Research Article

Optimization of $\text{Tb}^{3+}/\text{Gd}^{3+}$ Molar Ratio for Rapid Detection of Naja Atra Cobra Venom by Immunoglobulin G-Conjugated $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$ Nanorods

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In this report, $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ and Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ nanorods@silica- NH_2 conjugated with IgG antibody were synthesized by applying hydrothermal, sol-gel, and coprecipitation methods successively. The effects of $\text{Tb}^{3+}/\text{Gd}^{3+}$ molar ratios of reactants on the size, morphology, and luminescence of the synthesized samples were also investigated. For the optimized $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$ sample, uniform nanorods sizing from 10 to 30 nm in diameter and from 200 to 300 nm in length were obtained with the strongest luminescence in green color with narrow bands under the UV excitation (325 nm). The results revealed that, after being coated with silica- NH_2 and conjugated with IgG antibody, all luminescence characteristic peaks of $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$ corresponding to the process of energy transfer from Gd^{3+} to Tb^{3+} and then the emission from $^5\text{D}_4 \rightarrow ^7\text{F}_j$ ($j = 6, 5, 4, 3$) of Tb^{3+} were still clearly observed. The initial results of using the optimized Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ nanorods@silica- NH_2 conjugated with IgG antibody for rapid selective detection of Naja atra cobra venom were also reported.

1. Introduction

Rare earth-containing luminescent nanomaterials have drawn a special interest for telecommunication, imaging, lightning, fluorescent security labelling, and biomedical applications due to their attractive physical and optical properties, biocompatibility, environmentally friendly characteristics, and nontoxicity and especially due to their high efficiency [1–18]. These materials exhibited outstanding performances such as strong, stable luminescent intensity with a large *Stock* shift, narrow peak width, and long luminescent lifetime [6]. This luminescent lifetime of these materials is significantly longer, up to milliseconds, in comparison to that of quantum dots or organic luminescent materials. That

enables to minimize intrinsic luminescent background emitted from biological samples by employing the time-resolved signal recording process. Many targeted applications such as fluorescent labelling [19], drug delivery [20], and imaging for detecting biological species like virus, bacteria, cell, DNA, RNA molecules, or proteins [15, 18, 19] of new hybrid nanophosphors containing rare-earth elements (Eu, Tb, and Er) with various host materials (GdPO_4 , Gd_2O_3 , YVO_4 , and NaYF_4) have been carried out so far [20–25]. For the synthesis of these materials, numerous chemical methods of luminescent nanomaterials containing rare earth elements have been used such as coprecipitation [5], sol-gel Pechini [6], and hydrothermal/solvothermal method [26–29]. Among these hybrid nanophosphors, Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$

nanomaterials are found to be one of the most promising candidates for these abovementioned applications due to these nanomaterials that exhibit strong luminescent intensity, paramagnetic behavior, no toxicity, and ability to minimize photobleaching and photochemical decomposition [22, 23, 30–33]. It was reported in literature that Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ nanomaterials were usually synthesized by the hydrothermal method and the effects of the doping concentration on their luminescent properties were already performed [27, 31–33]. However, it was indicated that, to obtain the highest luminescence intensity of Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$, the doping Tb^{3+} concentration depended strongly on the hydrothermal synthesis parameters and no optimized procedures was provided. Furthermore, these materials have not been applied in biomedical applications to date. In details, the silica coating of Tb^{3+} -doped $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ followed by functionalization and conjugation with antibody have not been investigated.

In this paper, $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ nanorods were synthesized by controlling hydrothermal process and the effects of the $\text{Tb}^{3+}/\text{Gd}^{3+}$ molar ratio on the nanorod size, morphology, and luminescence of the as-prepared products were also investigated systematically. Moreover, $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ nanorods have been covered by silica as a shell, functionalized with NH_2 and conjugated with a specific antibody (IgG) to enable to set up a fluorescent immunoassay procedure for rapid detection and microscopic bioimaging Naja atra cobra venom *in vitro*.

