Silica Nanoparticles with Continuously Tunable Sizes: Synthesis and Size Effects on Cellular Contrast Imaging

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Controlling the size of silica nanoparticles (NPs) on a continuously variable scale was achieved by systematically varying the organic solvent(s) used in water-in-oil microemulsion synthesis. A number of individual as well as binary solvent mixtures were investigated for tuning silica NP size. The results demonstrated that the size of a silica NP was continuously tunable over as range of 20−100 nm by varying the alkane chain length of the organic solvent(s) being used. A simple physical model was proposed to describe the size effect and identify the principle factors needed for precisely controlling the size of a silica NP. In the model, the alkane chain length(s) of the organic solvent(s) primarily determined the average size of a silica NP when other synthetic conditions were fixed, whereas variance in size was affected by water droplet percolation with the precursor microemulsion. The significance and utility of these tunable silica NPs was evaluated for bioimaging applications. Specifically, dye-doped silica NPs of variable but precise size were used for in vitro contrast imaging of cells. The results demonstrated that precise control of silica NPs size can be used to reduce cytotoxicity, optimize luminescence signal intensity, and selectively discriminate between structures both inside and outside of cellular membranes.

1. Introduction

The size of silica-based nanoparticles (NPs) affects their physical, chemical, electrical, and optical properties. Traditionally, micrometer-sized silica particles have been used as catalyst substrates, pigments, stationary phase in chromatography columns, etc. Recently, nanometer-sized silica NPs have developed rapidly and become an important class of nanomaterial.[1–4] By themselves, the size-dependent properties of pure, nanoscale silica NPs are not remarkable. However, when these NPs are combined with various functional molecules, the impact of size becomes significant, especially for analysis applications.[5–10] The role of silica NP in these cases is usually as a supporting or entrapping matrix. Two major reasons make silica a useful matrix in this regard and particularly relevant in bioapplications. First, the surface of silica NPs is easily modified on the basis of well-established chemistry. With appropriate surface and internal functionality, these NPs can be linked to a variety of biorecognition agents in many different ways (e.g., antibodies, protein complexes, nucleic acids, aptamers, etc.).[1,2,11–15] Second, the negatively charged silica matrix itself provides numerous electrostatic binding sites to physically dope (i.e., adsorb) a wide variety of positively charged molecules.[12–20] When doped with dye molecules, these NPs become intensely luminescent reagents capable of sensitively signaling biological targets.[12–16] When doped with bioactive molecules and

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(1) Zhao, X.; Hilliard, L. R.; Wang, K.; Tan, W. In *Encyclopedia of Nanoscience and Nanotechnology*; Nalwa, H. S., Ed.; American Scientific Publishers: Stevenson Ranchm, CA, 2004; pp 255−268.
(2) Wang, L.; Wang, K.; Santra, S.; Zhao, X.; Hilliard, L. R.; Smith, J.; Wu, Y.; Tan, W. *Anal. Chem.* **2006**, *78*, 646–654.
(3) Yao, G.; Wang, L.; Wu, Y.; Smith, J.; Xu, J. Z.; , W.; Lee, E.; Tan, W. *Anal. Bioanal. Chem.* **2006**, *385*, 518–524.
(4) Burns, A.; Ow, H.; Wiesner, U. *Chem. Soc. Rev.* **2006**, *35*, 1028–1042.
(5) Sun, Y.; Yan, F.; Yang, W.; Zhao, S.; Yang, W.; Sun, C. *Anal. Bioanal. Chem.* **2007**, *387*, 1565–1572.
(6) Shang, W.; Nuffer, J. H.; Dordick, J. S.; Siegel, R. W. *Nano Lett.* **2007**, *7*, 1991–1995.
(7) Roach, P.; Farrar, D.; Perry, C. *J. Am. Chem. Soc.* **2006**, *128*, 3939–3945.
(8) Montalti, M.; Prodi, L.; Zaccheroni, N.; Battistini, G.; Marcuz, S.; Mancin, F.; Rampazzo, E.; Tonellato, U. *Langmuir* **2006**, *22*, 5877–5881.
(9) Vertegel, A. A.; Siegel, R. W.; Dordick, J. S. *Langmuir* **2004**, *20*, 6800–6807.
(10) Lundqvist, M.; Sethson, I.; Jonsson, B. H. *Langmuir* **2004**, *20*, 10639–10647.
(11) Wang, L.; Zhao, W.; O'Donoghue, M. B.; Tan, W. *Bioconjug. Chem.* **2007**, *18*, 297–301.
(12) Zhao, X.; Hilliard, L. R.; Mechery, S. J.; Wang, Y.; Bagwe, R. P.; Jin, S.; Tan, W. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15027–15032.
(13) Zhao, X.; Tapec-Dytioco, R.; Tan, W. *J. Am. Chem. Soc.* **2003**, *125*, 11474–11475.
(14) Santra, S.; Zhang, P.; Wang, K.; Tapec, R.; Tan, W. *Anal. Chem.* **2001**, *73*, 4988–4993.
(15) Santra, S.; Wang, K.; Tapec, R.; Tan, W. *J. Biomed. Opt.* **2001**, *6*, 160–166.
(16) Bagwe, R. P.; Hilliard, L. R.; Tan, W. *Langmuir* **2006**, *22*, 4357–4362.
(17) He, X. X.; Wang, K.; Tan, W.; Liu, B.; Lin, X.; He, C.; Li, D.; Huang, S.; Li, J. *J. Am. Chem. Soc.* **2003**, *125*, 7168–7169.
(18) Luo, D.; Saltzman, W. M. *Nat. Biotechnol.* **2000**, *18*, 893–895.
(19) Luo, D.; Han, E.; Belcheva, N.; Saltzman, W. M. *J. Controlled Release* **2004**, *95*, 333–341.
(20) Luo, D.; Saltzman, W. M. *Gene Ther.* **2006**, *13*, 585–586.

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medicines, these NPs can also serve as drug delivery vehicles capable of controlling the quantity and time of release.[17–20]

The size-dependent properties of silica-based nanomaterials are varied but can be tied to three mainly beneficial effects. In general, smaller sizes provide a higher surface area to volume ratio, better mobility, and faster reactivity than larger sizes. Meanwhile, a small size may lead to negative effects in some applications. For instance, a small size allows deeper penetration of nanomaterials into biological and environmental substrates, resulting in a toxic effect or environmental pollution. Therefore, a precise size of silica NPs on a continuous scale is needed for different applications.

