Fluorescent Tryptophan-Doped Silica Microparticles Prepared Through a Reverse Microemulsion Method

Janine Marriah G. Dela Cruz¹² and Christopher Jay T. Robidillo*¹

¹Department of Physical Sciences and Mathematics
College of Arts and Sciences, University of the Philippines Manila
²College of Medicine, University of the Philippines Manila

A reverse microemulsion method was utilized in synthesizing silica microparticles doped with tryptophan molecules as fluorophore. The fluorescent microparticles were formed inside reverse micelles dispersed in a cyclohexane continuous phase. The microparticles were produced within 24 hours and showed strong emission at a wavelength of 285 nm. The blue shift in their fluorescence maximum can be attributed to the caging and confinement effects of the silica network on the encapsulated tryptophan molecules. Successful encapsulation of tryptophan was confirmed through Fourier Transform Infrared Spectroscopy and Energy-Dispersive X-Ray Spectroscopy. Scanning Electron Microscopy and Dynamic Light Scattering Analysis revealed that the diameters of tryptophan-doped silica microparticles were in the range of 203 to 692 nm in the solid state, and in the range of 223 to 341 nm, with a narrow size distribution centered at 282 nm, in aqueous solution. Properties relevant to probe applications such as photostability and fluorophore leakage were also investigated. Tryptophan-doped silica microparticles were found to maintain their photostability even after six hours of continuous exposure to a 150 W halogen lamp and were observed to not undergo tryptophan leakage after three days of aqueous dispersion. This study has effectively extended dye encapsulation in silica to a biologically endogenous fluorescent amino acid, yielding fluorescent microparticles with desirable properties for fluorescent probes, namely, biocompatibility, photostability, non-leakage, monodispersity in solution, and fairly uniform sizes.

Key words: fluorophore, microparticles, reverse micelles, silica, tryptophan

INTRODUCTION
Fluorescence spectroscopy is a luminescent technique that has gained considerable interest due to its ease of implementation, and high sensitivity and selectivity. Innovations in fluorescent analytical techniques have been applied in the development of sensors for probing chemical and biological processes (Basabe-Desmonts et al. 2006). These probes were successfully used in biological research, clinical diagnosis, and detection of various kinds of substances (Lian et al. 2004; Liaud et al. 2015; Liu et al. 2015; Hodáková et al. 2015; Qin et al. 2016).

Fluorescent probes commonly utilize fluorophoric molecules such as polyaromatic carbocycles and heterocycles designed to respond to a specific stimulus or to localize within a specific region of a biological specimen. However, fluorophores are limited by their molecular instability. Most of them decompose upon exposure to a continuous light source and are susceptible to degradation and photobleaching in complex environments (Wang et al. 2004; Lian et al. 2015; Liu et al. 2015; Hodáková et al. 2015; Qin et al. 2016).
The demand for novel fluorescence technologies, then, gave rise to a new generation of luminescent molecules that are encapsulated in a silica network. Fluorophores such as tris(2,2’-bipyridyl)dichlororuthenium (II) hexahydrate, Alexa Fluor® 555, tetramethylrhodamine isothiocyanate, acridine orange, and combined fluorescein and phenosafranine, and others have been embedded in a silica matrix, with the resulting particles offering the following advantages over traditional fluorescent dyes: high intensity of the fluorescent signal, exceptional photostability attributable to the exclusion of oxygen by silica, efficient conjugation to many biomolecules due to an easily modified silica surface, and easy manufacturing process (Gao et al. 2002; Ow et al. 2005; Canton et al. 2011; Liu et al. 2014; Rouhani & Haghgoo 2015; Chen et al. 2015; Li et al. 2015; Kelleher et al. 2015; Nooney et al. 2015; Flynn et al. 2016; Tan et al. 2016).

