Bioimaging of M1 Cells Using Ceramic Nanophosphors: Synthesis and Toxicity Assay of Y$_2$O$_3$ Nanoparticles

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Abstract. Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles were synthesized by enzymatic and polymer-assisted homogeneous co-precipitation methods. Resultant particle size is about 30-40 nm with narrow size distribution whereas the particle size is smaller than those acquired by conventional homogeneous and alkali precipitation methods. The particles shows bright green (550 nm) and red (660 nm) upconversion (UC) as well as near infrared (NIR) fluorescence (1550 nm) under 980 nm excitation. Bioimaging of M1 cells using the nanoparticles were successfully attempted. It is observed that 0.5 mg/ml of nanoparticles is the nominal concentration for bioimaging of M1 cells under the physiological conditions. The cellular uptake of nanoparticles is evidenced from bright field, UC and NIR fluorescence images of live M1 cells. Our studies suggest that lower concentration of nanoparticles is sufficient for imaging when the particles are taken in the M1 cells and also the concentration can keep the cells alive. Further it was demonstrated that under the physiological conditions, Y$_2$O$_3$ nanoparticles emit UC and NIR fluorescence in M1 cells even after the surface modification with PEG-b-PAAc polymer. Moreover, surface modified nanoparticles shows lower toxic effect in M1 cells while compare to bare nanoparticles.

1. Introduction

Rare-earth (RE) doped inorganic nanostructure materials have attracted much attention in recent years because of their unique physical and chemical properties. Among the ceramic nanophosphors, Y$_2$O$_3$ is a promising candidate owing to its photo durability and its phonon energy that is low enough to reveal upconversion (UC) and near infrared (NIR) emission when trivalent RE ion doped in it [1,2]. Recently UC bioimaging using RE doped Y$_2$O$_3$ nanophosphors has been reported as a promising alternative candidate for fluorescent dyes in overcoming the color fading, photo toxicity, auto-fluorescence and light scattering problems that are encountered during fluorescence bioimaging of living cells with the use of ultraviolet or visible excitation lights [3]. The UC is a phenomenon that converts near infrared light into visible light by a multistep excitation process in discreet energy levels of trivalent RE ions in ceramic hosts. An important merit of the UC bioimaging is the usage of a NIR excitation light, which does not cause all of the above problems, such as color fading, photo toxicity etc. In order to apply the UC process to intra cellular bioimaging, the desirable size of the ceramic nanophosphors should be less than 100 nm and the size distribution should be narrow. To meet the requirements we have to control the three important synthesis conditions such as supersaturation, faster nucleation and slower diffusion of the starting materials as well as precipitated precursor particles during the homogeneous precipitation process. Because in a homogeneous method, water at 80 $^\circ$C is very active and the diffusion of starting
materials with respect to particle growth in such a water medium is fast. Instead of supplying activation energy through heating, one can use a biological catalyst such as enzymes in order to overcome such problems. Lower temperature (25 °C) of the reaction medium not only enhances the slower diffusion and growth of the nanoparticles but also avoids damage to the enzyme in an aqueous medium during the synthesis of precursor materials [4]. In another point of view, it is noteworthy that addition of capping agents such as polymers in an aqueous medium along with starting materials may enhance the viscosity and also creates some ionic interactions with metal ions which suppress the diffusion of starting materials as well as precipitated precursors during the homogeneous precipitation process [5]. Diffusion suppression ultimately leads to supersaturation of starting materials which favours the formation of large number of nucleation sites. In order to demonstrate the influence of polymer during the homogeneous co-precipitation of precursor particles [Y(OH)CO$_3$]$^{2-}$, we have investigated the effect of addition of poly cationic [polyallylamine (PAA)] and poly anionic [polyacrylic acid (PAAc)] polymers. Our experimental results suggest that the presence of PAAc during the homogeneous co-precipitation of precursor particles effectively controls the particle size than the presence of PAA.