The application highlighted in this work is the in vitro luminescence imaging of cellular structures using dye-doped silica NPs. In this case, the size of a dye-doped silica NP is determined by its rigid silica matrix. The presence of dye molecules has little effect on the NP size because the dye molecules are adsorbed to the porous network of the silica without extending or deforming the network. However, the size of a luminescent silica NP primarily determines both its luminescence intensity and intracellular mobility. Continuously tunable sizes of luminescent silica NPs are essential for optimal image contrast and detection sensitivity as well as for affecting a particular cellular or subcellular penetration.

The common methods of synthesizing silica particles are the Stöber method and the water-in-oil (w/o) microemulsion method.[11–16,21–37] Because of the small size and the narrow size distribution of the products, the w/o microemulsion method is preferred for the synthesis of nanodimensional particles. Accordingly, the synthetic conditions that affect the size and size distribution of silica NPs have been well-studied for this method. Studies include varying the amounts of water, ammonia hydroxide, and tetraethylorthosilicate (TEOS),[24–26] or changing the ratios of water to surfactant, surfactant to cosurfactant, surfactant to organic solvent, etc.[24,25] These modifications have produced different-sized silica NPs. However, the sizes are discrete and not variable on a continuous range. It has been a challenge to obtain silica NPs with a continuously tunable size.

An organic solvent − often an alkane − is an essential component to form a stable w/o microemulsion. The polarity and viscosity of this solvent is well-known to affect the size of silica NPs. However, little attention has been paid to the role that the size and structure of the organic solvent molecule itself has on silica NP size. Therefore, a systematic investigation of different dimensional organic solvents in the microemulsion is needed to explore potential functions of organic molecular dimensions on controlling sizes of silica NPs. The size and molecular structure of an organic solvent is expected to affect intermolecular forces and association free energy between the organic phase and surfactant molecules in a microemulsion. As such, the dimensional properties of the organic solvent should also influence the size of silica NPs. One possible method to tune the association free energy of solvent/surfactant in the w/o microemulsion and thereby produce silica NPs with a precise but variable size is to mix two or more organic solvents with different dimensional properties in variable proportions.

In this work, a systematic investigation of single and mixed organic solvents on the size of silica NPs was conducted. The results demonstrated that the usage of different alkane chain lengths of organic solvents permitted the size of silica NPs to be continuously tunable in the range of 20−100 nm. A physical model was also proposed to explain the results. The importance of the NPs with variable but precise size was demonstrated for luminescence imaging of living cells. This work should provide new insights into the manipulation of the size of silica-based nanomaterials and provide new avenues for improving detection and imaging in biological systems.

2. Experimental Section

2.1. Materials. Tetraethylorthosilicate (TEOS), polyoxyethylene(10)isooctylphenylether [Triton X-100, 4-(C_8H_{17})C_6H_4(OCH_2CH_3)$_{10}$-OH], and Igepal CO-520 (NP-5) were purchased from Aldrich. Ammonia hydroxide (28.0−30.0%), *n*-hexanol, cyclohexane, *n*-pentane, *n*-hexane, *n*-heptane, *n*-decane, *n*-hexadecane, acetone, ethanol (95%), and bovine serum albumin (BSA) were obtained from Fisher Scientific Co. Tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate, $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$, was purchased from ICN biomedicals Inc. *N*-(Trimethoxysilylpropyl)-ethylenediamine, triacetic acid trisodium salt (45% in water), was purchased from Gelest Inc. Human lung epithelial cells (A549 cells) and *Staphylococcus aureus* (SA) bacteria were obtained from American Tissue Culture Collection (ATCC). SA antibody (Mouse IgM ascites) was from Santa Cruz Biotechnology. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Pierce Inc. A 96-nonradioactive-cell proliferation assay (MTT) kit was purchased from Promega. Phosphate buffer (PB, 10 mM, pH 7.0) and 2-morpholinoethanesulfonic acid (MES) buffer (0.1 M, pH 6.0) were used during the cell imaging and bioconjugation.

2.2. Synthesis of the Luminescent Silica NPs in a Quaternary w/o Microemulsion. Luminescent silica NPs were synthesized using different organic solvents and surfactants in the w/o microemulsion. The procedure was similar as the reported method except for the variation of organic solvent.[24] In the quaternary w/o microemulsion system, besides water and an organic solvent, Triton

(21) Jin, Y.; Kannan, S.; Wu, M.; Zhao, X. *Chem. Res. Toxicol.* **2007**, *20*, 1126–1133.
(22) Wang, L.; Yang, C.; Tan, W. *Nano Lett.* **2005**, *5*, 37–43.
(23) Zhao, X.; Bagwe, R. P.; Tan, W. *Adv. Mater.* **2004**, *16*, 173–176.
(24) Bagwe, R. P.; Yang, C.; Hilliard, L. R.; Tan, W. *Langmuir* **2004**, *20*, 8336–8342.
(25) Chang, C.; Fogler, H. *Langmuir* **1997**, *13*, 3295–3307.
(26) Arriagada, F.; Osseo-Asare, K. *Colloids Surf.* **1999**, *154*, 311–326.
(27) Stöber, W.; Fink, A. *J. Colloid Interface Sci.* **1968**, *26*, 62–69.
(28) Wang, L.; Estévez, M. C.; O'Donoghue, M.; Tan, W. *Langmuir* **2008**, *24*, 1635–1639.
(29) Hartlen, K. D.; Athanasopoulos, A. P.; Kitaev, V. *Langmuir* **2008**, *24*, 1714–1720.
(30) Nakamura, M.; Shono, M.; Ishimura, K. *Anal. Chem.* **2007**, *79*, 6507–6514.
(31) Wang, L.; Tan, W. *Nano Lett.* **2006**, *6*, 84–88.
(32) Lin, W.; Huang, Y. W.; Zhou, X. D.; Ma, Y. *Toxicol. Appl. Pharmacol.* **2006**, *217*, 252–259.
(33) Rossi, L. M.; Shi, L.; Quina, F. H.; Rosenzweig, Z. *Langmuir* **2005**, *21*, 4277–4280.
(34) Ow, H.; Larson, D.; Srivastava, M.; Baird, B.; Webb, W.; Wiesner, U. *Nano Lett.* **2005**, *5*, 113–117.
(35) Nozawa, K.; Gailhanou, H.; Raison, L.; Panizza, P.; Ushiki, H.; Sellier, E.; Delville, J. P.; Delville, M. H. *Langmuir* **2005**, *21*, 1516–1523.
(36) Green, D. L.; Lin, J. S.; Lam, Y. F.; Hu, M. Z.; Schaefer, D. W.; Harris, M. T. *J. Colloid Interface Sci.* **2003**, *266*, 346–358.
(37) Montalti, M.; Prodi, L.; Zaccheroni, N.; Zattoni, A.; Reschiglian, P.; Falini, G. *Langmuir* **2004**, *20*, 2989–2991.

