Surface PolyPEGylation of Eu^{3+} doped luminescent hydroxyapatite nanorods through the combination of ligand exchange and metal free surface initiated atom transfer radical polymerization

Guangjian Zeng^{a, #}, Meiyang Liu^{a, #}, Chunling Heng^{a, c}, Qiang Huang^a, Liucheng Mao^a, Hongye Huang^a, Junfeng Hui^c, Fengjie Deng^{a, *}, Xiaoyong Zhang^{a, *}, Yen Wei^{b, *}

^a Department of Chemistry, Nanchang University, 999 Xuefu Avenue, Nanchang 330031, China.

^b Department of Chemistry and the Tsinghua Center for Frontier Polymer Research, Tsinghua University, Beijing, 100084, P. R. China.

^c Shaanxi Key Laboratory of Degradable Biomedical Materials, Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical and Engineering, Northwest University, Xi'an 710069, PR China

These authors contributed equally to this work

Abstract

The Eu^{3+} doped luminescent hydroxyapatite (HAp) nanorods with uniform size and morphology can be synthesized by hydrothermal route. However, these HAp nanorods are coated by hydrophobic oleylamine, which makes them difficult to be dispersed in aqueous solution and impede their biomedical applications. In this work, Eu^{3+} doped luminescent polymers functionalized HAp nanorods were prepared through the combination of ligand exchange reaction and metal free surface initiated atom transfer radical polymerization (ATRP) method. In this procedure, the amino group functionalized HAp nanorods were first prepared by ligand exchange reaction using adenosine monophosphate (AMP) as ligand. Then the Br-containing initiators (HAp-Br) were introduced onto the surface of HAp-AMP nanorods through the amidation reaction. Finally, polymers functionalized HAp nanorods were prepared by metal free ATRP method using poly(ethylene glycol) methacrylate (PEGMA) as monomer and 10-phenylphenothiazine (PTH) as organic photocatalyst. The properties of these obtained HAp nanocomposites (HAp-polyPEGMA nanorods) were characterized by means of transmission electron microscopy, Fourier transformed infrared spectroscopy, X-ray photoelectron spectroscopy and thermogravimetric analysis in detail. The cell imaging of these HAp-polyPEGMA nanorods was examined using laser scanning confocal microscope to evaluate their biomedical applications. We demonstrated for the first time that hydrophobic luminescent HAp nanorods can be functionalized with polyPEGMA through the combination of ligand exchange reaction and metal free surface initiated ATRP. As compared with the traditional ATRP, the metal free ATRP can overcome the toxic and fluorescence quenching effects of metal catalysts such as copper ions. More importantly, the strategy described in this work should also be utilized for fabrications of many other luminescent polymer nanocomposites due to its good monomer adoptability.

Key words: luminescent hydroxyapatite nanorods, metal free ATRP, ligand exchange reaction, photocatalysts, bioimaging applications

Introduction

Recently, hydroxyapatite ($\text{Ca}_{10}(\text{OH})(\text{PO}_4)_6$, HAp) as an important class of biomaterials has attracted much considerable interest for biomedical applications thanks to its superior properties, such as favorable biocompatibility, biological activity and biodegradability.[1-20] On the other hand, the HAp nanocrystals could be served as good host materials for doping lanthanide ions.[21-27] So these materials can be endowed with fluorescent properties and used as novel fluorescence probes for cell imaging and drug delivery applications. In order to avoid the fluorescence quenching of the excited state of the Eu^{3+} ions by the hydroxyl group in the lattice, the hydroxyl group can be replaced by fluorine. Although HAp nanocrystals have many excellent properties, the application is seriously hindered for the difference between the different HAp nanocrystals. Previous reports have suggested that hydrothermal treatment could obtain HAp nanocrystals with uniform size and morphology. However, these HAp nanocrystals are normally coated with hydrophobic organic molecules to maintain their good dispersibility only in organic solvents. This makes the direct usage of these hydrophobic luminescent HAp nanocrystals for biomedical applications problematic. It is well known that the prerequisite of materials for biomedical applications is their water dispersibility. Therefore, it is utmost important to develop novel surface modification strategy to overcome these issues described above. Previously, some reports have been devoted to the surface modification of HAp through different strategies. For example, we have demonstrated that hydrophobic Ln ions doped HAp nanorods can be transferred into hydrophilic ones through direct surface self-assembly with commercial available amphiphilic polymers (F127).[28] This makes the biomedical applications of Ln ions doped luminescent HAp nanomaterials possible. Furthermore, the surface coating of luminescent HAp nanomaterials with synthetic amphiphilic polymers has also achieved by our group.[29] Through living radical polymerization, we could adjust the structure and properties of these amphiphilic polymers. However, these self-assemblies may not be stable due to the weak interaction between the hydrophobic oleylamine coatings and these amphiphilic polymers.[30] This will inevitably influence the usefulness for biomedical applications. Therefore, the development of novel surface modification strategies for preparation of stable surface coatings is still of great importance.