Silica is often the matrix of choice for fluorophore encapsulation. silica coating prevents photobleaching, thereby increasing the photostability of the encapsulated molecules (Wolfbeis 2015). Also, its surface is an excellent substrate for many surface immobilization techniques ideal for biomolecular conjugation (Zhang et al. 2015). Furthermore, silica has low cytotoxicity and is effectively dispersed in water making it highly soluble in aqueous environments and suitable for solution-based biological assays (Lian et al. 2004; Balaure et al. 2016; Gu et al. 2016). Fluorophore-doped silica particles can be prepared through two general synthetic routes: the Stöber and the microemulsion methods (Bagwe et al. 2004; Wang et al. 2006). It is of great interest to extend the scope of the encapsulation methods to biocompatible fluorescent molecules. Hence, the water-in-oil microemulsion method has been used to encapsulate tryptophan in a silica network in this study. Tryptophan is one of the endogenous fluorophores that is responsible for the absorption and fluorescence of proteins in the ultraviolet range. This molecule has an absorption maximum at 280 nm. Moreover, it fluoresces at around 320-350 nm (Teale 1960; Burstein et al. 1973; Chen & Barkley 1998).

The fluorescence quantum yield of tryptophan in water is 0.13 at an excitation wavelength of 280 nm (Chen 1967). Fluorescence from tryptophan proves that it is an inviting candidate for probe applications because of its sensitivity to the polarity of the local environment. Its emission wavelength varies from 308 nm to 335 nm depending on its degree of solvent exposure (Vivian & Callis 2001). Unlike toxic commercial organic dyes, tryptophan is a biologically essential molecule. Hence, the inadvertent and potentially harmful exposure of the biological tissue to the fluorophore during bioassays resulting from unwanted fluorophore leakage is dramatically reduced, if not completely eradicated. Furthermore, the low frequency with which tryptophan is incorporated into proteins (Ohta & Kimura 1970) lessens the background signal expected from its autofluorescence during actual imaging of biological systems.

**MATERIALS AND METHODS**

**Microparticle Synthesis**

Tetraethyl orthosilicate (TEOS), Triton X-100 (TX-100), tryptophan, absolute ethanol, and n-hexanol were obtained from Sigma Aldrich. All of the aforementioned chemicals were of analytical grade and were used as received, without further purification. Distilled, deionized water was used all throughout the experiments.

A water-in-oil microemulsion was prepared by mixing 1.77 mL of TX-100, 7.5 mL of cyclohexane, 1.8 mL of n-hexanol, and 0.5 mL of 50 mM tryptophan aqueous solution, and was used to synthesize tryptophan-doped silica microparticles (trp-SMPs). No fluorophore was added to the microemulsion in the preparation of blank silica microparticles (SMPs). The mixture was stirred for 30 minutes in order to allow the tryptophan molecules to disperse into the water droplets. Then, 100 μL of TEOS was added as a precursor for silica shell formation, followed by the addition of 60 μL of NH₄OH (28-30 weight %) to initiate polymerization. The reaction was allowed to proceed for 24 hours at room temperature with constant stirring. When the polymerization was complete, an equal volume of acetone was added followed by vortexing the mixture in order to break the microemulsion state. The microparticles were then collected by centrifugation at 4000 rpm for 20 minutes. The solidified particles were washed three times with absolute ethanol. The particles were washed three times with absolute ethanol and then dried in an oven at 60 °C for 12 hours (Santra et al. 2001).

**Characterization and Analysis**

Fluorescence measurements were performed using a Thermo Scientific™ Varioskan™ flash multimode reader at the Institute of Chemistry, University of the Philippines Diliman. Surface morphologies of the tryptophan-doped silica microparticles coated in gold were viewed using a JEOL 5310 scanning electron microscope (SEM) housed at the Solid State Physics Group Laboratory, De La Salle University. Dynamic light scattering analysis for the measurement of size and size distribution was performed using a Malvern Zetasizer Nano 530 at the Institute of Chemistry, University of the Philippines Los Baños. The infrared spectra of the microparticles were obtained on a PerkinElmer FTIR spectrometer and absorbances for the photostability test...
were measured using a PerkinElmer Lambda 650 UV-Vis spectrophotometer, both at the Department of Physical Sciences and Mathematics, University of the Philippines Manila. Energy-Dispersive X-ray (EDX) analysis was carried out using a Hitachi SU 3500 Floortype SEM-EDX instrument at the Nanotech Analytical Services and Training Corp. Laboratories.