X-100 and *n*-hexanol were employed as a surfactant and a cosurfactant, respectively. A 7.5 mL aliquot of an organic solvent or solvent mixture, 1.8 mL of *n*-hexanol and 1.77 mL of Triton X-100 were mixed together under stirring for 20 min. Then, 480 μL of 1.70 mM $Ru(bpy)_3$ aqueous solution, 80 μL of TEOS and 60 μL of 28% ammonia hydroxide were added in 20 min intervals. The reaction proceeded under darkness for 24 h. Then, 20 μL of TEOS and 20 μL of *N*-(trimethoxysilylpropyl)-ethylenediamine, triacetic acid trisodium salt (45% in water), were added to the microemulsion for an additional 24 h to form a −COOH group on the NP surface to prevent aggregation. Finally, the NPs were collected by centrifuging at 11 000 rpm for 15 min.

2.3. Synthesis of the Luminescent Silica NPs in a Ternary Microemulsion. A ternary microemulsion is composed of three components: a surfactant (NP-5), an organic solvent, and the water. Ten milliliters of organic solvent and 460 μL of NP-5 were combined and stirred for 20 min. Then, 90 μL of 1.70 mM $Ru(bpy)_3$ aqueous solution was added. Succeeding steps were the same as those described for the quaternary microemulsion.

2.4. Characterization of the Luminescent Silica NPs. A Hitachi 7500 transmission electron microscope (TEM) was used to take images of the developed NPs. The luminescence intensities of the NPs were measured using a Jobin Yvon Horiba Fluorolog spectrofluorometer. Carl Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc.) was used to monitoring the process of NP penetration into cells and to visualize luminescent NP labeled bacterial cells. The detection of the absorbance in MTT assay was performed by a UV/vis absorption plate reader (Multskan Spectrum, Thermo Electron Corporation).

2.5. Conjugation of Antibody onto Luminescent Silica NPs. SA antibodies were conjugated to the NP surface using a previous method.[11,12] One-tenth a milligram of a powder of NPs in 2 mL of MES buffer was activated by 1.0 mL of 0.8 mg/mL EDC and 1.0 mL of 1.5 mg/mL sulfo-NHS in the MES buffer (pH 6.0) for 15 min. The activated NPs were washed and redispersed in the PB. A 50 μL aliquot of antibody solution was added to the mixture. After 2 h, the antibody conjugated NPs were washed and dispersed in PB. The NPs were then blocked with 0.05% (v/v) BSA for 0.5 h to prevent any nonspecific bindings. Finally, the NPs were stored in a 1% BSA solution at 4 °C before use.

2.6. In situ Monitoring of the NP Penetration into Cells. Two sizes of $Ru(bpy)_3$-doped silica NPs (large NPs, 85 ± 5 nm; and small NPs, 23 ± 3 nm) were used. A549 cells were cultured on a glass-bottomed plate with 1000 μL of Dulbecco's modified Eagle's medium (DMEM). The cells were first observed using a confocal fluorescence microscope at 37 °C with moisture. Then, 200 μL of luminescent silica NP solution (5 mg/mL in PB) was added into the cell culture medium. The penetration process of the NPs into the cells was monitored in situ, and the luminescence images were taken every 5−10 min. The luminescent NPs were excited at 488 nm (by an argon laser), and a 585−620 nm band-pass filter was selected to block the background signal. Because the luminescence intensities between the large NPs and the small NPs were significantly different, two settings of the amplification and the pinhole were applied to obtain the comparable luminescence images (for the large NPs, 720 of amplification gain and 76.4 nm of pinhole; for the small NPs, 850 of amplification gain and 160 nm of pinhole).

2.7. Cell Proliferation Assay to Determine Cytotoxicity. The cytotoxicity of the NPs was investigated using the cell proliferation assay (MTT assay). Human alveolar epithelial cells (A549) were cultured onto a 96-well culture plate at about 1000 cells per well. NPs of the same size but without luminescent dyes were used as controls. After 48 or 72 h incubation with the NPs, the cell samples were tested by the MTT assay. A 15 μL aliquot of MTT reagent was added into each well of the plate. The samples were incubated at 37 °C for 4 h. Then, a 100 μL of stop solution was added into each well. After the sample was incubated at 37 °C overnight, the absorbance was recorded at 565 nm to determine the cell proliferation rate.

Table 1. Variable Sizes of Silica NPs Synthesized by Different Molar Volumes of Organic Solvents

	physical properties		diameter of NPs (nm)	
organic phase	molar volume (mL/mol)	dielectric constant	quaternary system	ternary system
cyclohexane	108	2.02	53 ± 3	26 ± 2
n-pentane[a]	115	1.84	59 ± 5	20 ± 1
n-hexane	131	1.89	71 ± 5	44 ± 4
n-heptane	147	1.92	74 ± 2	44 ± 6
n-decane	195	1.99	85 ± 4	N/A
n-hexadecane	293	2.05	108 ± 8	N/A

[a] The reaction proceeded at 4−6 °C to prevent the evaporation of pentane.

3. Results and Discussion

3.1. Controlling the Sizes of Silica NPs in w/o Microemulsions. A w/o microemulsion is an isotropic and thermodynamically stable single-phase solution. It consists of a small amount of water, a large volume of organic solvent (oil), and a surfactant. The surfactant molecules lower the interfacial tension between water and the organic solvent, resulting in the formation of a transparent solution. Water droplets are formed in the bulk organic solvent and serve as nanoreactors for the synthesis of NPs from various silane precursors and with a variety of dopants. TEOS is a typical water soluble precursor for the synthesis of silica NPs. Upon the polymerization of TEOS, a silica core is formed in the water droplet. As the polymerization progresses, the silica core grows, and finally, a stable silica NP is produced in the water droplet. Several factors that affect the size of the produced silica NPs have been well-studied, including the type of surfactant, the water-to-surfactant ratio, and the amount of TEOS. This work focused on the effect of dimension of organic solvents on the silica NP size.