Surface initiated polymerization methods, including surface initiated atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer polymerization have been widely used for surface modification of different materials and surface for different purposes. However, the traditional ATRP required using transition metal ions as catalysts, which are toxic to living organisms and will quench the fluorescence. Therefore, it is a great challenge for surface modification of fluorescent materials through traditional SI-ATRP. As compared with the traditional ATRP methods, the new metal free polymerization

does not require heavy metal catalysts (such as copper ions) and expensive organic ligands. Since the metal free ATRP method proposed, it has attracted more and more attention of the scientists from chemistry, material and interdisciplinary fields.[31, 32] It has been reported that the metal free ATRP process could be carried out at room temperature and UV irradiation using a organic molecule as catalyst, which can be synthesized by a simple one-step conjugation reaction.[33] However, the utilization of the metal free ATRP for fabrication of functional polymer nanocomposites has rarely been reported thus far.

Poly(oxyethylene) (PEG) is a kind of popular amphipathic polymer for the surface modification of materials (such as CNTs, SiO₂, magnetic nanoparticle, nanodiamond, etc.) and enhanced their dispersibility and biocompatibility.[34-43] In this work, the Eu³⁺ doped luminescent HAp nanorods were surface functionalization of PEG through the combination of ligand exchange reaction and metal free ATRP method under a rather mild reaction conditions using 10-phenylphenothiazine (PTH) as organic photocatalyst. The detailed synthetic process for the preparation of PEGlated HAp nanorods was shown in **Scheme 1**. First, NH₂-contained HAp was prepared via ligand exchange reaction using adenosine monophosphate (AMP) as ligand. Then the Br-contained initiators (HAp-Br) were introduced onto the surface of HAp-AMP nanoparticles through the amidation reaction. Finally, the polyPEGylated HAp nanorods were prepared by metal free ATRP method using the PEGMA as monomer. The properties of these obtained HAp nanorods were characterized by a series of characterization methods. The polyPEGylated HAp nanorods showed favorable dispersibility in both organic and aqueous solution. On the other hand, the HAp nanorods with excellent luminescent properties could be applied to live cell imaging.

Scheme 1 Schematic representation for the synthesis of HAp-polyPEGMA.

Experiment section

2.1 Materials and characterization

Eu³⁺ doped HAp (HAp-Eu³⁺) nanoparticles were synthesized according to our previous report.[44] Adenosine monophosphate (AMP) was purchased from Shanghai Sangon Biological Engineering Technology And Service Co., Ltd., Poly(ethylene glycol) methylethermethacrylate (PEGMA, MW: 950 Da,

98%), 2-bromoisobutyryl bromide (BIBB, 98%) were purchased from Aladdin (Shanghai, China). All other reagents and solvents were obtained from Aladdin and used without further purification. The organic photocatalyst was synthesized according to the report by Hawker et al.[33] The characterization information of HAp samples was described in supporting information.

2.3 Synthesis of amino-terminated HAp nanoparticles (HAp-AMP)

The amino-terminated HAp nanorods (HAp-AMP) were synthesized through ligand exchange reaction. Namely, 50 mL water-acetone solution was added into the round-bottom flask. Then 200 mg HAp-Eu³⁺ nanorods and 100 mg AMP were suspended in the solution under ultrasonic processing for 20 min. The reaction was carried out at 70 °C for 7 h. The obtained solid was separated with centrifugation at 6000 rpm for 10 min. And the precipitations were washed with deionized water and acetone several times. Then the solid products were dried at 60 °C to a constant weight.

2.4 Synthesis of HAp-Br initiators

The Br-contained initiators (HAp-Br) were introduced onto the surface of HAp-AMP nanoparticles through the amidation reaction. 450 mg HAp-AMP nanoparticles, 60 µL TEA, and 35 mL dry toluene were added into 100 mL dry Schlenk flask. The mixture was treated with sonication for about 20 min. Subsequently, 20 µL BIBB dissolved in 5 mL of dry toluene was injected slowly at 0 °C. After 2 h, the HAp-Br initiators were separated with centrifugal machine at 6000 rpm and then rinsed with acetone several times for remove excess BIBB. The products were dried at 60 °C for 12 h.

