Facile, productive and cost-effective synthesis of a novel tetrazine-based iron oxide nanoparticle for targeted image contrast agents and nanomedicines

Marco M. Meloni • Stephen Barton • Alexandru Chivu • Juan C. Kaski • Wenhui Song • Taigang He

Abstract We have developed an operationally simple, time and cost-effective protocol to produce a novel tetrazine-based iron oxide nanoparticle using commercially available and inexpensive materials. Our protocol proceeds at room temperature and uses Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium, a well-known, widely used reagent for the large-scale industrial production of important pharmaceuticals. The nanoparticles obtained have a diameter range between 16 and 21 nm and showed no toxicity against endothelial cell lines. The tetrazine moiety on the nanoparticle surface could potentially allow further attachment of specific targeting vectors by using so-called copper-free click chemistry. We therefore anticipate that our protocol can represent a significant breakthrough in the future development and commercialization of improved, tissue-specific contrast agents and drug delivery for clinical diagnosis, monitoring and therapy of diseases at an asymptomatic stage.

Keywords Diagnosis • Magnetic Resonance Imaging • Iron Oxide Nanoparticles • Copper-free click Chemistry • Bioconjugation • Synthesis

Introduction

Since their discovery (Ohgushi, 1978), Iron Oxide Nanoparticles (IONPs) have emerged as powerful tools for a wide scope of applications such as in the fields of biolabeling, medical diagnostics, and therapy (Colombo et al. 2012). In particular as contrast agents for magnetic resonance imaging (MRI), IONPs have attracted enormous attention. IONPs have a transverse relaxivity which increases at higher magnetic fields, resulting in enhanced signal-to-noise ratio with lower dosages (Mishra et al. 2016). The use of IONPs for diagnostic imaging is therefore expected to increase steadily. Unlike Gd-based contrast agents (CAs), IONPs have proven safe with some systems already approved for clinical use (Schmitz et al. 2001; Trivedi et al. 2004). With the recent advent of molecular imaging (Meloni et al. 2017), IONPs are increasingly used for the preparation of targeted CAs, allowing non-invasive visualization of biomolecules which are the signatures of diseases in specific body tissues, both *in vivo* and in real time. The preparation of these targeted CAs firstly involves coating of the IONPs surface with a biocompatible, clinically approved polymer, followed by its functionalization with a targeting vector which allows tissue specificity (A. M. Morawski et al. 2005).

This process, so-called standard bioconjugation, uses well-established synthetic chemistry and involves the formation of stable chemical bonds. Despite being reliable, this method can lead to low yields and produce toxic byproducts, resulting in expensive, time-consuming purifications and toxicological screenings. A number of years may therefore be required for the translation, large scale production and commercialization of targeted, nanoparticle-based CAs into the clinical market.

Copper-free click reactions recently emerged as a powerful, time-saving bioconjugation strategy to prepare targeted CAs (Devaraj and Weissleder 2011; McKay and Finn 2014). Copper-free click reactions are operationally simpler and proceed at room temperature. They eliminate the use of cytotoxic copper catalysts (Baskin JM et al. 2007) and are inert to both water and oxygen. They also generate minimal, inoffensive byproducts, providing the best yield with the highest reaction rates. Copper-free click reactions can proceed in living organisms, allowing the targeting of biomolecules with high specificity without altering any native biochemical process. Among the arsenal of available copper-free click reactions, the Inverse Electron Demand Diels Alder (IEDDA) reaction stands out as the most promising one for its rapid reaction rate and generation of safe, inert nitrogen as the only byproduct (Oliveira et al. 2017). IEDDA reactions also involve tetrazines and cycloalkenes, both of which are easier substrates to prepare if compared to azadibenzocyclooctynes (DIBACs) (Debets et al. 2010) and biarylazacyclooctynones (BARACs) (Kuzmin et al. 2010). Despite such significant advantages, the preparation of targeted, nanoparticle-based CAs using IEDDA is a relatively unexplored field. A possible reason is a shortage of reliable protocols to prepare tetrazine-based IONPs. Therefore, the

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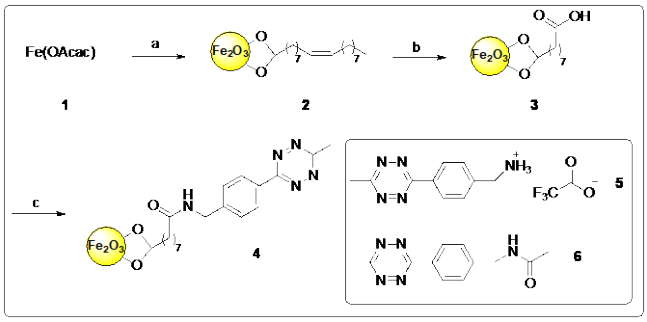
identification of novel, green and efficient ways to prepare these functional nanoparticles can accelerate the development of improved contrast agents for diagnosis and therapy. In this paper we designed and synthesized a novel route to a tetrazine-bound nanoparticle **4** (Scheme 1).



