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

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3

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

22

23 **Keywords** Diagnosis • Magnetic Resonance Imaging • Iron

24 Oxide Nanoparticles • Copper-free click Chemistry •

25 Bioconjugation • Synthesis

26

27 **Introduction**

28

29 Since their discovery (Ohgushi, 1978), Iron Oxide  
30 Nanoparticles (IONPs) have emerged as powerful tools for a  
31 wide scope of applications such as in the fields of biolabeling,  
32 medical diagnostics, and therapy (Colombo et al. 2012). In  
33 particular as contrast agents for magnetic resonance imaging  
34 (MRI), IONPs have attracted enormous attention. IONPs  
35 have a transverse relaxivity which increases at higher  
36 magnetic fields, resulting in enhanced signal-to-noise ratio  
37 with lower dosages (Mishra et al. 2016). The use of IONPs  
38 for diagnostic imaging is therefore expected to increase  
39 steadily. Unlike Gd-based contrast agents (CAs), IONPs have  
40 proven safe with some systems already approved for clinical  
41 use (Schmitz et al. 2001; Trivedi et al. 2004). With the recent  
42 advent of molecular imaging (Meloni et al. 2017), IONPs are

43 increasingly used for the preparation of targeted CAs,  
44 allowing non-invasive visualization of biomolecules which  
45 are the signatures of diseases in specific body tissues, both *in*  
46 *vivo* and in real time. The preparation of these targeted CAs  
47 firstly involves coating of the IONPs surface with a  
48 biocompatible, clinically approved polymer, followed by its  
49 functionalization with a targeting vector which allows tissue  
50 specificity (A. M. Morawski et al. 2005).

51

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

60

61 Copper-free click reactions recently emerged as a powerful,  
62 time-saving bioconjugation strategy to prepare targeted CAs  
63 (Devaraj and Weissleder 2011; McKay and Finn 2014).  
64 Copper-free click reactions are operationally simpler and  
65 proceed at room temperature. They eliminate the use of  
66 cytotoxic copper catalysts (Baskin JM et al. 2007) and are  
67 inert to both water and oxygen. They also generate minimal,  
68 inoffensive byproducts, providing the best yield with the  
69 highest reaction rates. Copper-free click reactions can  
70 proceed in living organisms, allowing the targeting of  
71 biomolecules with high specificity without altering any native  
72 biochemical process. Among the arsenal of available copper-  
73 free click reactions, the Inverse Electron Demand Diels Alder  
74 (IEDDA) reaction stands out as the most promising one for  
75 its rapid reaction rate and generation of safe, inert nitrogen as  
76 the only byproduct (Oliveira et al. 2017). IEDDA reactions  
77 also involve tetrazines and cycloalkenes, both of which are  
78 easier substrates to prepare if compared to  
79 azadibenzocyclooctynes (DIBACs) (Debets et al. 2010) and  
80 biarylazacyclooctynones (BARACs) (Kuzmin et al. 2010).

81 Despite such significant advantages, the preparation of  
82 targeted, nanoparticle-based CAs using IEDDA is a relatively  
83 unexplored field. A possible reason is a shortage of reliable  
84 protocols to prepare tetrazine-based IONPs. Therefore, the

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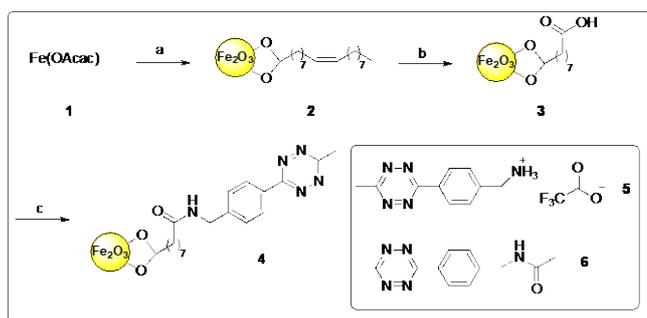
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1 identification of novel, green and efficient ways to prepare  
 2 these functional nanoparticles can accelerate the development  
 3 of improved contrast agents for diagnosis and therapy. In this  
 4 paper we designed and synthesized a novel route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

6



7  
 8 **Scheme 1** (a) Oleic acid, oleylamine, phenyl ether (b) BTAC,  
 9  $\text{KMnO}_4$  (c). Tetrazine **5**, HATU, DIPEA,  $\text{CH}_2\text{Cl}_2$ , at room  
 10 temperature.

11  
 12 As illustrated above, our strategy involved the loading of a  
 13 tetrazine moiety to nanoparticles **3** using  
 14 Hexafluorophosphate Azabenzotriazole Tetramethyl  
 15 Uronium (HATU), a readily available, inexpensive and well-  
 16 known reagent. Our overall approach to nanoparticles **4** can  
 17 be exploited for the future production of improved contrast  
 18 reagents, with potential application also in the fields of  
 19 nanomedicine.