Bioimaging of M1 cells (macrophages) using Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles by UC as well as NIR fluorescence were observed under 980-nm excitation. UC bioimaging of M1 cells under NIR fluorescence is not reported to date. M1 cells are key players in the immune response to foreign invaders such as infectious microorganisms when it enters in our body. Likewise, they help to destroy bacteria, protozoa, and tumour cells [6-8]. Thus, we demonstrated the experimental work in M1 cells, which is commercially available cell line of mouse macrophage. The influence of various experimental parameters such as concentration of Y$_2$O$_3$ nanoparticles with respect to the survivability of macrophages (toxicity), incubation period (time required to uptake the nanoparticles by M1 cells), particle size effect, toxicity of bare and surface modified nanoparticles were investigated in order to improve the imaging process. Therefore, the present study demonstrates the synthesis, characterization of Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles as well as its UC and NIR fluorescence in M1 cells under 980-nm excitation along with the estimation of the cell toxicity of the nanoparticles in the cells.

2. Experimental

2.1. Preparation of Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles

The Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles were prepared by calcination of precursors at 1100 °C for 1 h. The precursors [Y(OH)CO$_3$]$^{2-}$ were precipitated with enzymatic and polymer assisted homogeneous co-precipitation methods. A mixture of urea (0.4 mol), nitrates of yttrium and erbium were dissolved into distilled water (300 ml) as a starting solution. The amount of Er$^{3+}$ source corresponds to the final composition of the particles to be Y$_{0.93}$ Er$_{0.07}$ O$_{1.5}$. For enzymatic decomposition method, urease enzyme (30 mg) was added to the starting solution and for homogeneous co-precipitation method, PAAc or PAA (0.1 mmol) was dissolved into the starting solution. By heating (for homogeneous precipitation) the solution at 80 °C for 2 h, the urea decomposed into CO$_3^{2-}$ and NH$_4^+$ as precipitants for precursor precipitation. For enzymatic decomposition of urea, the solution was kept for stirring under room temperature (25 °C) for 2 h. For conventional alkali precipitation method, aqueous NH$_4$OH (12 ml from 1.0 mol/L) was added to the starting solution and kept for stirring at 80 °C for 2 h. The precipitated precursors were washed three times with distilled water centrifugally and subsequently washed one time with alcohol and then dried at 100 °C for 12 h. The dried precursor materials were calcined in air atmosphere at 1100 °C for 1 h. Precipitated precursor materials as well as calcined Y$_2$O$_3$ nanoparticles were characterized by XRD, FT-IR, TG-DTA, DLS, FE-SEM and TEM observations.
2.2. UC and NIR emission spectrum measurements

UC and NIR emission spectrum of the samples was measured using a spectrofluorophotometer equipped with a diode laser at 980-nm with 260 mW power as an excitation source.

2.3. UC and NIR fluorescence bioimaging

UC and NIR fluorescence images of the samples were observed with biological microscope equipped with a near infrared excitation (980-nm) as described above and Si and InGaAs-CCD cameras for the UC and NIR fluorescence imaging respectively.

2.4. Bioimaging of M1 cells

Commercially available M1 cell lines of mouse were used for the experiments. For the cellular uptake of Y_2O_3 nanoparticles by M1 cells, various concentrations (0.1-3 mg/ml) of nanoparticles with an average size of 200 nm was mixed with culture medium (mixture of D-MEM cell culture medium and 5% horse serum). After that, matured M1 cells are added to the culture medium and incubated for 12, 24 and 48 h at 37 °C for the cellular uptake of nanoparticles. UC and NIR fluorescence of the M1 cells were observed with above described biological fluorescence microscope.