Six alkane molecules with different alkane chain lengths were used for the synthesis of silica NPs with w/o microemulsions (Table 1). Because the chain length of an alkane is proportional to its molar volume, the effect of organic solvent dimension is referred to hereafter as the effect of molar volume. A common quaternary w/o microemulsion containing four components − Triton X-100, *n*-hexanol, water, and an organic solvent − was employed. All synthesis conditions were fixed except for the usage of different organic solvents. As the molar volume of the single organic solvent increased from 108 mL/mol (cyclohexane) to 195 mL/mol (*n*-decane), the NP size increased significantly from 53 ± 3 to 85 ± 4 nm (Figure 1A-E). When molar volumes of the organic solvent differed only slightly, such as with cyclohexane (108 mL/mol) and *n*-pentane (115 mL/mol), the sizes of resultant NPs were changed slightly as well (53 ± 3 nm, Figure 1A; 59 ± 5 nm, Figure 1B). Similar results were observed for *n*-hexane and *n*-heptane (Table 1). Therefore, the size of a silica NP can be tuned by varying the molar volume of an organic solvent. A smaller molar

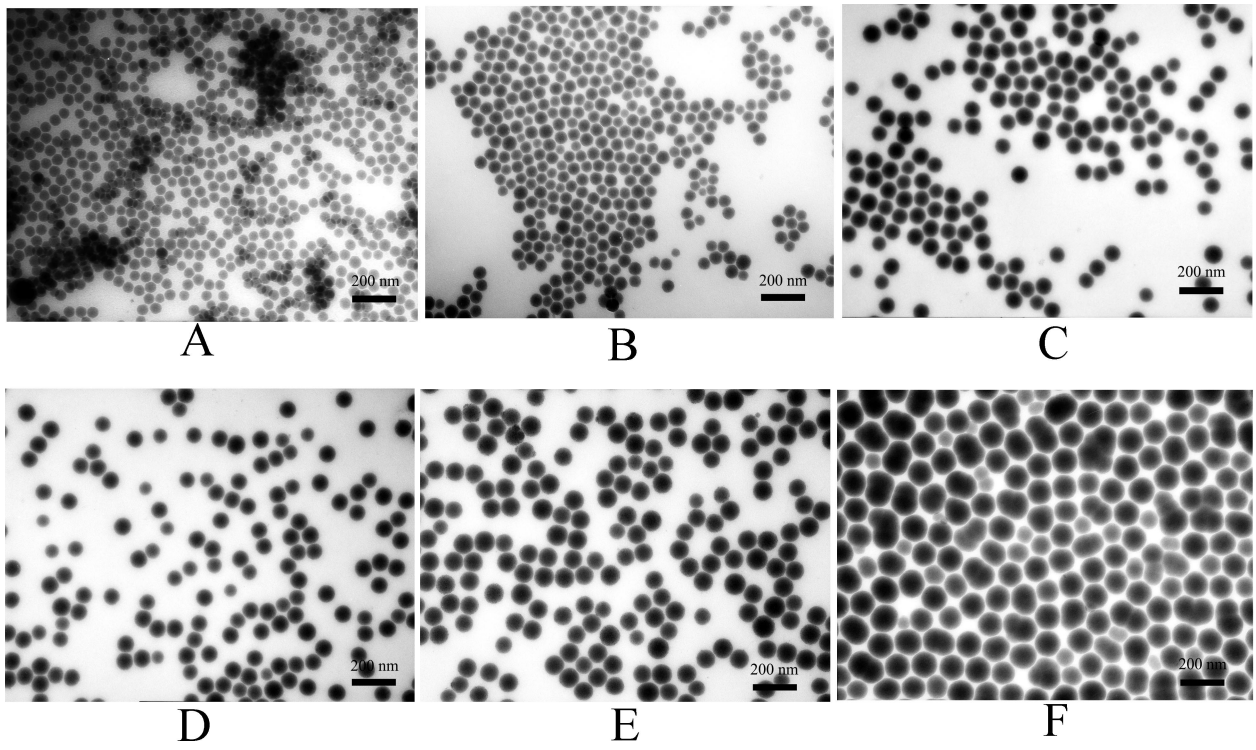


Figure 1. TEM images of variable sizes of silica NPs obtained from a quaternary w/o microemulsion using different organic solvents: (A) cyclohexane, (B) *n*-pentane, (C) *n*-hexane, (D) *n*-heptane, (E) *n*-decane, and (F) *n*-hexadecane.

volume of organic solvent produces a smaller size of silica NP. With significantly larger organic solvents, such as *n*-hexadecane (293 mL/mol), the water droplets became much larger and the microemulsion turned into unstable. A turbid solution was formed instead of a transparent microemulsion during the synthesis process. In this case, solvent polarity may have become a determining factor and the longer alkane chain made *n*-hexadecane was too hydrophobic to form a stable microemulsion with water. Although both a surfactant and a cosurfactant were used in this case, it could not lower the interfacial tension between the water and the *n*-hexadecane enough to produce a stable microemulsion. The larger water droplets of these semistable microemulsions significantly increase the chance of interwater droplet percolation resulting wide size distributions of silica NPs (Figure 1F, 99 ± 26 nm). The effect of organic solvent molar volume on NP size was also probed for a ternary w/o microemulsion, which was made of water, an organic solvent, and NP-5 as the surfactant. The same six organic solvents evaluated for quaternary microemulsion were used. Because there was no cosurfactant to assist in lowering interfacial energy between water and the organic solvent, the two largest alkyl chain organic molecules, *n*-hexadecane and *n*-decane, could not form microemulsions. The other four organic solvents did form stable microemulsions and produced uniformly sized NPs (Figure 2 and Table 1). The same trend as in the quaternary microemulsion was observed, that is, as the molar volume of the organic solvent increased, the size of the NP became larger. However, the average NP size was smaller than NP produced with quaternary microemulsions in which Triton X-100 was employed as surfactant. This difference was mainly caused by the different types of surfactants. Overall, these results demonstrated a strong correlation between molar volume of the organic solvent to NP size, regardless of the type of w/o microemulsion used in their synthesis.

