was estimated to be 3.6 nm using Scherrer formula^[25] using the width of the (111) peak in Figure 5(a). The TEM micrographs of the SnS QDs with Gly/Cys=40 before and after heat treatment are shown in Figure 5(b) and (c). A high resolution TEM micrograph is shown in Figure 5(c) which shows the crystalline fringes of the SnS QDs after heat treatment. The size of the SnS QDs was estimated from Figure 5(c) to be $13 \pm 6\text{ nm}$, which was smaller than the $22.4 \pm 4.1\text{ nm}$ measured by Zetasizer. However, TEM images generally do not include the capping molecules. Given the size of cysteamine, a TEM size of $13 \pm 6\text{ nm}$ was consistent with the hydrodynamic size of $16.7 \pm 3.2\text{ nm}$ for SnS QDs with Gly/Cys=0. The TEM images in Figure 5 were not very sharp resulting in a larger standard deviation. Therefore, if we use the standard deviation as a measure of the width of the size distribution, the results from TEM give a larger size distribution than that from Zetasizer.

With the crystallite size estimated to be 3.6 nm from XRD, this indicates that each SnS QD was consisted of several crystallites.

The results of MTS tests are shown in Figure 6 where the relative viability of the 3T3 cells incubated with un-modified SnS QDs (full squares) for 24 hours and that of 3T3 cells incubated with MPA-neutralized SnS QDs (open circles) for 24 hours are plotted versus the concentration of the QDs. As can be seen, with untreated SnS QDs, the viability of 3T3 cells dropped sharply well within the 100 μM range with an LC50 (lethal concentration at 50% viability) of about 43 μM . The high cytotoxicity of the untreated SnS QDs was attributed to the positive charges on the QD surface as positively charged particles are known to exhibit greater cell penetration and internalization than negatively charged particles.^[26] To reduce cytotoxicity, we modified the SnS QDs by adding 0.01 M of negatively charged MPA to the suspension to neutralize the positive charges of the amine groups on the SnS QD surface. As a result, the zeta potential changed from 32 ± 3 mV for the unmodified SnS QDs to 1 ± 2 mV for the MPA-modified SnS QDs (see supporting information Figure S6). Clearly, the MPA-neutralized SnS QDs exhibited much reduced cytotoxicity with the relative cell viability remained above 90% for concentrations below 250 μM and an LC50 approaching 750 μM —a 17-fold increase from that of untreated SnS QDs. Note that a typical SnS QD concentration used for immunostaining was 80 μM , which was well below the concentration of 250 μM where the relative cell viability was above 90%. Clearly, MPA-modified SnS QDs were not cytotoxic under immunostaining conditions. Cytotoxicity of MPA-neutralized SnS QDs was also tested for a longer time, and the LD50 was found to be 500 μM after 72 hours of incubation (see the supporting information Figure S7).

The MPA neutralization of surface charge also plays an important role in the stability of SnS QDs in cell culture. Figure 7 shows the size versus time of the MPA-neutralized SnS QDs and that of the unmodified SnS QDs in cell culture medium. Clearly, the unmodified SnS QDs were unstable whose size increased with time in cell culture medium. However, MPA-neutralized SnS QDs were stable in culture medium—where the size of the QDs remained constant with time. This was likely due to the fact that charge-neutral particles have less interaction with proteins such as bovine serum albumin (BSA) as demonstrated by Park *et al.*^[13]

For immunostaining, we used streptavidin (SA) to conjugate MPA-neutralized SnS QDs to stain the biotinylated secondary antibodies that were linked to the primary antibodies bound to the antigen on the cell membrane through the streptavidin-biotin linkage. To optimize the immunostaining results, the molar ratio SA:QD was examined in the range of 6:1 to 26:1 targeting the VEGF on the 3T3 cells for imaging. Figure 8(a)–9(d) show the immunostaining images of VEGF on 3T3 cells for SA:QD molar ratio=6:1, 14:1, 22:1, and 26:1, respectively. As can be seen, the staining using a SA:QD=22:1 in Figure 8(c) exhibited the brightest image. We further analyze the fluorescent intensity per unit area within each cell using ImageJ.^[27] In Figure 8(e) we show the average fluorescent intensity per unit area on each 3T3 cell versus the SA:QD ratio. Indeed, the fluorescent intensity per unit area was optimal at about 69 ± 10 with SA:QD =22:1, as consistent with the images shown in Figure 8(a)–8(d).