2.5 Preparation of the hydrophilic HAp-polyPEGMA nanoparticles

The preparation of hydrophilic HAp-polyPEGMA nanoparticles was carried out by the new method of metal free ATRP process. 150 mg HAp-Br, 500 mg PEGMA and 19 mL dry DMF were added into 100 mL dry Schlenk flask which was equipped with a magnetic stir bar. The mixture was treated with freeze-pump-thaw circle for three times. The reaction system was backfilled with N₂. Then the photocatalyst (0.1 mol%) was dissolved in 1 mL DMF and injected into the reaction system slowly by syringe. The reaction flask was placed into the 380 nm LEDs light box which made by ourselves while cooling with mini fan to maintain room temperature. The reaction was carried out for 4 h. After that, the products were collected with centrifugation at 6000 rpm for 6 min and washed with water and ethanol to remove unreacted PEGMA monomer. The resulting products were dried at 60 °C for 12 h.

2.6 Cell imaging and cell viability evaluation of HAp-polyPEGMA

The experimental procedures about the cell imaging and cell viability of HAp-polyPEGMA were provided in the supporting information in detail.

Results and discussion

^1H NMR spectra were employed to characterize the PEGMA monomer and the obtained HAp composites. The ^1H NMR spectrum of PEGMA monomer was shown in **Fig. S2**. The peaks at 5.55 and 6.10 ppm were attributed to the protons that were connected to the C=C bond in $\text{CH}_2=\text{C}(\text{CH}_3)$ (b). The peak at 4.27 ppm was attributed to the protons of COOCH_2 (c). The peak at 3.62 ppm was attributed to the protons of $\text{CH}_2\text{CH}_2\text{O}$ repeat units of PEGMA (d). The ^1H NMR spectrum of HAp-polyPEGMA was shown in **Fig. 1**. A series of characteristic peaks with chemical shift at 0.8-4.0 ppm were observed, which attributed to the protons of polyPEGMA. More importantly, the peaks at 5.55 and 6.10 ppm were disappeared, confirming that the PEGMA monomer was successfully polymerized and introduced into the sample of HAp-Br. Additionally, As compared with the ^1H NMR spectrum of AMP, two characteristic signals with chemical shift at 8.0-8.5 ppm were observed. These peaks can be corresponded to the protons of adenine of AMP. A peak at 5.93 ppm was observed and it also could be attributed to the protons of AMP, confirming the successful fabrication HAp-AMP composites. All these results were further suggesting that the polyPEGMA was successfully grafted from the surface of HAp by metal free ATRP method.

Fig. 1 ^1H NMR spectrum of HAp-polyPEGMA dissolved in D_2O .

The morphology and structure of the samples of HAp nanorods before and after surface modification have been investigated in details by using TEM. As shown in **Fig. 2**, it can be observed that the Eu^{3+} ion deposited HAp nanoparticles are relatively straight nanorods with a diameter of about 15 nm and the lengths between 50 and 80 nm. After coating with polyPEGMA, the size and morphology of HAp nanorods has not obvious change, because the PEGylation process is rather gentle. It could be that this method for grafting from surface of HAp is pretty gentle. And the sparse distribution of polyPEGlated HAp is consistent with the dispersibility of HAp-polyPEGMA. The dispersibility of HAp samples are shown in **Fig. S1**. It can be observed that the HAp nanorods are easily to disperse in water after surface modification with PEGMA.

Fig. 2 TEM images of the HAp-Eu³⁺ (A), HAp-AMP (B), HAp-Br (C) and HAp-polyPEGMA (D).

FT-IR spectra were employed to characterize the characteristic peak in the samples of HAp for proving the successful synthesis of HAp-polyPEGMA. As shown in **Fig. 3**, after grafting PEGMA on the surface of HAp, the new characteristic peaks emerged at about 2950 and 1648 cm⁻¹, which could be attributed to the alkyl C-H stretching vibration and C=O stretch vibration, respectively. As compared with the spectra of HAp-Br and HAp-AMP, the intensity of characteristic peaks located at about 2950 and 1648 cm⁻¹ were obviously increased, indicating that the PEGMA could be grafted from the surface of HAp-Br under the 380 nm light UV irradiate by metal free ATRP method. In addition, as compared with HAp-Eu³⁺, the characteristic peaks located 564 and 604 cm⁻¹ haven't changed a lot in the samples of HAp-AMP and HAp-Br, which could be attributed to the bending vibration of PO₄³⁻ group. But it could be observed that the intensity was obviously decreased in the sample of HAp-polyPEGMA. We suspect that could be caused by the hydrolyzation of polyPEGMA and the formation of chemical bond of -C-O-P.

Fig. 3 FT-IR spectra of the HAp-Eu³⁺, HAp-AMP, HAp-Br and HAp-polyPEGMA.