Scheme 1 (a) Oleic acid, oleylamine, phenyl ether (b) BTAC, $KMnO_4$ (c). Tetrazine **5**, HATU, DIPEA, CH_2Cl_2, at room temperature.

As illustrated above, our strategy involved the loading of a tetrazine moiety to nanoparticles **3** using Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU), a readily available, inexpensive and well-known reagent. Our overall approach to nanoparticles **4** can be exploited for the future production of improved contrast reagents, with potential application also in the fields of nanomedicine.

Results and discussion

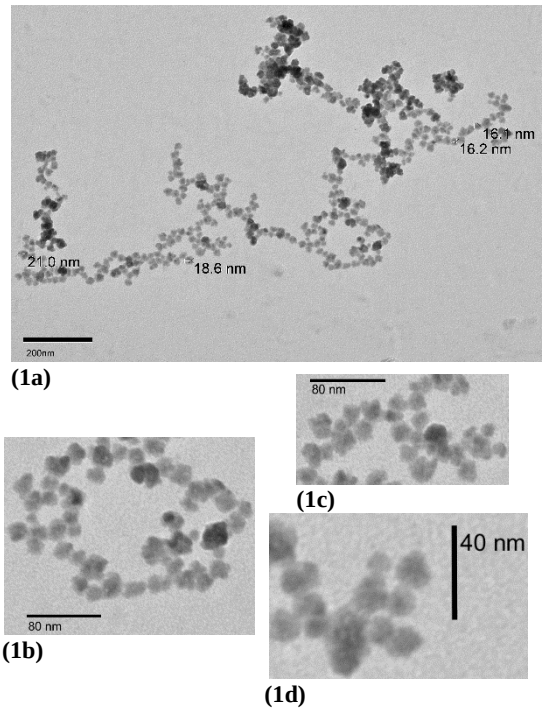
In this work, we successfully designed and developed an operationally simple, economical and productive approach to produce a novel tetrazine-decorated IONP **4** (Scheme 1, page 2). Our protocol is reproducible by taking an advantage of well-established chemistry and usages of building blocks **3** and **5**, both accessible via established published protocols (Herranz et al. 2008; Evans et al. 2014). Nanoparticles **4** were obtained via coupling between carboxylic acid IONPs **3** and tetrazine-based amine **5** (Scheme 1, page 2). Our method builds upon the reaction between a carboxylic acid and an amine, which produces extremely stable chemical bonds even in a biological environment and is one of the most robust, known reactions in synthetic chemistry (Jaradat, 2018). During this process a challenge also emerged due to the presence of the paramagnetic core both in **3** and **4**, which excluded quantitative reaction monitoring via NMR. Therefore, we first decided to validate the chemistry using acetic acid as a surrogate for nanoparticles **3**. We wanted a fast, high yielding protocol to obtain model compound **6**. After unsuccessful attempts to prepare this latter using N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in water (Herranz et al. 2008), we found that the system HATU/DIPEA/CH_2Cl_2 afforded **6** in 92% yield after only 3 hours.

We therefore adopted this method for the synthesis of nanoparticles **4**. Coupling between **3** and **5** was performed with HATU and DIPEA in CH_2Cl_2, affording the desired nanoparticles **4**. We found this method both safe and operationally simple for many reasons. Firstly, HATU is an established chemical which is widely used during large scale synthesis of pharmaceuticals. Several reports of HATU applications have showcased its use in the synthesis of promising drug candidates (Dunetz et al. 2016 and references cited herein). Secondly, a simple filtration/washing cycle can be used to purify and isolate nanoparticles **4**. Thirdly, the reaction also proceeded both at room temperature and in presence of relatively safe chemicals. Finally, our approach is highly reproducible. As a proof, we repeated the synthesis three times, obtaining nanoparticles **4** with the same zeta potentials in all cases (see Table 1). Yields of nanoparticles **4** were 91%, 88% and 90% for repetitions 1-3 respectively. We therefore believe that our approach to nanoparticles **4** can be feasible for a scale-up production. The tetrazine grafted on IONP render a nano-platform for conjugating a diverse range of biomolecules or drugs via copper-free click chemistry. This strategy has advantages of simplifying chemical synthesis, avoiding cytotoxic copper catalysts and generating a system to detect biomarkers in live cells with improved sensitivity. One such example is the nanoparticle-antibody conjugate developed to image extracellular receptors in cancer cells (Haun et al. 2010).

Table 1. Zeta potentials found for nanoparticles precursor **3**, tetrazine bound IONPs **4** and yields obtained.