2. Materials and Methods

All used chemicals were of analytical grade, including $\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich, 99.9%), $\text{Tb}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich, 99.9%), $\text{NH}_4\text{H}_2\text{PO}_4$ (Merck), and NH_4OH 28% (Merck). The deionized (DI) water was used through all the preparation steps of nanomaterials. Naja atra cobra venom antigen was supplied by the Immunology Department of Vietnam Military Medical University.

2.1. Synthesis of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ Materials. For the synthesis of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ nanorods, 20 ml of 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ solution was added into a 100 ml round-bottom flask containing 0.05 M $\text{Gd}(\text{NO}_3)_3$ and 0.05 M $\text{Tb}(\text{NO}_3)_3$ with molar ratios of $\text{Tb}^{3+}/\text{Gd}^{3+}$ are 1%, 3%, 5%, 7%, and 9% during stirring. The pH value of the obtained solution was adjusted to 2 by using 1 M NH_4OH solution. After being stirred for 1 h, the mixture was transferred into a 100 ml teflon-lined stainless-steel autoclave. The autoclave was sealed and heated at 200°C for 24 hours. The white precipitate was centrifuged, washed with DI water and ethanol, and dried at 70°C for 24 hours.

2.2. $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ Materials Coated with Silica Layer via the Sol-Gel Process. Briefly, 0.1 g $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ nanorods were dispersed in 20 ml of ethanol (EtOH) by using a Vortex mixing equipment to obtain mixture A. Subsequently, 60 μl of deionized water and 60 μl of CH_3COOH were added into a 250 ml round-bottom flask containing 50 ml of EtOH and 30 μl TEOS during stirring for 15 minutes

to receive mixture B. Mixture A was then dropped into mixture B, and the final mixture was stirred for 24 hours. Finally, the resulting precipitate was centrifuged and washed with distilled water and ethanol several times. The white precipitate was dispersed in 30 ml EtOH solution for the next functionalization process.

2.3. The Surface Functionalization with $-\text{NH}_2$ ($\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{Silica-NH}_2$). For the surface functionalization of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ by the NH_2 group, 20 μl APTES (3-aminopropyltriethoxysilane) and 40 μl CH_3COOH was added into a 250 ml round-bottom flask containing 50 ml of EtOH. The solution was stirred by magnetic stirring at room temperature for 2 hours. The mixture of 0.3 g $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ and 30 ml EtOH was then dropped into the above solution, and the stirring was remained for 2 hours. The resulting products were collected, centrifuged, and washed with ethanol and distilled water several times. The white precipitate was kept in 30 ml deionized water for the binding process with biological elements.

2.4. Conjugation between $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{Silica-NH}_2$ with Specific IgG Antibody to Detect Naja Atra Cobra Venom. Glutaraldehyde (GDA) was used to form a complex between $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{silica-NH}_2$ with IgG antibody due to the fact that it is a homobifunctional cross-linker to bridge both homogenous aldehyde groups, resulting in conjugation.

The specific IgG antibody conjugation process is as follows: The $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{silica-NH}_2$ solution obtained above (in 2.3.) was centrifuged and cleaned three times with 25 mM sodium phosphate (pH 7.0). The resulting products were dissolved in 5 ml of sodium phosphate solution. Then, 10 ml of glutaraldehyde 0.5% was added and mixed by a vortex mixer for 1 hour at room temperature to form a uniform suspension. 10 μl of anti-venom rabbit antibody was mixed with 100 μl of the solution obtained above in a ratio of 1 : 10 then mixed well and kept at room temperature for 2 to 4 hours.

Excess glutaraldehyde linkers were blocked by adding 1.5 M ethanolamine in phosphate-buffered saline (PBS) reacting with 10% volume of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{silica-NH}_2$ -IgG solution for 2 hours at room temperature. The residual ethanolamine was then removed by washing and centrifugation three times with 0.1 mM PBS solution. The resulting products were dissolved in 5 ml of 25 mM sodium phosphate (pH 7.0) and stored at 4°C for the next steps. The final obtained product was $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{silica-NH}_2$ -IgG solution.