3.2. Fine-Tuning the Size of Silica NPs Using a Binary Organic Solvent Mixture. The size of silica NPs showed in Table 1 was still discrete. To continuously tune the size of NPs in nanoscale, binary solvents were employed in the microemulsion. Cyclohexane and *n*-hexadecane were the smallest and the largest organic molecules among the six chosen solvents in this study. Because their effects on the formation of microemulsion were distinct, they were chosen to evaluate the combinational effect of two solvents (Figure 3). Compared to the single organic solvent system in which *n*-hexadecane did not produce a stable microemulsion, the addition of cyclohexane assisted in the microemulsion formation process. For instance, with an organic solvent mixture of 30% (v/v) cyclohexane and 70% *n*-hexadecane, a transparent and stable microemulsion was obtained. Using this ratio, silica NPs 87 ± 4 nm in diameter were formed. When the volume percentage of hexadecane was reduced and the cyclohexane was increased, the size of the isolated NPs decreased significantly (Figure 3). Below 20% hexadecane, the size of the silica NPs changed very little and remained about 50 nm. The result suggested that small amounts of *n*-hexadecane had little influence on the nanoenvironment of the water doplets in the microemulsion and that cyclohexane dominated the organic interactions with the surfactant layer. Overall, the cyclohexane/*n*-hexadecane binary solvent system provided an adjustable silica NP size range of 50−100 nm in diameter by simply changing the volume ratio of *n*-hexadecane to cyclohexane. When a ternary microemulsion was used, the size of silica NP can be continuously adjusted in a smaller size range of 20−50

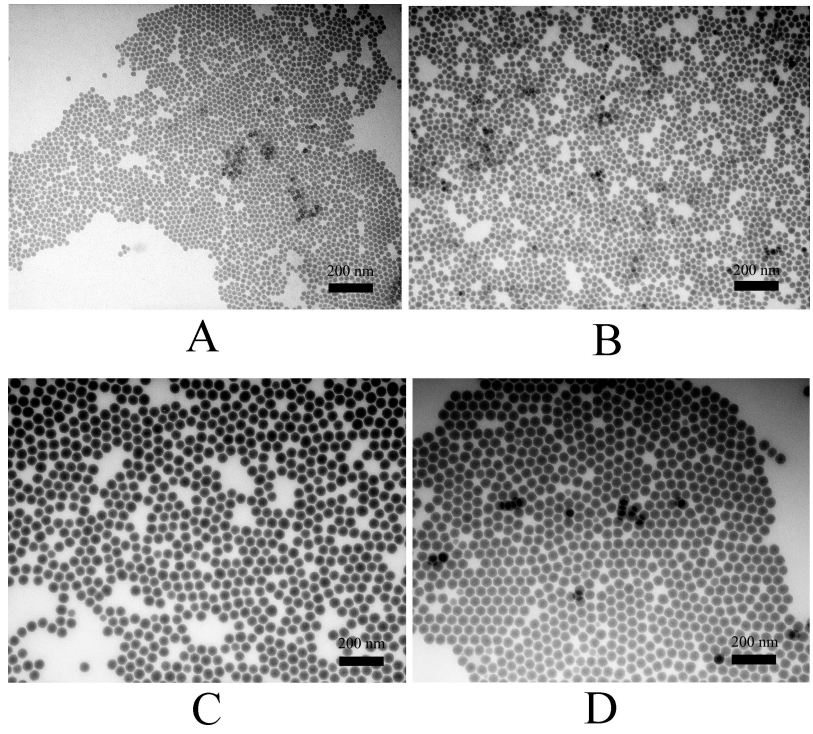


Figure 2. TEM images of variable sizes of silica NPs obtained from a ternary w/o microemulsion using different organic solvents: (A) *n*-pentane, (B) cyclohexane, (C) *n*-hexane, (D) *n*-heptane.

nm using a binary solvent system. It is very likely that this same size-tuning principle can be extended to other binary solvent systems for the reverse microemulsion synthesis of other size ranges and with other NP materials.

3.3. Model for Solvent Effect on Silica NP Size. The size of NPs produced by the w/o microemulsion method is directly influenced by the water droplet size. Hence, to explain the effect of molar volume of the organic solvent on NP size, it is necessary to explain the same effect on water droplet size in the w/o microemulsion. For this purpose, a simple physical model was adopted that describes the geometric constraints imposed on water droplets of a stable w/o microemulsion.[38] Equation 1 describes that the radius (R) of a water droplet in relation to three factors when R is below the critical value.

$$R = \frac{3\varphi}{C_s\Sigma} \tag{1}$$

The geometric constrains in this model are the number of surfactant molecules per unit volume (C_S) and the area occupied per surfactant molecule (Σ). Also considered is the volume fraction (ϕ) of the dispersed (water) phase. The term ϕ/C_S is usually referred to as the water to surfactant ratio and is fixed by stoichiometry. However, the Σ term can provide useful insight into the effect of organic solvent, particularly through its changes to the surface area occupied by surfactant molecules (Scheme 1A).

If organic solvent molecules partially or entirely penetrate into the hydrophobic layer of surfactant on a water droplet surface, Σ should vary based on the extent of penetration and the molar volume of the organic solvent molecule(s). A small organic molecule can penetrate into the surfactant layer much deeper than a large molecule, resulting in a larger area per surfactant occupied (Σ, the shadow area in Scheme 1A, (1)). Conversely, a larger organic molecule cannot easily penetrate into a surfactant layer, resulting in a smaller Σ (Scheme 1A, (2)). The combination of a larger and a small organic molecule can produce variable Σ values based on the ratio of two molecules (Scheme 1A, (3)). According to eq 1, if ϕ/C_S is fixed, R is inversely proportional to Σ. From this simple physical model, it can be seen that difference in molar volume of an organic solvent can change the size of water droplets, and thus the change in the size of silica NPs.

In addition to the above effect, the molar volume of an organic solvent also affects the number of silica cores formed in the w/o microemulsion synthesis. As the amount of TEOS,