We expect the same optimal PL intensity at SA:QD ratio of 22:1 would apply for HT29 cells as well. This is because even though the amount of Tn-antigen on the HT29 may be different from that of VEGF on the 3T3 cells and required a different amount of SA to saturate the available biotins of the bound antibodies, the number of QDs that could be conjugated with SA would be limited by the saturated SA:QD ratio. Therefore, we expect the saturated PL intensity for HT29 cells would be 22:1, the same as with 3T3 cells. Consequently, we used the same SA:QD ratio of 22:1 to stain both VEGF on 3T3 cells and Tn antigen on HT29 cells and compared the stained fluorescent image and fluorescent intensity per unit area on each cell with the negative control. Figure 9 shows the fluorescence images of VEGF staining on 3T3 cells (row I), Tn-antigen staining on HT29 cells (row II) stained first with the primary antibody, followed by biotinylated secondary antibody and SA-linked SnS QDs conjugated with a SA:QD=22:1, and the negative control staining on HT29 cells (row III) which had the secondary antibody and SA-linked SnS QDs conjugated with a SA:QD=22:1 but without the primary antibody binding step. The first, second, and third columns (from left to right) of each row show the images of DAPI staining, QD staining, and the overlay of DAPI and QD staining, respectively. As can be seen, the SA-conjugated QDs could image VEGF on 3T3 cells (row I) and Tn antigen on HT29 (row II) brightly while the negative control that skipped the primary anti-Tn antigen antibody step show no QD staining on the HT29 cells. Furthermore, the average fluorescent intensities per unit area due to QD staining within each cell as determined using ImageJ in arbitrary units (a.u.) was 69 ± 10 (a.u.) for VEGF on 3T3 cells (row I), 73 ± 4 (a.u.) for Tn antigen on HT29 (row II), and 2 ± 2 (a.u.) for the negative control with no primary antibody step on HT29 (row III). As can be seen, the average fluorescent intensity of the negative control was low with small standard deviation. In addition to effective blocking of non-specific binding of the SA-conjugated QDs to cells the low fluorescent intensity of the negative control could be attributed to the fact that there was little cellular autofluorescence in the NIR range. Although the average fluorescent intensity of the negative control was only obtained on the HT29 cells it is likely that similarly low average fluorescent intensity per unit area will be obtained for the 3T3 cells. For these reasons, we will use the average fluorescent intensity per unit area on the negative control from HT29, 2 ± 2 (a.u.) as the noise level for both the VEGF staining on 3T3 cells (row I) and the Tn antigen staining on HT29 cells (row II) and the obtained signal to noise ratio (SNR) of the VEGF staining of 3T3 cells and that of the Tn antigen staining of HT29 cells were 34 and 35, respectively, well above the accepted minimal SNR of 3.

3. Conclusions

In summary, we have successfully synthesized charge-neutral, stable SnS QDs by first making Cys-capped SnS seed QDs in glycerol in acidic conditions followed by lengthening the capping molecule by repeatedly reacting with Gly to form peptide bonds in the presence of EDC and NHS in neutral pH and subsequent heat treatment at 200°C for 4 hours. The obtained SnS QDs exhibited a band gap of 1.5 eV as determined by the Tauc plot and a strong, narrow NIR photoluminescence peak at 830 nm wavelength with a quantum yield of 4.6%. With a Gly/Cys = 40, the obtained SnS QDs suspension were stable for more than 1 month against aggregation with hydrodynamic size of 22.4 nm. The positively-charged amine groups on the QD surface were further neutralized by adding MPA. The MPA