2.5. Detection of Cobra Venom by the Nanorods-Antibodies ($\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{Silica-NH}_2$ -IgG). Cobra venom for detection was conducted as follows: six-well plates (polystyrene plate) were coated with 50 μl /well of Naja atra venom (20 $\mu\text{g}/\text{ml}$) then diluted in 0.05 M carbonate buffer at pH = 9.6. After incubation at 4°C for 12 h, the plates were washed three times with 0.15 M PBS–0.05% Tween-20 (PT) and blocked with 50 μl /well PBS, 1% BSA, 0.05% Tween-20, and 0.02% sodium azide (PBTN) for 1 hour at room

FIGURE 1: FESEM images of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ with molar ratios of $\text{Tb}^{3+}/\text{Gd}^{3+}$ are 0, 1, 3, 5, 7, and 9%.

temperature. After three times of washing cycle in PT, 50 μl antibody nanorods diluted in PBTN were added into each well and incubated at room temperature for 2 hours. Specific antibody-bound $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+} @ \text{silica-NH}_2$ nanorods were used to bind cobra venom antigens. After the plates were washed four times with PT to remove the nonsticking components, the emission color of the nanorods was used to detect the presence of specific expressed venom found under excitation UV light.

2.6. Measurements. The morphology of these nanomaterials was observed by field emission scanning electron microscopy (FESEM, Hitachi S4800). The structure of the materials was determined with the X-ray diffraction system (Siemens D5000). The fluorescence emission and excitation spectra of the materials were measured with a high-resolution microscope (FL 3-22 HORIBA). The ability to detect venom of the nanoparticles is indicated by emitting a green color under a fluorescence microscope (ZEISS Primo Star iLED).

3. Results and Discussion

3.1. The Morphology of Synthesized Samples. The FESEM images of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ with different molar ratios of $\text{Tb}^{3+}/\text{Gd}^{3+}$ from 1 to 9 mol % are given in Figure 1. FESEM images of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ show no significant change in the shape and size of the nanorods with different molar ratios.

It can be seen that $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ nanorods with the molar ratio of 7% are the most uniform rods (average length of 300–500 nm and width of 10–30 nm).

3.2. X-Ray Diffraction Investigation of $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$. Figure 2 shows that the all diffraction peaks of $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$ and $\text{GdPO}_4 \cdot n\text{H}_2\text{O} : \text{Tb}^{3+}$ have high intensity, narrow width at 2θ angles of 15.24, 20.61, 25.81, 30.00, 32.19, 38.71, 43.09, 48.1, 49.86, and 53.15 and can be distinctly indexed to a standard pattern coded 98-004-3396 of gadolinium orthophosphate hydrate.

FIGURE 2: X-ray diffraction patterns of GdPO₄·nH₂O and GdPO₄·nH₂O:Tb³⁺.

3.3. Photoluminescence Properties. In order to investigate the luminescence intensity and determine the radiative transfer between the energy levels of the electrons in these materials, the excitation and fluorescence spectra of these samples were measured under 543 nm and 272 nm excitations, respectively (Figure 3).

In the excitation spectra of GdPO₄·nH₂O:Tb³⁺ (Figure 3(a)), the characteristic transition peaks exist around 310 nm and 272 nm which belong to ⁶P_J → ⁸S_{7/2} and ⁸S_{7/2} → ⁶I₁ of Gd³⁺, respectively [34]. The energy level between ⁶P_J and ⁸S_{7/2} of Gd³⁺ is similar to Tb³⁺, so that the excitation energy of Gd³⁺ can be transferred to Tb³⁺. Under the 272 nm excitation, the photoluminescence spectra of the GdPO₄·nH₂O:Tb³⁺ (1-9%) samples emitted in the green light region with the characteristic transitions of Tb³⁺: ⁵D₄ → ⁷F_n (*n* = 6, 5, 4, 3) (shown in Figure 3(b)). The transition of ⁵D₄ → ⁷F₆, ⁵D₄ → ⁷F₅, ⁵D₄ → ⁷F₄, and ⁵D₄ → ⁷F₃ of Tb³⁺ can be observed at 488, 543, 586, and 620 nm, respectively, and the strongest emission peak was found at 543 nm. The results showed that GdPO₄·nH₂O:Tb³⁺ nanorods with 7% exhibit the strongest green luminescence. It can be seen that the fluorescence of GdPO₄·nH₂O doped with Tb³⁺ was greatly enhanced due to the energy transfer process from Gd³⁺ to Tb³⁺ [34, 35]. The uniform nanorods were obtained by changing the ratios of Gd³⁺ and Tb³⁺ which were functionalized and dispersed in the water and ready for biological applications.