The percentage of polymer was determined and the thermostability of HAp samples was investigated by TGA (**Fig. 4**). It could be seen that the weight of HAp-Eu³⁺ is tended to be a constant when the heating temperature at 550 - 800 °C. And the weight loss of HAp-Eu³⁺ can be calculated to be 15.7%. After ligand exchange with AMP, it could be observed that much more weight loss between 250 and 550 °C which suggested that the AMP have been successfully exchanged onto the surface of HAp particles. As compared

with HAp-AMP, no obvious weight loss was observed in the sample of HAp-Br. This indicated that the weight percentage of BIBB immobilized on the surface of HAp nanorods is few. Furthermore, after the PEGMA was grafted from the surface of HAp-Br, the weight loss of HAp-polyPEGMA was increased to 33.8%. Therefore, the mass percentages of polyPEGMA conjugated on the HAp particles were calculated to be 18.1% for HAp-polyPEGMA. A significant mass decrease can be observed in the samples of HAp-polyPEGMA when the temperature is located at 250-400 °C, which could be attributed to the decomposition temperature of PEGMA.

Fig. 4 TGA curves of HAp-Eu³⁺, HAp-AMP, HAp-Br and HAp-polyPEGMA.

Apart from the characterizations of TEM, FT-IR, TGA and ¹H NMR, XPS was also utilized to characterize the chemical compositions of HAp-Eu³⁺, HAp-AMP, HAp-Br and HAp-polyPEGMA. As shown in **Fig. 5A**, the elements including Ca, P, O, Eu, C, N and Br could be observed in the HAp samples. The high resolution XPS spectra of Ca2p, P2p, O1s, Eu3d, C1s, N1s and Br3d were shown in **Fig. 5B-H**. It could be observed that the intensity of Ca2p, P2p and O1s were decreased after the samples of HAp reacting with other agents. After ligand exchange with AMP, a new peak between 395 and 405 eV relating to N1s was emerged in the samples of HAp-AMP. It is similar to the HAp-AMP, a new peak between 66 and 72 eV relating to Br3d can be observed in the samples of HAp-Br, indicating that the Br-contained initiators were successfully introduced onto the surface of HAp-AMP nanorods through the amidation reaction. Especially to deserve to be mentioned, we can get more detail information about the samples of HAp from the C1s region of XPS in **Fig. 5F**. Curve fitting of the C1s spectra was performed using Lorentzian-Gaussian peak

shape after performing a Tougaard background correction (**Fig. S3**). It can be seen that the photoelectron peak of C in the sample of HAp-Eu³⁺ is mainly located at about 284.6 eV, which can be attributed to the C-C and C-H bonding of oleic acid on the surface of HAp nanorods. After reaction with AMP, the binding energy located at 285.7, 286.3 and 287.0 eV are typically assigned for the C-N, C-O and C=N bond, respectively. As we expect that the peaks between 286.03–288.83 eV were obviously increased after modification with polyPEGMA, which can be ascribed to the C-O bond in the repeat unit of PEG of the polyPEGMA, indicating that polyPEGMA can be grafting from the surface of HAp nanorods through metal free ATRP method.

Fig. 5 (A) The XPS full scan spectra of HAp-Eu³⁺, HAp-AMP, HAp-Br and HAp-polyPEGMA ranging from 0 to 1200 eV. (B) the Ca2p region, (C) the P2p region, (D) the O1s region, (E) the Eu3d region, (F) the

C1s region, (G) the N1s region and (H) the Br3d region.

Based on the XPS analysis, the weight percentages of Br, C, Ca, N, O, P and Eu were also calculated and listed in **Table 1**. The percentage of Eu element in the sample of HAp-Eu³⁺ was 2.30%. After reaction with AMP and BIBB, the weight percentages of Ca element were decreased from 12.48% to 8.68% and 9.91% with the contents of organic component increase in the sample of HAp-AMP and HAp-Br, respectively. More importantly, a new element N (4.49%) was found in the sample of HAp-AMP, which can be attributed to the adenine group in AMP. These results suggest that the AMP was successfully immobilized on the surface of HAp nanorods using ligand exchange reaction. Furthermore, a new element Br (0.23%) was found in the sample of HAp-Br. The obtained HAp-Br can be evidenced by the content change of Br element in the same way. After grafting polymerization with PEGMA under UV light irradiation, the O and C contents were further increased to 37.94% and 42.87%, respectively. Well consistent with the results, the percentages of Br, Ca, N, P and Eu were decreased. These results provide further powerful evidence for the successful preparation of HAp-polyPEGMA by metal free ATRP method.

