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Al(OH)$_3$ facilitated synthesis of water-soluble, magnetic, radiolabelled and fluorescent hydroxyapatite nanoparticles†

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Magnetic and fluorescent hydroxyapatite nanoparticles were synthesised using Al(OH)$_3$-stabilised MnFe$_2$O$_4$ or Fe$_3$O$_4$ nanoparticles as precursors. They were readily and efficiently radiolabelled with $^{18}$F. Bisphosphonate polyethylene glycol polymers were utilised to endow the nanoparticles with excellent colloidal stability in water and to incorporate cyclam for high affinity labelling with $^{64}$Cu.

so that they share similar in vivo behaviour, and chemically stable to ensure that the signal of each modality reflects the same anatomic position.

Hydroxyapatite (HA) has attracted much interest as the basis of multifunctional probes very recently, because of its biocompatibility and high affinity for fluoride which allows facile labelling with the positron emitter $^{18}$F. Fluorescent HA can be obtained by either doping with rare earth cations or by conjugation with organic dyes. HA is not an ideal fluorescent host matrix, so luminescent rare earth doped HA nanocrystal requires up to 20% replacement of OH$^-$ by F$^-$ (maximum theoretical value for fluoride substitution), to minimise the quenching of the excited state of rare earth cations. As a result, such HA is no longer suitable for $^{18}$F radiolabelling. It has been reported that magnetic iron oxide NPs can be deposited on the surface of HA aggregates or NPs via thermolysis or a wet chemistry approach. One problem that remained unsolved for both synthetic approaches is how to effectively isolate the desired Fe$_3$O$_4$–HA composites from the unwanted iron oxides and HA nanoparticles. Moreover, all these multifunctional HA NPs suffer from the problem of aggregation or large size to some extent, which is an obstacle for their biological or medical applications. In this work, we present a novel synthesis of magnetic and fluorescent HA nanocomposites with uniform size and morphology, and excellent colloidal stability in water by using Fe$_3$O$_4$ nanoparticles stabilised with Al(OH)$_3$ as a template. The radiolabelling, magnetic and optical properties were investigated, to demonstrate potential for application as tri-modal probes for MR, PET and optical imaging.

Our strategy is to synthesise HA using water-soluble magnetic Fe$_3$O$_4$@Al(OH)$_3$ or MnFe$_2$O$_4$@Al(OH)$_3$ NPs as templates. The advantages of this approach is the small hydrodynamic size of the template particles and their excellent colloidal stability, provided by the Al(OH)$_3$ layer as reported previously. More importantly, the layer of Al(OH)$_3$ can be readily removed as it is soluble under basic pH conditions. The design incorporated bisphosphonate polyethylene glycol (BP-PEG) polymers (Scheme 1) to stabilise NPs after the formation of HA on the surface, to take advantage of the outstanding binding affinity of bisphosphonates to HA.
Bisphosphonate amine 1 was obtained via a slightly modified version of the previously reported protocol. PEG carboxylic acids were obtained by oxidation of corresponding PEG polymers with CrO3/H2SO4 via the reported protocol. The bisphosphonate (BP) or 1,4,8,11-tetraazacyclotetradecane (cyclam) were grafted to PEG polymeric chain via amide formation mediated by N,N'-dicyclohexylcarbodiimide (DCC), followed by deprotection with bromotrimethylsilane (TMBS). BP-PEG-Me and BP-PEG-cyclam were purified by dialysis for over 24 hours using a membrane with a cut-off size of 3500 Da to remove unconjugated small molecules such as bisphosphonate amine 1 and 1,4,8,11-tetraazacyclotetradecane. The conjugation of bisphosphonate and PEG was confirmed by the change in chemical shift in the 31P NMR spectrum (from 20 ppm for free bisphosphonate to 12.8 ppm for BP-PEG-Me, see ESI†).

Nanoparticulate precursors MnFe2O4@Al(OH)3 and Fe3O4@Al(OH)3 were obtained via a method reported by our group previously. Typically, 4 ml Fe3O4@Al(OH)3 colloids (concentration of Fe3O4 ca. 8 mg ml⁻¹) and 200 mg BP-PEG-Me polymers were placed in a 500 ml flask containing 300 ml water. Under stirring, 4 ml 0.1 mol l⁻¹ NaH2PO4 and 4 ml 0.2 mol l⁻¹ CaCl2 aqueous solutions were added sequentially. The pH of the reaction was adjusted to 7.5 after the addition of NaH2PO4 solution, from 42.5 mV to 27.1 mV after adding the CaCl2 solution, indicating a reaction of calcium cations and phosphate anions. Similar results were also observed for MnFe2O4@HA NPs colloids instead of MnFe2O4@Al(OH)3 as precursors, a simple mixture of magnetic MnFe2O4 and non-magnetic HA NPs was obtained, identifiable as two kinds of NPs with apparently different morphology and size on TEM images. Both MnFe2O4@HA and Fe3O4@HA NPs were coated by BP-PEG-Me polymers 2 during the synthesis, as confirmed by ca. 18% mass loss on thermogravimetric analysis (TGA). Due to the strong interactions between the bisphosphonate group of 2
and MnFe₂O₄@HA and Fe₃O₄@HA NPs, both NPs exhibit long-term colloidal stability in aqueous solution, even in high ionic strength environment such as PBS. The hydrodynamic size of Fe₃O₄@HA and MnFe₂O₄@HA NPs remained at 50.7 nm and 60.3 nm, respectively, for over two months (Fig. 2a). The excellent colloidal stability and small hydrodynamic size of MnFe₂O₄@HA and Fe₃O₄@HA NPs make them potentially suitable for biological or medical applications.