**Fluorescence Studies**
A standard curve was constructed from fluorescence measurements of tryptophan standard solutions that were prepared by dissolving tryptophan in water and serial dilution to the following final concentrations: 75 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL, and 1 µg/mL. Linear regression was performed to obtain an equation for the quantification of encapsulated tryptophan in 1 mg/mL aqueous solution of trp- SMPs. The excitation wavelength used was 280 nm and the fluorescence intensities were evaluated at the wavelength of maximum emission. The instrument used was only capable of measuring fluorescence at wavelengths higher than 285 nm.

**Fourier Transform Infrared Spectroscopy (FTIR)**
Dried SMPs and trp-SMPs were ground using mortar and pestle. Subsequently, the FTIR spectra of both dried SMPs and trp-SMPs were obtained through the KBr window method.

**Energy-Dispersive X-Ray Spectroscopy (EDX)**
Dried trp-SMP samples were prepared by fixing the powder on the microscope holder. The samples were then subjected to EDX measurements. Quantitative elemental analysis of samples was performed by collecting EDX data.

**Scanning Electron Microscope Analysis (SEM)**
The microparticles were imaged on a SEM to assess the particle size and shape. SMPs and trp-SMPs were finely pulverized prior to coating with gold.

**Dynamic Light Scattering Analysis (DLS)**
The mean diameter of the microparticles in suspension was obtained from cumulative fits of the autocorrelation functions. Samples were prepared by sonication of water suspension and progressive dilution to stay in the instrumental linearity range.

**Photostability Test**
Aqueous solutions of tryptophan and trp-SMPs were prepared and concentrations were adjusted to ensure their UV absorbances at 280 nm were less than 1.00. The samples were exposed to light coming from a halogen lamp (150 W) with the distance between lamp and samples being 30 cm. The absorbance of the samples at 280 nm were recorded after every 30 min of continuous light exposure for six hours at room temperature (Chen et al. 2012).

**Leakage Test**
A 2 mg/mL aqueous suspension of trp-SMPs was prepared. The initial fluorescence spectrum was obtained. Before the succeeding fluorescence spectrum was measured, the supernatant was discarded, and the particles were redispersed in water to a final concentration of 2 mg/mL. The fluorescence spectra were obtained in 24-hour intervals for 3 days at room temperature (Gao et al. 2009).

**RESULTS AND DISCUSSION**

**Microparticle Synthesis**
The microparticles were synthesized using a reverse or water-in-oil microemulsion method. The first step was the preparation of the microemulsion mixture composed of cyclohexane, \( n \)-hexanol, Triton X-100 (TX-100), and an aqueous solution of tryptophan. This mixture was stirred afterwards to facilitate the dispersion of the aqueous phase into the organic phase. The reverse microemulsion formed when water became dispersed in cyclohexane. The added polar components, specifically the aqueous solution of tryptophan, were compartmentalized into the central cores of the reverse micelles. Dispersion of the aqueous phase in the organic phase was aided by the nonionic surfactant, TX-100. Surfactant molecules minimize surface energy by assembling into reverse micelles (Oh et al. 2002). The next step was the addition of the silica precursor, tetraethyl orthosilicate (TEOS), and \( \text{NH}_3\text{OH} \) to the transparent mixture. TEOS slowly penetrates into the surfactant membrane surrounding the aqueous droplets and provides the silicates needed to form the shell of the microparticles. Aqueous ammonia ensures alkalinity as it is dispersed into the \( n \)-hexanol medium and stabilized by the surfactant. Furthermore, it also functions as a catalyst for the hydrolysis of TEOS (Yoo & Pak 2013). Ultimately, the synthesis of microparticles occurs within the micelles, which effectively function as “microreactors” (Gao et al. 2007). The clear microemulsion mixture turned colloidal after stirring for 24 hours. The fluorescent microparticles were recovered as white precipitate (Figure 1) from the microemulsion by the addition of acetone. Figure 2 shows the suspensions of tryptophan-doped silica microparticles and silica microparticles in water under ambient light conditions and upon ultraviolet light irradiation.