## 21 Results and discussion

22  
 23 In this work, we successfully designed and developed an  
 24 operationally simple, economical and productive approach to  
 25 produce a novel tetrazine-decorated IONP **4** (Scheme 1, page  
 26 2). Our protocol is reproducible by taking an advantage of  
 27 well-established chemistry and usages of building blocks **3**  
 28 and **5**, both accessible via established published protocols  
 29 (Herranz et al. 2008; Evans et al. 2014). Nanoparticles **4** were  
 30 obtained via coupling between carboxylic acid IONPs **3** and  
 31 tetrazine-based amine **5** (Scheme 1, page 2). Our method  
 32 builds upon the reaction between a carboxylic acid and an  
 33 amine, which produces extremely stable chemical bonds even  
 34 in a biological environment and is one of the most robust,  
 35 known reactions in synthetic chemistry (Jaradat, 2018).  
 36 During this process a challenge also emerged due to the  
 37 presence of the paramagnetic core both in **3** and **4**, which  
 38 excluded quantitative reaction monitoring via NMR.

39 Therefore, we first decided to validate the chemistry using  
 40 acetic acid as a surrogate for nanoparticles **3**. We wanted a  
 41 fast, high yielding protocol to obtain model compound **6**.  
 42 After unsuccessful attempts to prepare this latter using N-  
 43 hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-  
 44 dimethylaminopropyl)carbodiimide (EDC) in water (Herranz  
 45 et al. 2008), we found that the system HATU/DIPEA/ $\text{CH}_2\text{Cl}_2$   
 46 afforded **6** in 92% yield after only 3 hours.

56 cited herein). Secondly, a simple filtration/washing cycle can  
 57 be used to purify and isolate nanoparticles **4**. Thirdly, the  
 58 reaction also proceeded both at room temperature and in  
 59 presence of relatively safe chemicals. Finally, our approach is  
 60 highly reproducible. As a proof, we repeated the synthesis  
 61 three times, obtaining nanoparticles **4** with the same zeta  
 62 potentials in all cases (see Table 1). Yields of nanoparticles **4**  
 63 were 91%, 88% and 90% for repetitions 1-3 respectively. We  
 64 therefore believe that our approach to nanoparticles **4** can be  
 65 feasible for a scale-up production. The tetrazine grafted on  
 66 IONP render a nano-platform for conjugating a diverse range  
 67 of biomolecules or drugs via copper-free click chemistry.  
 68 This strategy has advantages of simplifying chemical  
 69 synthesis, avoiding cytotoxic copper catalysts and generating  
 70 a system to detect biomarkers in live cells with improved  
 71 sensitivity. One such example is the nanoparticle-antibody  
 72 conjugate developed to image extracellular receptors in  
 73 cancer cells (Haun et al. 2010).

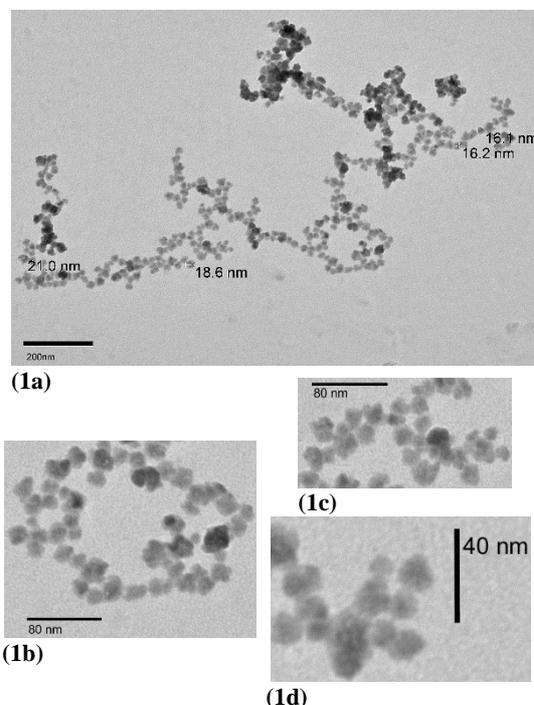
74  
 75 **Table 1.** Zeta potentials found for nanoparticles precursor **3**,  
 76 tetrazine bound IONPs **4** and yields obtained.

77

Sample (repeat)	IONPs <b>3</b> (mV)	IONPs <b>4</b> (mV)
<b>1</b>	-37.6	-20.5
<b>2</b>	-41.1	-16.1
<b>3</b>	-42.9	-18.1

78

79 With nanoparticles **4** successfully prepared, we then decided  
 80 to assess their shape and average size. These parameters are  
 81 relevant in assessing IONPs sensitivity for biosensing  
 82 applications. As a proof, in 2013 Kolhatkar et al.  
 83 demonstrated that cubic and spherical nanoparticles display  
 84 different sensitivities due to their different contact areas. With  
 85 this in mind, we obtained TEM images of IONPs **4** (Figures  
 86 1a-d). It can be seen that the particles are spherical in shape  
 87 with a size range between 16 and 21 nm.