Unsurprisingly, because of the high affinity of fluoride for HA, both NPs exhibit a high radiolabelling efficiency with ¹⁸F-fluoride, up to 88.3 ± 0.5% for 0.3 mg MnFe₂O₄@HA NPs and 92.1 ± 0.1% for 0.3 mg Fe₃O₄@NaYF₄ NPs (Fig. 2b). Labelling and purification was readily achieved in less than 23 min. To provide a means of incorporating the positron emitter ⁶⁴Cu, the NPs were sonicated in 1 mg ml⁻¹ BP-PEG-cyclam solution for 30 minutes to allow replacement of a fraction of BP-PEG-Me by BP-PEG-cyclam, and free BP-PEG-polymer were removed by centrifugation before mixing with radioactivity. The resulting particles showed a high ⁶⁴Cu radiolabelling efficiency in a short time (<5 minutes) (Fig. 2b). Both NPs display essentially the magnetic properties of Fe₃O₄ or MnFe₂O₄ NPs and are active on MR images (Fig. 2c and d). The transverse (r₂) and longitudinal (r₁) relaxivities of Fe₃O₄@HA NPs were measured to be 150.2 ± 3.2 mM⁻¹ s⁻¹ and 1.9 ± 0.1 mM⁻¹ s⁻¹, respectively, at 3 T magnetic field. As expected, the relaxivities of NPs could be improved by altering the ratio of magnetic component and non-magnetic HA, since r₂ is proportional to the volume fraction of magnetic component. For example, the r₂ of MnFe₂O₄@HA NPs could be dramatically improved from 105.7 ± 3.5 mM⁻¹ s⁻¹ to 246.5 ± 15.9 mM⁻¹ s⁻¹ by doubling the amount of MnFe₂O₄@Al(OH)₃ while keeping the amount of NaH₂PO₄ and CaCl₂ solutions the same during the synthesis. High transverse relaxivity of these magnetic hydroxyapatite NPs as well as a high ratio of r₂/r₁ demonstrate their potential application as T₂ contrast agents on MR imaging.

Fluorescent HA is normally produced either by doping with rare earth cations (Eu or Tb), or by conjugation with organic dyes. Here we conjugated the fluorescent dyes Maria blue and Alexa Fluor-covalently to the NPs surface using sodium pamidronate as an aminobisphosphonate linker (see ESI†). The amine group of pamidronate is reactive for NHS ester dyes to form stable amide bonds while its bisphosphonate group interacts strongly with the Ca or Fe at the surface of NPs; dyes are thus linked to NPs without the risk of leakage. Fluorescent spectra in Fig. 3 show an emission at 455 nm for the conjugates of Fe₃O₄@HA and Maria blue under excitation at 365 nm, and an emission at 517 nm for the Alexa Fluor conjugates under excitation at 488 nm. More importantly, the fluorescence of these conjugated NPs is stable and strong even after being stored at room temperature for over one month, implying the potential applications as optical contrast.

In summary, we have presented a facile approach to synthesis magnetic and fluorescent hydroxyapatite nanoparticles with a well-defined morphology and uniform size, using Al(OH)₃-stabilised Fe₃O₄ or MnFe₂O₄ NPs as templates. The change from the highly positively charged surface and base-solubility of the Al(OH)₃ layer to the neutral or slightly negative zeta potential and acid solubility of the Fe₃O₄@HA particles is likely to offer alternative biological properties. These NPs are promising candidates for development as tri-modal probes for MR, PET and optical imaging, since they display excellent colloidal stability and high radiolabelling efficiency both for ⁶⁴Cu and for ¹⁸F, as well as fluorescent and magnetic properties. Radiolabelling with other metallic radioisotopes will also be achievable by replacing cyclam with corresponding chelators. This synthesis approach allows us to tune the magnetic properties of particles by altering the ratio of precursors, without decreasing the radiolabelling efficiency or fluorescent property. The flexible conjugation method ensures that dyes with different wavelengths could be selected for different applications. The synthesis strategy for conjugation of BP-PEG-cyclam can also be applied to the conjugation of BP-PEG with peptides or antibodies, leading to the application in targeted imaging.

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Notes and references