The absorbance of the water-soluble SiOH-based molecules that condense
within the aqueous droplets. Thus, the aqueous droplets restrict the growth of the silica particles (Oh et al. 2002). The mechanism of formation of tryptophan-doped silica microparticles in reverse microemulsions consists of the same steps as those in the formation of other colloidal dispersions, namely, nuclei formation and particle growth. The concentration of silica monomers present in the droplets must exceed the nucleation threshold before seed formation can occur. The concentration of monomers in the aqueous droplets varies with their sizes. Nucleation occurs in the micelles through intramicellar condensation of monomers into silica polymers. The monomers, then, deposit onto and react with the seeds, leading to the formation of bigger particles. (Chang & Fogler 1997; Joumaa et al. 2008).

The size of the synthesized microparticles depends on the sizes of aqueous droplets dispersed in cyclohexane. The prepared microparticles may be prone to aggregation as their surfaces were not modified with compounds such as epoxides and hydrophilic polymers that are effective in reducing the surface free energy of the particles and in controlling agglomeration due to mutual exclusion and steric hindrance (Park et al. 2010). The tendency of unmodified silica particles to agglomerate is high because of their large hydrodynamic radii (>10 nm) and large surface area (Bagwe et al. 2004).

**Fluorescence Studies**

The maximum emission wavelength of tryptophan and the trp-SMPs were 290 and 285 nm respectively, with the same excitation wavelength of 280 nm as shown in Figure 3. It is evident from the figure that the maximum emission wavelength mentioned for the trp-SMPs is only an estimate and that the real emission maximum may actually be at an even lower wavelength. Nonetheless, there was an apparent blue-shift in emission maximum. Previous studies have shown that caging effects of the silica matrix on embedded fluorophores can cause a blue shift in the fluorescence emission (Montalti et al. 2006; Fales et al. 2011). The main reason for the shift is the difference in the polarity of the environment around tryptophan molecules dissolved in water and those entrapped within the silica matrix (Baria et al. 2014). The silica cage, which primarily consists of Si-OH and Si-O-Si groups is less polar compared to water (Avnir et al. 1984). Presumably, the caging effects alter the energies of the molecular orbitals of the tryptophan molecules resulting to a change in the energy of the emitted light.

Fluorescence measurements were used to estimate the concentration of tryptophan encapsulated in 1 mg/mL of trp-SMPs. Shown in Figure 4 is the standard curve depicting fluorescence intensity (FI) as a linear function of tryptophan concentration. A 1-mg/mL suspension of trp-SMPs with an FI of 9580 ± 1207 au corresponds to 67.10 ± 5.40 μM tryptophan, which is equivalent to 67 nmol of tryptophan in 1 mg of trp-SMPs. This value can be considered only as an estimate since it does not take into consideration the effect of the silica matrix on the FI of the encapsulated tryptophan molecules.

**Figure 3.** Fluorescence spectra of tryptophan-doped silica microparticles and tryptophan (λex=280 nm).

**Fourier Transform Infrared Spectroscopy and Energy-Dispersive X-Ray Spectroscopy**

The encapsulation of tryptophan in silica has been confirmed through Fourier Transform Infrared Spectroscopy (FTIR) and Energy-Dispersive X-Ray Spectroscopy (EDX)
Spectroscopy (EDX). The peaks characteristic of either an amino group or a nitrogen and a carbon atom of tryptophan were observed in both FTIR and EDX spectra.