3. Results and Discussion

3.1. Physico-chemical characterization of nanoparticles

Figure 1 shows FE-SEM and TEM images of precursors as well as calcined Y_2O_3 nanoparticles obtained by various preparation methods. Among the methods enzymatic and polymer assisted co-precipitation methods by using PAAc yield amorphous precursors with a size of 20-30 nm within a narrow size distribution. Figure 2 shows the particle size distribution of precursor particles and calcined Er^{3+} doped Y_2O_3 nanoparticles. We observed that even after calcination process there is no greater change in size and morphology of the nanoparticles which were evident from DLS, FE-SEM and TEM observations when the enzymatic and polymer-assisted methods are used, while obvious growth of the particles was observed when used with homogeneous and alkali precipitation methods. The authors have reported that there is not an obvious change in the morphology and size when the precursors were calcined at 900 °C for 1 h [9]. The calcination at 1100 °C for 1 h, causes the crystal growth though it is proved that the growth can be avoided by the use of enzymatic and polymer-assisted methods. It was observed that the physico-chemical characterization of precursor particles results were in good agreement with our previous report [9]. On the basis of the characterization studies, we concluded that the precipitated precursors are in the form of amorphous yttrium hydroxyl carbonate [Y(OH)CO_3\textsuperscript{2-}]. Moreover, precursor samples co-precipitated with PAAc shows the characteristic vibrations for the presence of CH\textsubscript{2} and COO\textsuperscript{-} molecules even after washing processes which were evident from FT-IR and TG-DTA analysis. This may be due to the fact that PAAc is an ionomer with many negatively charged carboxylate anion (COO\textsuperscript{-}) groups; it has the significant effect to attract Y\textsuperscript{3+} and Er\textsuperscript{3+} cations in an aqueous medium. Thus, we believe that the diffusion of cations is suppressed significantly by the presence of PAAc than the presence of PAA. Because PAA is a polycationic polymer; it makes repulsion with Y\textsuperscript{3+} and Er\textsuperscript{3+} cations in an aqueous medium. It was demonstrated that calcination process eventually removes the organic residues, carbonate and hydroxide species from the Y_2O_3 nanoparticles which were evident from FT-IR, TG-DTA and SEM-EDAX observations.
Figure 1 FE-SEM images of Y(OH)CO$_3^{2-}$ particles precipitated by (a) enzymatic, (b) homogeneous precipitation, (c) polymer-assisted homogeneous co-precipitation and (d) alkali precipitation method. FE-SEM (c1) and TEM (c2) images of Y$_2$O$_3$ nanoparticles were observed after the calcination of Y(OH)CO$_3^{2-}$ particles precipitated by the method (c) are also shown.

Figure 2 Particle size distributions of as-synthesized and calcined Y(OH)CO$_3^{2-}$ particles obtained by the DLS measurement. (a) and (a1) are as-synthesized and calcined Y$_2$O$_3$ nanoparticles by conventional homogeneous precipitation method respectively. (b) and (b1) are as-synthesized and calcined Y$_2$O$_3$ nanoparticles by polymer-assisted homogeneous co-precipitation method respectively.
3.2. UC and NIR emission of Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles

Figures 3 and 4 show the UC and NIR emission spectra as well as fluorescence images of Er$^{3+}$ doped Y$_2$O$_3$ nanoparticles (sample b1). It was confirmed that under physiological conditions Er$^{3+}$ ions emits UC and NIR fluorescence under 980-nm excitation.

3.3. UC and NIR imaging and toxicity studies in M1 cells

The efficiency of UC bioimaging of M1 cells depends upon the various factors including the viability of the M1 cells after up-taking the Y$_2$O$_3$ nanoparticles. Hence it is essential to optimise the Y$_2$O$_3$ concentration to the live M1 cells under physiological conditions in order to achieve the efficient bioimaging. It is observed that the amount of Y$_2$O$_3$ nanoparticles taken by M1 cells increases with increase of Y$_2$O$_3$ nanoparticles concentration up to 0.5 mg/ml (more than 80% cells are alive when compared to the control sample) and above the concentration cell viability decreased (Figure 5). Furthermore, we found that longer incubation period (upto 48 h) does not affect the viability of the M1 cell under the above optimised concentration (Figure 5). Besides, we investigated the size effect under the same experimental condition (0.5 mg/ml of Y$_2$O$_3$; 24 h incubation). We observed that the cell death rate marginally increased when the particle size decreases (Figure 6). This may be due to the fact that smaller size Y$_2$O$_3$ nanoparticles start to dissolve gradually when it retains longer time within the M1 cells. We suspect that, M1 cells food path is under acidic nature so that Y$_2$O$_3$ nanoparticles starts to dissolve and may increase the toxicity in M1 cells. This may also retards the fluorescence of nanoparticles when we want to observe the M1 cells after the longer incubation period like above 48 h.