Figure 4 presents the photoluminescence spectra of nanorod solutions: GdPO₄·nH₂O:Tb³⁺ (1); GdPO₄·nH₂O:Tb³⁺@silica (2), GdPO₄·nH₂O:Tb³⁺@silica-NH₂ (3), and GdPO₄·nH₂O:Tb³⁺@silica-NH₂-IgG (4) under 355 nm excitation. The green emission at 491, 544, 588, and 620 nm corresponding to ⁵D₄ → ⁷F₆, ⁵D₄ → ⁷F₅, ⁵D₄ → ⁷F₄, and ⁵D₄ → ⁷F₃ of Tb³⁺ transitions of Tb³⁺ ions, respectively. The emission intensity of these samples decreases with the

functional steps of GdPO₄·nH₂O:Tb³⁺ nanorods. However, luminescent intensity is not much changed after conjugating with IgG and the maximum emission peak is found at 543 nm. This is the good condition for labelling the cobra venom antigen identification in the next steps.

3.4. IR Properties. The functionalized nanorods were analyzed by IR spectra using the Nexus 670 infrared spectrometer (Nicolet).

The infrared spectra of GdPO₄·nH₂O:Tb³⁺ (a), GdPO₄·nH₂O:Tb³⁺@silica (b), GdPO₄·nH₂O:Tb³⁺@silica-NH₂ (c), and GdPO₄·nH₂O:Tb³⁺@silica-NH₂-IgG (d) samples are presented in Figure 5. It can be observed that oscillations of the O-H bond were found at around 1600 cm⁻¹ and near 3500 cm⁻¹ in lines (a) and (b). Oscillations at 545 and 622 cm⁻¹ are assigned to the stretching vibrations of (PO₄)³⁻ [36]. The stretching vibrations of the C-H bond were observed at 2898 cm⁻¹, 2976 cm⁻¹ and 2884 cm⁻¹, 2941 cm⁻¹ corresponding to GdPO₄·nH₂O:Tb³⁺@silica-NH₂ (c) and GdPO₄·nH₂O:Tb³⁺@silica-NH₂-IgG (d) samples. In these samples, the stretching vibrations of (PO₄)³⁻ are still observed at 622 and 880 cm⁻¹. The peaks in the range of 3300-3500 cm⁻¹ and 1590-1620 cm⁻¹ appear as stretching and bending vibrations of the N-H bond. These results show that the fabricated samples were successfully surface-functionalized with the NH₂ groups as a binding agent.

3.5. Application of Nanorods Conjugated with Rabbit Antibody to Detect Naja Atrra Cobra Venom Antigen

3.5.1. Evaluating the Results of Binding Nanorods with Rabbit Antibody by Infrared Spectra. The infrared spectra of the synthesized samples were used to elucidate the difference

FIGURE 3: Excitation (a) and photoluminescence spectra (b) of the $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$ samples with molar ratios of $\text{Tb}^{3+}/\text{Gd}^{3+}$ are 0, 1, 3, 5, 7, and 9%.

FIGURE 4: Photoluminescence spectra of $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$ with molar ratios of $\text{Tb}^{3+}/\text{Gd}^{3+}$ 7% conjugated with IgG.

between anti-venom antibody before and after conjugating with luminescence nanorods.