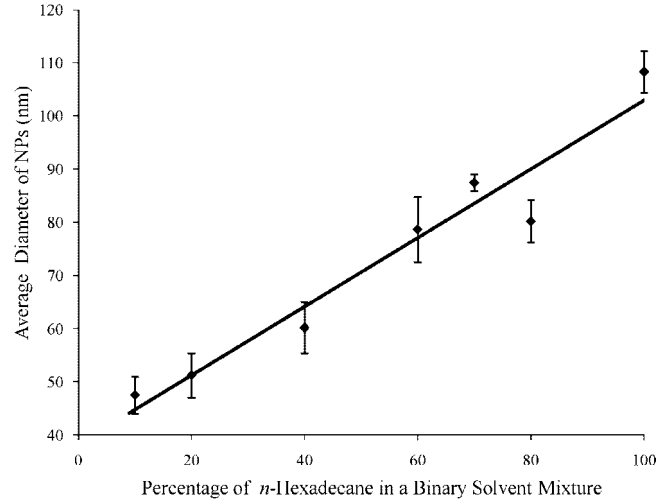
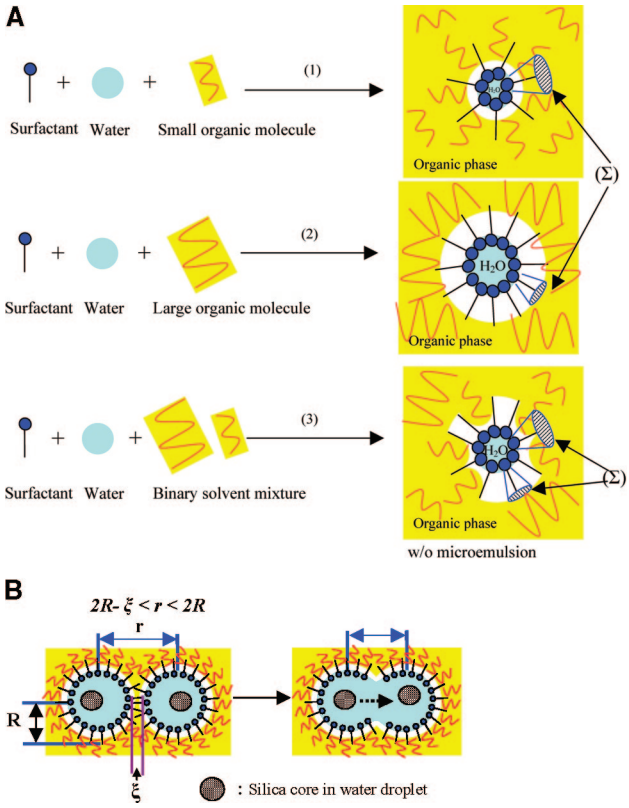


Figure 3. Size of the silica NPs is proportional to the ratio of *n*-hexadecane to cyclohexane in the binary organic solvent mixture.

(38) Langevin, D. *Annu. Rev. Phys. Chem.* **1992**, *43*, 341–369.

Scheme 1. Model for Organic Solvent Effect on Silica NP Size in W/O Microemulsion Synthesis: (A) Effect of Molar Volume of Organic Solvent on the Size of Water Droplet; (B) Effect of Organic Solvent on the Potential of Inter-Droplet Percolation



water-to-surfactant ratio, and the type of surfactant and cosurfactant are fixed, the size of the silica NPs is primarily determined by the number of the silica cores formed in the initial state. Because nearly all TEOS can be polymerized, the more silica cores are formed, the smaller the final size of the silica NPs is. Based on the NP formation process described above, the number of water droplets determines the number of silica cores, and the size of ultimate silica NPs is limited by the boundary of the water droplet.[20] Thus, both the number and the size of water droplets in the microemulsion are determining factors for the size of silica NPs. The theory of how to change the number of water droplets has been well-studied. After the initial silica cores are formed, the NPs start growing. Occasionally, the collision of two water droplets may result in silica core transfer between the water droplets as shown in Scheme 1B. This process is so-called as interdroplet percolation. The percolation causes the aggregation of silica cores and leads to the reduction of the number of silica cores. In consequence, the final size of silica NPs becomes larger, and the NPs have a broad size distribution. In the w/o microemulsion, the molar volume of an organic solvent affects the potential of interdroplet percolation as described in equation (2).[39]

$$U(r) = 0, r > 2R$$
$$U(r) = -kT\Delta\rho(2R - r)^2(2R + r/2)/6, 2R - \xi < r < 2R$$
$$U(r) = \infty, r < 2R - \xi \quad (2)$$

Here, $U(r)$ is the potential of interdroplet percolation, r is the distance between two water droplets, R is a radius of the water droplet, k is the boltzman constant, T is the Kelvin temperature, and ξ is a parameter to characterize the penetrable length of the interfacial layer. $\Delta\rho$ is related to molar volume of the organic solvent. A large molar volume corresponds to a larger $\Delta\rho$ value.[39] In the range of $2R - \xi < r < 2R$ (Scheme 1B), the value of $(2R - r)$ is very small. One could expect that the percolation potential increases with the increase of the size of droplet (R). Therefore, the interdroplet percolation occurs easily in the organic solvent with a large molar volume when the interdroplet distance (r) is in the range of $2R - \xi < r < 2R$ (Scheme 1B).[26] Thus, the size of a silica NP rises as the increase of molar volume of an organic solvent.

The above physical model from two angles explained the effect of molar volume of organic solvent on the size of silica NPs. The explanation provided a strong foundation to support the experimental results of this work.

4. Effect of NP Size on Cellular Contrast Imaging

The precise but variable size of silica NPs are needed in a variety of applications. Specifically, dye-doped luminescent silica NPs have demonstrated a great potential for in vitro contrast imaging of living cells. Thus, the significance and utility of these tunable silica NPs was evaluated for cellular imaging in three aspects: penetration ability, cytotoxicity, and luminescence signal intensity.

4.1. Effect of NP Size on the Ability to Penetrate Cellular Membranes. When used as signaling/labeling reagents in subcellular bioimaging, nanomaterials need to penetrate cell membranes. It has been reported that quantum dots (QDs) penetrate the cell membrane and accumulate in the cytosol. Because the size of QDs are smaller than 10 nm in diameter, they can further penetrate the cell nucleus.[40] The effect of size on the penetrating ability of luminescent silica NPs has not been studied previously. $Ru(bpy)_3$-doped silica NPs with two distinct sizes, 23 ± 3 and 85 ± 5 nm, referred to hereafter as small NPs and large NPs, were used to investigate the penetrating ability. Human A549 cells were used as the cell model based on previous work by our group.[28] Penetration of these cells by small and large NPs was performed at 37 °C with moisture and was monitored under a confocal fluorescence microscope as shown in Figure 4.

The small NPs clearly penetrated the cells much faster than the large NPs. After the first 10 min of incubation, the two differently sized NPs were well-dispersed in the cell perfusion medium. Luminescence signals were not detectable inside the cells, although there were a few aggregates of the NPs outside the cells (Figure 4 A, B). After 20 min of incubation, NPs began to penetrate the cellular membrane with no significant difference between the two sized NPs (Figure 4C). The difference became clear as the incubation time increased to 40 min (Figure 4D). At 60 min, the majority of large NPs were physically adsorbed on the outer cell surface and formed luminescence rings as observed by the

(39) Hou, M.; Shah, D. *Langmuir* **1987**, *3*, 1086–1096.
(40) Lovric, J.; Bazzi, H. S.; Cuie, Y.; Fortin, G. R. A.; Winnik, F. M.; Maysinger, D. *J. Mol. Med.* **2005**, *83*, 377–385.