The IR spectra for both SMPs and trp-SMPs were obtained and compared (Figure 5). Silanol groups on the surface of both microparticles, which are characteristic of silica particles, were detected at 3402 cm\textsuperscript{-1} for trp-SMPs and at 3435 cm\textsuperscript{-1} for SMPs. The bands at 1090 cm\textsuperscript{-1} and 742 cm\textsuperscript{-1} are attributed to the stretching vibrations of the silica (Si-O-Si) network, and the band at 1630-1635 cm\textsuperscript{-1} is related to silica lattice vibrations and bending of O-H groups (Gao et al. 2009). The difference in the spectra of trp-SMPs and SMPs is the presence of the peak at 3317 cm\textsuperscript{-1} in trp-SMPs, which corresponds to a secondary amine N-H stretch of the indole ring of tryptophan. The peaks at around 1600 cm\textsuperscript{-1} that merged with the 1631 cm\textsuperscript{-1} peak correspond to N-H bending and N-H deformation (Gao et al. 2009).

The FTIR spectra of trp-SMPs after three successive ethanol washes showed that the tryptophan indole N-H stretch persists even after the washes (Figure 6). This strongly suggests that the tryptophan molecules were encapsulated within the silica network and not just adsorbed on the surface where they could have easily been removed by subsequent washes.

EDX analysis was carried out on trp-SMPs in order to further confirm successful tryptophan encapsulation. EDX analysis showed the presence of all expected elements, C and N from tryptophan, and Si and O from the silica matrix with the following weight percentages: 43.9 % C, 2.3 % N, 23.5 % Si, and 30.3 % O (Figure 7).

**Scanning Electron Microscopy and Dynamic Light Scattering Analysis**

Figure 8 shows the SEM images of SMPs whereas Figure 9 shows the SEM images of trp-SMPs at 10,000 and 50,000 times magnification, respectively. The SEM images suggest that the microparticles synthesized through the reverse microemulsion method were roughly spherical in shape with some deposits over them. These small deposits may be due to contaminating oxides. The rough surface could also be attributed to the microparticles that have scaled down to smaller sizes due to their unperfected...
silica networks, which eventually collapsed and sunk, thus forming a loose layer around the stable particles (Xu et al. 2011). The sizes of synthesized SMPs and trp-SMPs were in the wide ranges of 74-592 nm and 203-692 nm. DLS analysis showed that the hydrodynamic diameter of SMPs and trp-SMPs were within the ranges of 67-310 nm and 223-341 nm, respectively (Figure 10 and 11). It is evident from Figure 10 and 11 that the SMPs have a bimodal size distribution while the trp-SMPs have a narrow monodispersed size distribution in water. The size distribution of silica particles formed through oil-in-water microemulsion synthesis depends, among others, on factors that encourage particle growth through intermicellar mixing of silica monomers (vide supra). We hypothesize that the presence of trp might have led to the formation of smaller micellar droplets as was observed when micelles formed from aqueous ammonia were compared with those formed from just water (Chang & Fogler 1997). Trp contains an amino group, which like ammonia (and ammonium ion) potentially weakens the interaction of water molecules with the poly(ethylene oxide) chains of the TX-100 surfactant molecules (Kahlweit et al. 1990, Kabalnov et al. 1995). This leads to decreased hydration swelling of surfactant head groups and decreased surfactant head-to-tail area ratio, which ultimately gives rise to smaller droplets (Chang & Fogler 1997). The diminished chances of micellar fusion due to smaller droplets might have led to the persistence of micelles of comparable silica precursor concentrations, thus, resulting in particles of unimodal dispersity. Alternatively, the unimodal size distribution of trp-SMPs can also be explained by the buffering action of trp. The pH of the aqueous environment within the micellar droplets containing trp is expected to be lower than the pH within the droplets that do not contain trp. Under this condition, TEOS hydrolysis occur more slowly than particle growth. As such, the silica monomers formed rapidly react with the preformed seeds instead of aggregating to form new ones, leading to the formation of uniformly sized particles through the size focusing effect (Reiss 1952, Yokoi et al. 2009, Shahabi et al. 2015).