In Figure 6(a), the strong and wide peak at 3460 cm^{-1} corresponds to the O-H bond. The peak at 2079 cm^{-1} belongs to the N-H bond. The wavelengths from 1640 cm^{-1} to 1443 cm^{-1} are the oscillating peaks of the C=O and N-H bonds in the first amine group, respectively.

Figure 6(b) shows that the peak at 1389 cm^{-1} indicates the forming the C-N bond through reaction of the aldehyde

group ($\text{O}=\text{C}-\text{H}$) of the GDA linker with NH_2 of IgG. Thus, it can be suggested that the conjugation (linkage) between luminescence nanorods with anti-venom antibodies was formed.

3.5.2. Evaluation the Ability of the $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}@\text{Silica}-\text{NH}_2\text{-IgG}$ (Nanorods-Antibodies) to Detect the *Naja Atracobra* Venom. As the experimental steps described above (in Section 2.5), in order to evaluate the ability of

FIGURE 5: IR spectra of $\text{GdPO}_4 \cdot n\text{H}_2\text{O}$, $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}$, $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}@\text{silica-NH}_2$, and $\text{GdPO}_4 \cdot n\text{H}_2\text{O}:\text{Tb}^{3+}@\text{silica-NH}_2\text{-IgG}$.

FIGURE 6: IR spectra of IgG antibody without (a) and with (b) luminescence nanorods.

detection of *Naja atra* cobra venom of nanorods, two kinds of well groups were organized. The wells coated with *Naja atra* cobra venom antigens were incubated with nanorods (1) and nanorods-antibodies (2). The image of *Naja atra* cobra venom antigens after coating on the plate under visible light of a 40x microscope is illustrated in Figure 7(a) while Figures 7(b) and 7(c) present the images of *Naja atra* cobra venom antigens conjugated with nanorods-antibodies observed without and with UV light of 40x fluorescence microscope, respectively.

The results showed that the wells incubated with nanorods-antibodies provided a green image and nanoparticles distributed uniformly throughout the field green light under a 40x fluorescence microscope (Figure 7(c)). This result shows that these wells have a specific association between the nanorods-antibodies and the *Naja atra* cobra antigen so that when it was washed, it does not wash away. Meanwhile, other emissions were not green due to the absence of a combination of antibody-coated nanoparticles and *Naja atra* cobra venom antigens so they were washed

FIGURE 7: The images of *Naja atra* cobra venom antigens under visible light (a); *Naja atra* cobra venom antigens conjugated with nanorods-antibodies without UV light (b) and with UV light (c) under 40x fluorescence microscope.

away during washing. Based on these results, it could be concluded that this *Naja atra* venom detection procedure can shorten the time needed for early diagnosis and improve the efficacy of treatment and thus could facilitate early treatment of snake bite and save lives. Thus, with respect to other green-emitting luminescent materials like Yb,Er-codoped NaYF_4 or $\text{TbPO}_4 \cdot \text{H}_2\text{O}$ -based nanomaterials [19, 20], our synthesized immunoglobulin G-conjugated $\text{GdPO}_4 \cdot \text{nH}_2\text{O} : \text{Tb}^{3+}$ nanorods are compatible for detection of antigen-antibody reaction.

4. Conclusions

$\text{GdPO}_4 \cdot \text{nH}_2\text{O}$ and $\text{GdPO}_4 \cdot \text{nH}_2\text{O} : \text{Tb}^{3+}$ nanorods with a tetragonal phase were synthesized by the hydrothermal method. Optical properties of nanorod materials were optimized by varying the concentration ratio of $\text{Tb}^{3+}/\text{Gd}^{3+}$. At 7 mol % of Tb^{3+} , the nanorods exhibited the strongest luminescence because of the energy transfer from gadolinium to terbium. The most uniform nanorods have dimensions of 300–500 nm in length and 10–30 nm in width. The surface functionalization of luminescent nanorods conjugated with rabbit IgG antibodies can be used for early and rapid detecting *Naja atra* cobra venom antigen.

Data Availability

The concerning data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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