In order to increase the chemical durability of Y$_2$O$_3$ nanoparticles we made the surface modification with PEG-\textit{b}-PAAc [poly (ethylene glycol)-\textit{b}-poly (acrylic acid)]. Recently our research group members are successfully reported the surface modification of Y$_2$O$_3$ nanoparticles using PEG-\textit{b}-PAAc [10-12]. It is also reported that another PEG modification on the Y$_2$O$_3$ nanoparticles by using a silane coupling agent is another effective surface modification method [12, 13]. We observed that the chemical durability of Y$_2$O$_3$ particles was improved significantly after the surface modification with PEG-\textit{b}-PAAc. Moreover, after the surface modification, agglomeration of the nanoparticles in M1 cells was significantly reduced under the physiological conditions when compare to bare particles. Hence, surface modified Y$_2$O$_3$ nanoparticles with PEG-\textit{b}-PAAc shows lower toxic effect in M1 cells than the bare Y$_2$O$_3$ nanoparticles (Figure 6).
The cellular uptake of nanoparticles is evidenced from the bright field (Figures 7a & 7b), UC and NIR fluorescence (Figure 7c and 7d) images of the live M1 cells incubated for 24 h. To compare the efficiency of Y$_2$O$_3$ nanoparticles with the conventional fluorescence agent, some of the experiments were done with commercially available latex beads. The cellular uptake of latex beads amount is higher when compared to Y$_2$O$_3$ nanoparticles under the identical experimental conditions. However, the emission intensity of the latex beads was diminished within several tens of minutes whereas the Y$_2$O$_3$ nanophosphors maintain a bright image for more than 12 h. Our studies suggest that even lower concentration of Y$_2$O$_3$ nanoparticles (0.5 mg/ml) is sufficient for the UC imaging when the particles are taken in the cells, and also the concentration can keep the M1 cells alive. In order to improve the efficiency of the UC as well as NIR bioimaging under 980-nm excitation further experimental investigations are under progress.

**Figure 5** Cell toxicity of Y$_2$O$_3$ nanoparticle with various concentrations. The number in the view graph denotes the Y$_2$O$_3$ nanoparticles in mg/ml. The result of the fluorescent latex beads is also shown for a comparison.

**Figure 6** Cell toxicity of bare and surface modified Y$_2$O$_3$ nanoparticle with various sizes. The concentrations of the nanoparticles are fixed to be 0.5 mg/ml.

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**Figure 7** Live M1 cells (a) and those up-taking Y$_2$O$_3$ nanoparticles (b, c and d). (a) and (b) are bright field images; (c) and (d) are UC and NIR fluorescence images under 980-nm excitation in biological microscopic view (scale bar: 25 µm).
4. Conclusions

Er$^{3+}$ doped Y$_2$O$_3$ ceramic nanophosphors materials were successfully prepared by enzymatic and polymer assisted homogeneous co-precipitation method. Resultant particles size is approximately 30 nm with narrow size distributions. Surface modification improves the chemical durability of nanoparticles as well as significantly reduces the agglomeration under physiological conditions. Surface modified nanoparticles generate lower toxic effect in M1 cells while compare to bare nanoparticles. After the intra cellular uptake by M1 cells nanoparticles shows green, red UC and NIR fluorescence under 980-nm excitation. Therefore, we concluded that surface modified Er$^{3+}$ doped Y$_2$O$_3$ ceramic nanophosphors are promising materials for UC and NIR bioimaging under NIR excitation.

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References

[1] Matsuura D 2002 Appl. Phys. Lett. 81 4526
[2] Matsuura D, Ikeuchi T and Soga K 2008 J. Lumin. 128 1267
[3] Hampl J, Hall M, Mufti M.A, Yao Y.M, Mac Queen D.B, Wright W.H. and Cooper D.E. 2001 Anal. Biochem. 288 176
[4] Unuma H 2007 Int. J. Appl. Ceram. Technol. 4 14
[5] Peng Z, Jiang Y, Song Y, Wang C and Zhang H 2008 Chem. Mater. 20 3153
[6] Shevach E.M. and Rosenthal A.S. 1973 J. Exp. Medicine. 138 1213
[7] Krombach F, Munzing S, Allmeling A.M, Gerlach J.T, Behr J and Dorger M. 1997 Environ. Health. Perspect. 105 1261
[8] http://en.wikipedia.org/wiki/Macrophage
[9] Venkatachalam N, Saito Y and K. Soga 2009 J. Am. Ceram. Soc. (JACE 02986; article in press)
[10] Kamimura M, Miyamoto D, Saito Y, Soga K and Nagasaki Y 2008 Langmuir 24 8864
[11] Kamimura M, Miyamoto D, Saito Y, Soga K and Nagasaki Y 2008 J. Photopolym. Sci. Technol. 21 183
[12] Zako T, Nagata H, Terada N, Utsumia A, Sakono M, Yohda M, Ueda H, Soga K and Maeda M 2009 Biochem. Biophys. Res. Comm. 381 54
[13] Zako T, Nagata H, Terada N, Sakono M, Soga K and Maeda M 2008 J. Mater. Sci. 43 5325