Sample (repeat)	IONPs **3** (mV)	IONPs **4** (mV)
1	-37.6	-20.5
2	-41.1	-16.1
3	-42.9	-18.1

With nanoparticles **4** successfully prepared, we then decided to assess their shape and average size. These parameters are relevant in assessing IONPs sensitivity for biosensing applications. As a proof, in 2013 Kolhatkar et al. demonstrated that cubic and spherical nanoparticles display different sensitivities due to their different contact areas. With this in mind, we obtained TEM images of IONPs **4** (Figures 1a-d). It can be seen that the particles are spherical in shape with a size range between 16 and 21 nm.



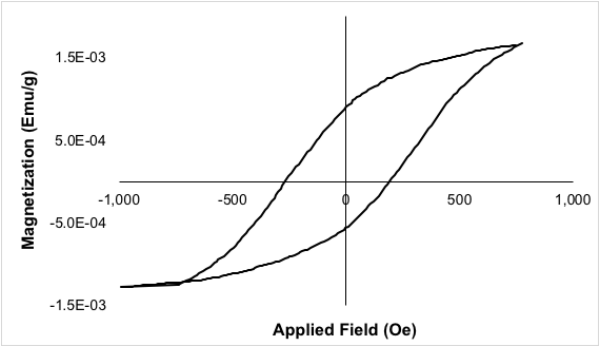
(1a) **(1c)** **(1b)** **(1d)**

Figures 1a-d. TEM Images of nanoparticles **2**

Once that both shape and size of the nanoparticles were assessed, we moved to establish their magnetic properties.

We were particularly interested in the saturation magnetization value because this is another key indicator in assessing IONPs sensitivity for biosensing applications (Colombo et al. 2012).
Magnetization curves were performed on a solid sample. IONPs **4** showed a paramagnetic behaviour with a saturation magnetization value of 1.49E-03 emu/g (Figure 2), confirming also that IONPs **4** present a degree of surface functionalisation.

Fig 2. Magnetization curve of IONPs **4** at 310K



To further demonstrate the potential applicability of nanoparticles **4** in pre-clinical and clinical fields, we felt mandatory to perform toxicological screenings. After being cultured in the presence of nanoparticles **4** at various concentrations for one to three days, both metabolic activity (Figure 3) and total DNA of HUVECs (Figure 4) were performed, with no cytotoxicity or changes in cell viability and morphology encountered.

Fig. 3 Metabolic profiles of HUVEC cells treated at different concentrations of IONPs **4**.

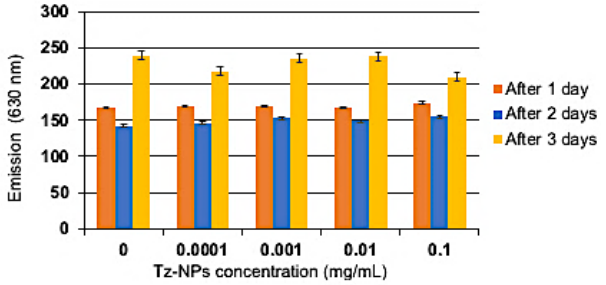
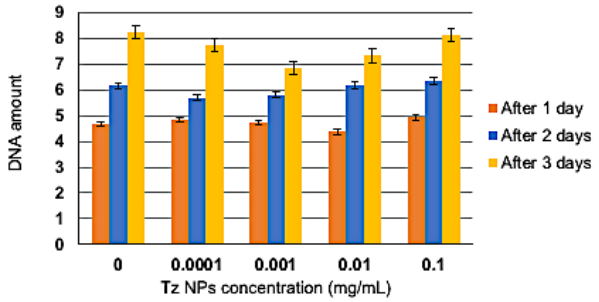


Fig. 4 Total DNA of HUVEC cells treated at different concentrations of IONPs **4**.



These results strongly suggest that targeted contrast reagents based on IONPs **4** can be safely used in pre-clinical and clinical imaging or magnetic-nanomedicine.
The relaxation rate is another key parameter in the applicability of nanoparticles **4** in preclinical and clinical fields. We plan to obtain this parameter as a part of our future applications *in vivo*, and the results will be reported in due course.

Conclusions

We have developed an operationally simple, high-yielding and cost-effective protocol to produce a novel class of low toxicity, tetrazine-based IONPs. Our protocol is reproducible and the tetrazine motif can be exploited for the preparation of conjugated biomolecules or drug molecules for targeted-based contrast agents or nanomedicine by using all the strengths of the well-known copper-free click chemistry. Targeted magnetic nanoparticle-based contrast agents and nanomedicines are rapidly emerging into the preclinical and clinical fields. We therefore anticipate that our method can represent an invaluable breakthrough and provides a multi-functional nanoparticle platform for the large-scale production and commercialization of improved, tissue specific contrast agents or magnetic nanomedicine for the clinical diagnosis and therapy of diseases at an asymptomatic stage.

Conflicts of interest

None to declare

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