neutralized SnS QDs were shown to be non-cytotoxic up to a QD concentration 6-times higher than a typical concentration for immunostaining. The MPA-neutralized SnS QDs were optimally conjugated to SA at a SA:QD molar ratio of 22:1 and demonstrated highly effective immunofluorescent-imaging of VEGF on 3T3 cells and Tn antigen on HT29 cells with a high SNR of 34 and 35, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) The day 0 size distribution and (b) the average cluster size versus time of SnS QDs after lengthening with Gly/Cys = 0 (■), 10 (●), 20 (▲), 30 (◆), and 40 (★) ratios and heat treatment in glycerol at 200 °C for 4 h. To show what the suspensions look like, as examples, inset I and II in (a) respectively show the photograph of the suspension with Gly/Cys = 0 at day 0 and that of the suspension with Gly/Cys = 40 at day 0.

Figure 2.

FT-IR spectra of glycine, SnS QDs before lengthening (Gly/Cys=0) and SnS QDs after lengthening with Gly/Cys= 10 and Gly/Cys =40.

Figure 3. Photoluminescence (right) and UV-vis absorbance (left) of SnS QDs suspensions. The inset shows the band gap of 1.5 eV (827 nm) as estimated using the Tauc equation based on UV-vis absorbance.

Figure 4. Integrated fluorescence intensity versus integrated absorption of SnS QDs (●) and Rhodamine 101 (■). The quantum yield (QY) of SnS QDs was determined as the ratio of the slope of the integrated fluorescence intensity versus absorbance of the QDs to that of Rhodamine 101.

Figure 5.

(a) A typical X-ray diffraction (XRD) pattern of SnS QDs obtained after capping-molecule lengthening with Gly/Cys=40 and heat treatment at 200°C for 4 h that showed a cubic zincblende structure; (b) TEM images of the SnS QDs after capping-molecule lengthening with Gly/Cys=40 (b) before and (c) after heat treatment. The inserts in (b) and (c) indicated that the SnS with Gly/Cys =40 addition in capping molecule lengthening had a size of 5 ± 2 nm and 13 ± 6 nm before and after heat treatment, respectively.

Figure 6. Viability of 3T3 cells versus concentration of unmodified SnS QDs and MPA-neutralized SnS QDs after 24 h. The dash line at 80 μM indicates the $<100 \mu\text{M}$ concentration range used for imaging study.

Figure 7. Particle size versus time of positively-charged unmodified SnS QDs (■) and MPA-modified SnS QDs (●) in cell culture media.

Figure 8. Fluorescent images of 3T3 cells immuno-stained with SnS QDs with an SA:QD ratio of (a) 6:1, (b) 14:1, (c) 22:1, and (d) 26:1, and (e) average fluorescent intensity per unit area exhibited by the 3T3 cells versus the SA:QD ratio of the SA-QD conjugates used for staining.

Figure 9.

Fluorescence images of VEGF staining on 3T3 cells (row I), Tn-antigen staining on HT29 cells (row II) first with the primary antibody, followed by biotinylated secondary antibody and SA-conjugated SnS QDs with a SA:QD=22:1, and negative control staining on HT29 cells (row III) which had the secondary antibody and SA-conjugated SnS QDs but without the primary antibody, as illustrated by the schematic at the end of each row. The first, second, and third columns (from left to right) of each row shows the images of DAPI staining, QD staining, and the overlay of DAPI and QD staining, respectively.

Scheme 1.

A schematic of (a) a cysteamine-capped SnS QD, (b) the reaction of the COOH of glycine to the NH₂ of the cysteamine on the QD surface in the presence of EDC and NHS to lengthen the capping molecule, and (c) further lengthening by repeated reaction of the COOH of glycine with the NH₂ of the capping molecules on the QD surface.