The disparity in the measured hydrodynamic radius and the actual particle diameter suggests that the agglomerated microparticles in the solid state became dispersed when solvated with water molecules. Alternatively, this may suggest that the loose layer of collapsed unperfected -SiOH networks responsible for the rough surfaces of the microparticles have dissolved in water (vide supra).

Photostability and Leakage Tests
Properties relevant to fluorescent probe applications such as photostability and potential fluorophore leakage were also tested. Compared to free tryptophan, the absorbance of trp-SMPs were observed to be more stable. The absorbance of trp-SMPs in aqueous solution changed from 0.149 ± 0.004 to 0.143 ± 0.020 after 6 hours of continuous exposure to a 150 W halogen lamp. Statistical analysis of the sequential absorbance measurements showed that this decrease in absorbance was not significant. Its photostability was comparable with free tryptophan, whose absorbance also did not significantly change after the same exposure time. However, it can be seen in Figure 8.
that the absorbance of free tryptophan fluctuated more compared to that of trp-SMPs. Shifts in absorbances must be avoided especially during actual real-time applications as in bioanalysis and imaging where they are used in complex biological environments for extended period of time against the background intrinsic cellular emissions. Photostability is conferred to tryptophan molecules in trp-SMPs by protecting them from dissolved oxygen and preventing their photo-oxidation to non-fluorescent products (Miletto et al. 2010).

The leakage test was performed by measuring the fluorescence intensity of 2-mg/mL trp-SMP solution at 24 hour intervals for 3 days. Figure 13 shows the fluorescence intensities of the fluorescent microparticles as a function of immersion time. The fluorescence intensities of the microparticles exhibited insignificant variations within the 3-day immersion time. This shows that tryptophan molecules did not leak out of the silica microparticles even after 3 days of being immersed in aqueous solution. This finding is noteworthy, especially since the fluorophore molecules were embedded in the silica network only through noncovalent interactions. This observation is of crucial importance to the trp-SMPs’ potential application in bioimaging since leakage of encapsulated fluorophores leads to reduction in fluorescent intensity, amplification of the background signal, and fluorophore bleaching (Auger et al. 2011).

Autofluorescence of trp residues in proteins (Teale 1960, Burstein et al. 1973, Chen & Barkley 1998) present in tissues can complicate the potential use of trp-SMPs in biological imaging. However, this hurdle can, in principle, be overcome by encapsulating high concentrations of trp molecules in silica microparticles as the much more intense fluorescence signal coming from the trp-SMP probe can be readily distinguished from the relatively weaker tissue background fluorescence.

**CONCLUSION**

Tryptophan, a fluorescent amino acid, has been successfully incorporated into silica microparticles using the reverse microemulsion method, adding to the variety of fluorescent compounds that can be used for preparing fluorescent material probes. The synthesis was accomplished within 24 hours, yielding particles with diameters ranging from 203 to 692 nm. The fluorescent microparticles were aggregated in the solid state, however, these aggregates became dispersed when suspended in water. The tryptophan-doped silica microparticles exhibited fluorescence at a wavelength of 285 nm. The fluorescent microparticles were photostable and displayed minimal fluctuations in absorbance compared to free tryptophan molecules in solution. Furthermore, tryptophan leakage was not observed even after 3 days of immersion in water despite the use of a non-covalent approach to fluorophore encapsulation. The tryptophan-doped silica microparticles prepared in this study can be employed as fluorescent probes for *in vitro* and *in vivo* bioimaging and bioanalysis upon surface-functionalization with moieties recognized by proteins, DNA, lipid assemblies, and even live cells.

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