Table 1 Element contents (%) of HAp samples based on XPS analysis.

Samples	Br3d	C1s	Ca2p	N1s	O1s	P2p	Eu3d
HAp-Eu³⁺	0	41.42	12.48	0	35.93	7.87	2.30
HAp-Amp	0	43.82	8.68	4.49	35.33	6.08	1.60
HAp-Br	0.23	37.26	9.91	6.20	37.23	6.99	2.18
HAp-polyPEGMA	0.19	42.87	6.53	5.08	37.94	6.06	1.33

The emission spectrum of the HAp-polyPEGMA nanorods is recorded from 420 to 750 nm and the excitation wavelength was set at 405 nm (**Fig. 6**). There are two main characteristic peaks corresponding to its red luminescence at the wavelengths of 591 and 615 nm can be observed in the sample, which are actually attributed to ⁵D₀→⁷F₁, ⁵D₀→⁷F₂ transitions of Eu ions, respectively.[45, 46] Besides, the HAp-polyPEGMA nanorods also displayed a main characteristic peak corresponding to the blue luminescence at around 450 nm.[47, 48] Fortunately, the luminescent properties unchanged after grafting with PEGMA, which could be attributed to the radiative transition of rare earth ions is f-f electronic transition. The biocompatibility of biomaterials is very important for their potential biomedical applications. Therefore, the possible adverse effects of HAp-polyPEGMA nanorods towards A549 cells were evaluated

using CCK-8 assay. As shown in **Fig. S5**, no obvious cell viability decrease was found after A549 cells incubated with HAp-polyPEGMA nanorods for up to 36 h. As evidenced by CCK-8 assay, we demonstrated that the cell viability values of A549 cells are still greater than 93%. The cell viability examination implied that HAp-polyPEGMA nanorods possess desirable biocompatibility. Therefore, the method for preparation of hydrophilic luminescent HAp nanorods provides the possibility for potential biomedical applications.

Fig. 6 The emission spectrum of HAp-polyPEGMA nanorods dispersed in cyclohexane under excitation at 405 nm.

The cell uptake behavior of HAp-polyPEGMA nanorods was evaluated using CLSM. As shown in **Fig. 7**, many fluorescent signals were found in **Fig. 7A**. These fluorescent signals were encapsulation of some areas with absent of fluorescence. **Fig. 7B** shows the bright field of CLSM images. It can be seen that cells still adhered very well to the cell plates. And the cells still kept their normal morphology after they incubated with HAp-polyPEGMA nanorods for cell imaging. This indicated that the HAp-polyPEGMA nanorods possess excellent biocompatibility. Furthermore, the emerged CLSM image from **Fig. 7A** and **Fig. 7B** was displayed in **Fig. 7C**, it can be seen that the locations of cells and fluorescent signal areas are in the same locations. In other words, the fluorescent signals were exactly overlapped on the cells. On the other hand, we also found that the center of cells is absent of fluorescent signals. This implied that the HAp-polyPEGMA nanorods were mainly accumulated in the cytoplasm after internalization and could not enter cell nucleus. The cell uptake behavior suggested that HAp-polyPEGMA nanorods can be used as probes for biological imaging and imaging guided drug delivery applications.

Fig. 7 CLSM images of A549 cells after they were incubated with $100\ \mu\text{g mL}^{-1}$ of HAp-polyPEGMA nanorods for 3 h. (A) fluorescent image, (B) bright field, (C) merged image of A and B. The scale bar =20 μm .

Conclusions

In this work, the polyPEGylated HAp nanorods were successfully synthesized by metal free ATRP method. To achieve this, the NH_2 -contained HAp was first prepared by ligand exchange reaction using AMP as ligand. Then the Br-contained initiators were introduced onto the surface of HAp-AMP nanorods through the amidation reaction. Finally, the hydrophilic HAp nanorods were prepared via metal free SI-ATRP using organic dye as photocatalyst PEGMA as the monomer. HAp samples have been characterized by a series of characterization techniques, including TEM, FT-IR, ^1H NMR, TGA, XPS and FL spectra. Because the PEGylated HAp nanorods showed strong luminescence, biodegradability and biocompatibility, they could be promising candidate materials for cell imaging and drug delivery applications. Compared with the traditional ATRP method, the strategy mentioned in this work is rather simple and effective for grafting polymer from the surface of materials. Furthermore, the metal free ATRP method is effectively avoided the negative effect caused by the metal ions against living organisms. Additionally, the Br-contained initiators can be synthesized through the amidation and esterification. So this method is also a convenient strategy for the surface modification of other materials and enhancing their biocompatibility, water solubility for their applications in biomedicine fields.

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