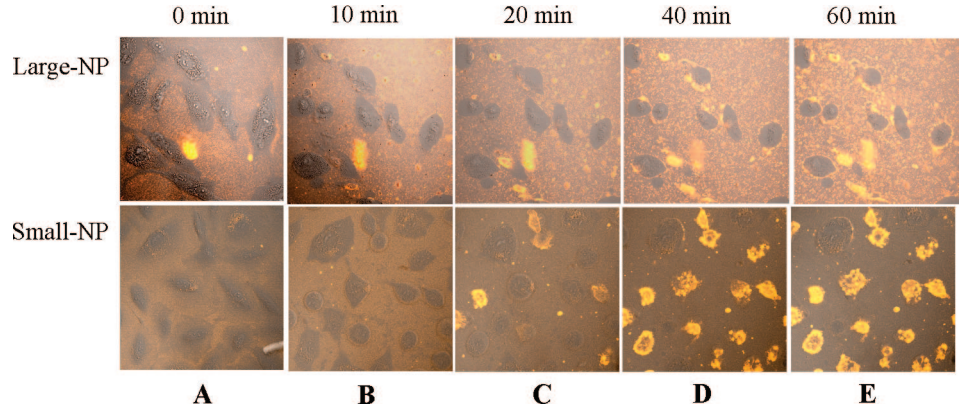


Figure 4. Penetration of different-sized NPs through cell membranes. A549 cells. Big NPs, 85 ± 5 nm; small NPs, 23 ± 3 nm.

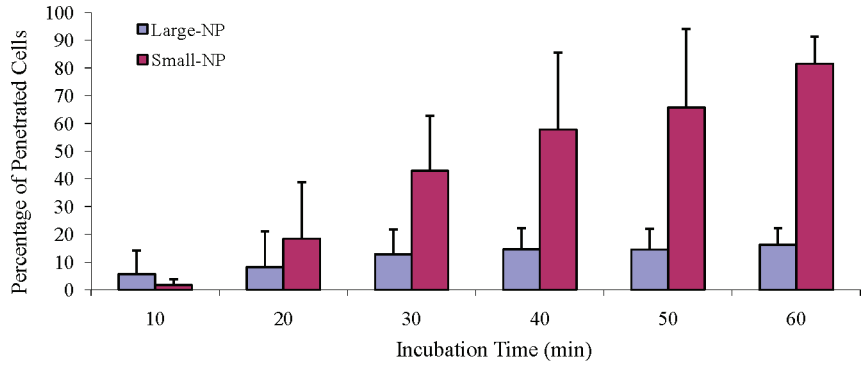


Figure 5. Percentage of cells penetrated by NPs at different time periods. The data are average of three experiments.

confocal microscope (Figure 4E). In contrast, a large portion of the small NPs penetrated the cell membrane and accumulated evenly throughout the cytosol (Figure 4E).

To quantify the mobility difference between NPs, a statistical analysis of these cell images was carried out by calculating the percentages of cells penetrated by NPs (Figure 5). At 20 min of incubation, although the percentage of cells penetrated by small NPs was higher than that in the larger NPs, the two averages had no significant difference at the early phases based on student *t* test ($p > 0.95$). The difference appeared when the incubation time was longer than 30 min. As the incubation time reached to 40 min, 14% of the cells were penetrated by the large NP, whereas 58% of the cells had accumulated the small NPs. The largest difference appeared at 60 min. Here the small NPs penetrated 81% of A549 cells, whereas the large NPs penetrated only 16% of the cells over the same time. These results clearly demonstrated that the penetrating ability of silica NPs was strongly correlated with size, and particularly that smaller NPs tend to penetrate cellular membranes much faster.

4.2. Effect of NP Size on Cytotoxicity. In a previous study, we demonstrated a low cytotoxicity of luminescent silica NPs with a diameter of a 50 ± 3 nm.[21] However, the effect of NP size variation on cytotoxicity has not been previously investigated. In this work, the toxicity effects of small (23 ± 3 nm) and large (85 ± 5 nm) NPs were compared using a cell proliferation assay. These NPs were added to A549 cells in a 96-well plate and incubated for 48 or 72 h. To also compare our previous published data, silica NPs were applied at concentrations of 0.01, 0.1, and 0.5 mg/mL.

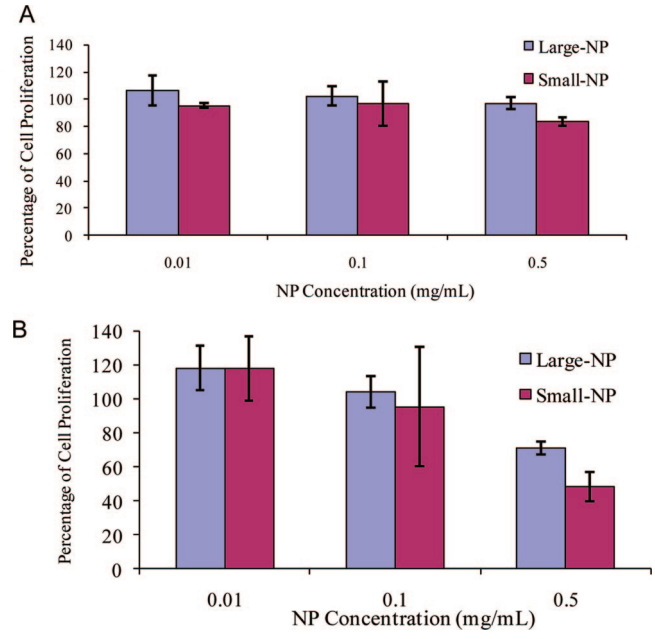


Figure 6. Comparison of cytotoxicity induced by different size NPs using cell proliferation assay. The incubation time of the cells with NPs was (A) 48 and (B) 72 h.