88  
 89  
 90 **Figures 1a-d.** TEM Images of nanoparticles **2**

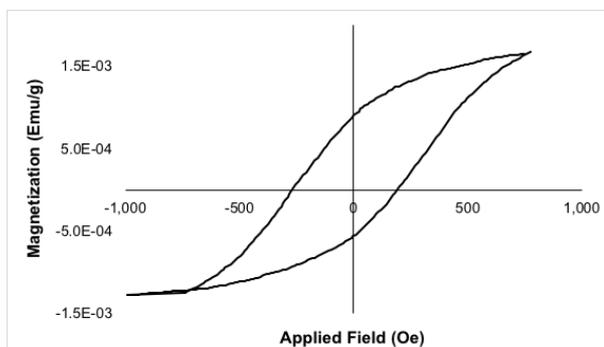
91

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

1 We were particularly interested in the saturation  
2 magnetization value because this is another key indicator in  
3 assessing IONPs sensitivity for biosensing applications  
4 (Colombo et al. 2012).

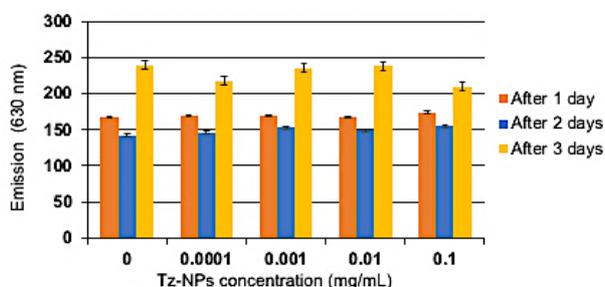
5 Magnetization curves were performed on a solid sample.  
6 IONPs 4 showed a paramagnetic behaviour with a saturation  
7 magnetization value of 1.49E-03 emu/g (Figure 2),  
8 confirming also that IONPs 4 present a degree of surface  
9 functionalisation.

10  
11 **Fig 2.** Magnetization curve of IONPs 4 at 310K

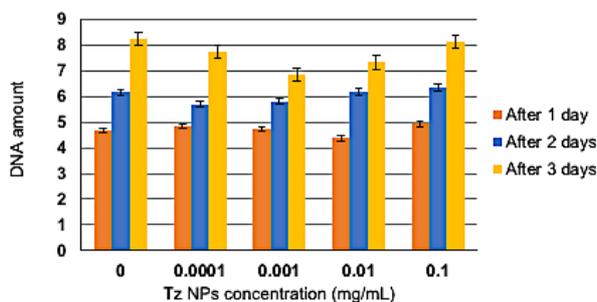


12  
13 To further demonstrate the potential applicability of  
14 nanoparticles 4 in pre-clinical and clinical fields, we felt  
15 mandatory to perform toxicological screenings. After being  
16 cultured in the presence of nanoparticles 4 at various  
17 concentrations for one to three days, both metabolic activity  
18 (Figure 3) and total DNA of HUVECs (Figure 4) were  
19 performed, with no cytotoxicity or changes in cell viability  
20 and morphology encountered.

21  
22 **Fig. 3** Metabolic profiles of HUVEC cells treated at different  
23 concentrations of IONPs 4.



25  
26 **Fig. 4** Total DNA of HUVEC cells treated at different  
27 concentrations of IONPs 4.



29  
30  
31 These results strongly suggest that targeted contrast reagents  
32 based on IONPs 4 can be safely used in pre-clinical and  
33 clinical imaging or magnetic-nanomedicine.

34 The relaxation rate is another key parameter in the  
35 applicability of nanoparticles 4 in preclinical and clinical

36 fields. We plan to obtain this parameter as a part of our future  
37 applications *in vivo*, and the results will be reported in due  
38 course.

## 39 40 Conclusions

41  
42 We have developed an operationally simple, high-yielding  
43 and cost-effective protocol to produce a novel class of low  
44 toxicity, tetrazine-based IONPs. Our protocol is reproducible  
45 and the tetrazine motif can be exploited for the preparation of  
46 conjugated biomolecules or drug molecules for targeted-  
47 based contrast agents or nanomedicine by using all the  
48 strengths of the well-known copper-free click chemistry.  
49 Targeted magnetic nanoparticle-based contrast agents and  
50 nanomedicines are rapidly emerging into the preclinical and  
51 clinical fields. We therefore anticipate that our method can  
52 represent an invaluable breakthrough and provides a multi-  
53 functional nanoparticle platform for the large-scale  
54 production and commercialization of improved, tissue  
55 specific contrast agents or magnetic nanomedicine for the  
56 clinical diagnosis and therapy of diseases at an asymptomatic  
57 stage.

## 58 59 Conflicts of interest

60  
61 None to declare

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