Cell proliferation results are shown in Figure 6, which demonstrated that smaller NP size, longer incubation time, and higher NP concentration were all factors that lead to higher cytotoxicity. Below a concentration of ca. 0.1 mg/mL, NP size and incubation time had no discernible effect on cytotoxicity ($p > 0.95$). This finding supports our previous observation that silica NPs have relatively low cytotoxicity and a great potential for bioimaging application. Only at the

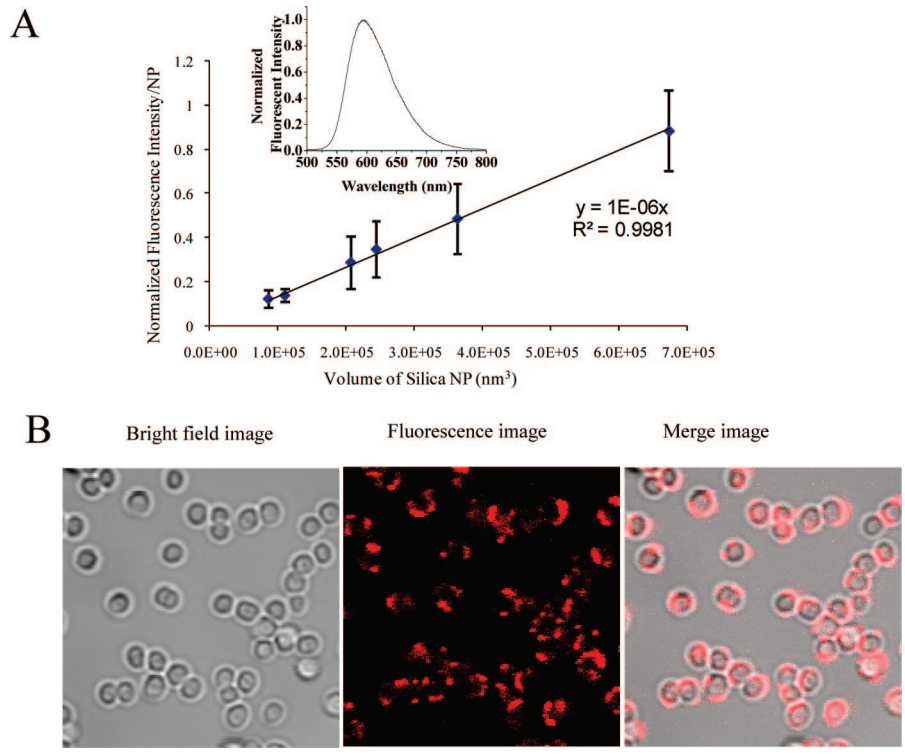


Figure 7. (A) Luminescence intensity of a $Ru(bpy)_3$-doped NP is proportional to the NP volume. Inset: Spectrum of the $Ru(bpy)_3$-doped NP. (B) SA bacterial cells were labeled by the $Ru(bpy)_3$-doped NPs with a diamter of 57 ± 4 nm.

higher NP concentration (0.5 mg/mL) was cell proliferation affected. In this case, only small NPs caused a discernible but small loss of proliferation of the shorter incubation time (small NPs, 0.5 mg/mL, 48 h incubation time, Figure 6A). At the higher incubation time of 0.5 mg/mL NPs, both NP sizes significantly slowed cell proliferation (Figure 6B). However, this loss of proliferation was much greater for the smaller NPs compared to the larger NPs (proliferation rate: 29% (small NPs) vs 52% (large NPs), respectively).

In our previous work, a low cytotoxicity was observed with NPs having a diameter of 50 nm. Toxicity at the highest concentration (0.5 mg/mL) was attributed to the accumulation of the silica NPs, which may physically hamper the local environment and metabolism of the cells, particularly after a long period of exposure. On the basis of additional experiments in this work (Figures 4 and 5), the small NPs are shown to penetrate the cell membrane and accumulate inside the cytosol more rapidly and effectively than the large NPs. Large NPs merely accumulate on the cell membrane, whereas small NPs distribute both on the surface and inside the cells. As such, penetration of the cellular membrane appears to cause the greatest cytotoxicity, and careful selection of NP concentration and size are needed for cellular imaging.

4.3. Effect of NP Size on Luminescence Signaling Intensity. The luminescence intensity of dye-doped silica NPs is proportional to the number of dye molecules doped in the silica matrix. Higher numbers usually lead to a stronger emission signal except at the concentration limit where self-quenching occurs. To improve the emission intensity of dye-doped NPs, one can either switch to a dye molecule with a higher luminescence quantum yield or change the NP size. Using different dye molecules has been previously reported for signal enhancement. Here, the effect of size on luminescence intensity is demonstrated.

A large number of dye molecules ($\sim 1 \times 10^4$/NP) can be doped inside the silica NP matrix. $Ru(bpy)_3^{2+}$, which emits 592 nm with an optimum excitation wavelength of 450 nm (Figure 7A, inset), was used in this work. To vary the luminescence emission per NP, we synthesized differently sized $Ru(bpy)_3$-doped NPs using the method described above. As expected, the larger NPs gave higher luminescence signals and the luminescence intensity was proportional to the NP volume (Figure 7A).

4.4. Tuning the NP Size for Cellular Contrast Imaging Applications. An important perspective provided by this work is that the size of luminescent silica NPs must be carefully evaluated and tuned for a particular bioimaging application. For the purpose of subcellular imaging, small NPs are most effective at penetrating the cellular wall but they can suffer from low sensitivity and require long incubation times to achieve adequate contrast. For the purpose of imaging structures or biochemistry occurring on the outer cellular wall, large NPs with high luminosity can be easily employed because wall penetration is not need. However, if the number of binding sites for a NP label is limited on the cell wall, smaller NPs could prevent the blocking of adjacent sites and provide a higher image contrast.

Here, we gave an example of labeling SA bacterial cells using $Ru(bpy)_3$-doped nanoparticles. After the surface modification of the NPs with a specific SA antibody, the NPs were able to recognize SA antigen on the SA bacterial cell surface and emitted luminescence signals for determination

of the bacteria. The size of 57 ± 4 nm in diameter gave fairly high luminescence signals for each single bacterial cell as shown in Figure 7B. The optimal NP size will be varied based on cell types.

5. Conclusions

In summary, the size of the luminescent silica NPs is tunable through changing the organic solvents in a w/o microemulsion. The organic solvents with different molar volumes, ranging from 108 to 293 mL/mol, have produced NPs with sizes between 20 and 100 nm in diameter. A model of the effect of molar volume of organic solvent on the silica NP size is proposed. The pathway of the molar volume effect is likely through controlling of the size of water droplet and the potential of interdroplet percolation. The luminescent silica NPs can be used as a highly intense labeling reagent for imaging of cells. The size of the NPs affects their signaling capacity, cell penetration, and cytotoxicity. The smaller NPs showed faster penetration, low luminescence intensity, and yet, detectable cytotoxicity only at a very high concentration. Despite the toxicity produced by the small NPs at the high concentration, all the NPs exhibit the low-toxicity nature, allowing them to be used in biological research. All the size-related properties suggest that the size of the luminescent silica NPs significantly affects their performance in cellular imaging.

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