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Item Type	Article
Authors	Kakwere, Hamilton; Pernia Leal, Manuel; Materia, Maria-Elena; Curcio, Alberto; Guardia, Pablo; Niculaes, Dina; Marotta, Roberto; Falqui, Andrea; Pellegrino, Teresa
Citation	Functionalization of Strongly Interacting Magnetic Nanocubes with (Thermo)responsive Coating and their Application in Hyperthermia and Heat-Triggered Drug Delivery 2015:150403101542007 ACS Applied Materials & Interfaces
Eprint version	Post-print
DOI	<a href="https://doi.org/10.1021/am5088117">10.1021/am5088117</a>
Publisher	American Chemical Society (ACS)
Journal	ACS Applied Materials & Interfaces
Rights	Archived with thanks to ACS Applied Materials & Interfaces
Download date	21/10/2020 11:22:20
Link to Item	<a href="http://hdl.handle.net/10754/350276">http://hdl.handle.net/10754/350276</a>

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*ACS Appl. Mater. Interfaces*, **Just Accepted Manuscript** • DOI: 10.1021/am5088117 • Publication Date (Web): 03 Apr 2015

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# Functionalization of Strongly Interacting Magnetic Nanocubes with (Thermo)responsive Coating and their Application in Hyperthermia and Heat-Triggered Drug Delivery

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**Keywords:** Smart materials, cubic magnetic nanoparticles, thermo-responsive, pH-responsive, drug release, hyperthermia

**Abstract:** Herein we prepare nanohybrids by incorporating iron oxide nanocubes (cubic-IONPs) within a thermo-responsive polymer shell that can act as drug carriers for doxorubicin(doxo). The cubic-shaped nanoparticles employed are at the interface between superparamagnetic and ferromagnetic behavior and have an exceptionally high specific absorption rate (SAR) but their functionalization is extremely challenging compared to bare superparamagnetic iron oxide nanoparticles as they strongly interact with each other. By conducting the polymer grafting reaction using reversible addition-fragmentation chain transfer (RAFT) polymerization in a viscous solvent medium, we have here developed a facile approach to decorate the nanocubes with stimuli-responsive polymers. When the thermo-responsive shell is composed of poly(*N*-isopropyl acrylamide-*co*-polyethylene glycolmethylether acrylate), nanohybrids have a phase transition temperature, the lower critical solution temperature (LCST), above 37 °C in physiological conditions. Doxo loaded nanohybrids exhibited a negligible drug release below 37 °C but showed a consistent release of their cargo on demand by exploiting the capability of the nanocubes to generate heat under an alternating magnetic field (AMF). Moreover, the drug free nanocarrier does not

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3 exhibit cytotoxicity even when administered at high concentration of nanocubes (1g/L of iron) and  
4 internalized at high extent (260 pg of iron per cell). We have also implemented the synthesis protocol to  
5 decorate the surface of nanocubes with poly(vinylpyridine) polymer and thus prepare pH-responsive  
6 shell coated nanocubes.  
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## 10 11 **1. Introduction**

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13 Emerging technologies in preparation of magnetic nanoparticles have afforded materials with a wide  
14 range of potential applications including magnetic resonance imaging (MRI) agents, carriers for  
15 drug/gene delivery, hyperthermia agents and biosensors.<sup>1-4</sup> Non-aqueous synthesis protocols allow for  
16 the preparation of iron oxide nanoparticles (IONPs) with control over the crystallinity and thus obtaining  
17 particles with desirable magnetic properties for the distinct applications.<sup>5</sup> However, these methods yield  
18 hydrophobic nanoparticles capped with organic surfactants which hinder their direct application in  
19 biomedicine.<sup>4, 6</sup> With the persistent developments in synthesis of magnetic nanoparticles/IONPs  
20 targeted for various biologic applications, there is accordingly the need to continuously develop  
21 strategies for their functionalization.  
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30 Functionalization of IONPs has been conducted via modification of the IONPs surfaces with small  
31 molecules as well as with polymers.<sup>7</sup> The combination of polymers, specifically stimuli-responsive  
32 polymers, with inorganic particles represents a fascinating route for generation of new types of  
33 materials and devices integrating properties of inorganic particles with those of polymers.<sup>8</sup> The main  
34 strategies employed for polymer functionalization of IONPs include the *grafting from* and *grafting to*  
35 techniques<sup>6, 9</sup> which lately have been employed in combination with living radical polymerization (LRP)  
36 techniques.<sup>10-11</sup> LRP techniques allow rapid access to well-defined functional (co)polymers of varying  
37 molecular architecture and thus offer good control over the size of hybrid nanostructures.<sup>12</sup> Herein, we  
38 report a potentially generic and facile approach for the functionalization of strongly interacting cubic  
39 shaped IONPs (cubic-IONPs) via the *grafting from* approach using an LRP technique (reversible addition-  
40 fragmentation chain transfer (RAFT)) polymerization.<sup>13</sup> Although several reports on functionalization of  
41 spherical-IONPs (mainly prepared by co-precipitation methods) *via* RAFT polymerization and other LRP  
42 techniques have demonstrated the feasibility to synthesize thermo-responsive shell on top of IONPs,<sup>14-  
43 18,19-20</sup> the protocols previously developed have mainly used nanoparticles showing superparamagnetic  
44 behavior at room temperature. These nanoparticles are, by their nature, non-interacting particles, as  
45 they miss any remanence magnetization at room temperature and in absence of an external magnetic  
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3 field. Very recently, our group has reported the non-hydrolytic synthesis of 19-22 nm cubic-IONPs having  
4 a magnetic behavior at the interface between the superparamagnetic and ferromagnetic regime at  
5 room/body temperature. In a comparative study, we have observed that cubic-IONPs with sizes of 19-22  
6 nm have the best heating performance as they show very high specific absorption rates (SAR) values  
7 whilst above or below this nanoparticle size range, the SAR values decreases and the heating  
8 performance becomes lower. This makes the 19-22 nm cubic-IONPs very promising for hyperthermia  
9 applications.<sup>21</sup> Further, they are composed of biocompatible species (only iron and oxygen) and at the  
10 same time, they exhibited efficient heat dissipation at frequency and field conditions which are  
11 applicable to patients.<sup>22</sup> Given the high SAR values, lower doses of cubic-IONPs could be applied *in vivo*  
12 for reaching therapeutic temperatures.<sup>23</sup> Furthermore, *in vivo* degradation in liver of those iron oxide  
13 nanocubes has been reported by some of us.<sup>24</sup> However, because of their size and magnetic properties  
14 those cubic-IONPs strongly interact with each other and many challenges are encountered when trying  
15 to functionalize such nanoparticles.<sup>9, 11</sup> Amphiphilic polymer enwrapping and ligand exchange protocols  
16 with pegylated molecules have been adapted to allow the nanoparticle water transfer.<sup>22</sup> However,  
17 having a functional coating like a thermo-responsive or pH-responsive shell could further enhance their  
18 applicability. Herein, using a direct approach, we employ cubic-IONPs as a blueprint of strongly  
19 aggregating nanoparticles and we develop protocols at the nanoparticle surface for an *in situ*  
20 polymerization by LRP techniques of responsive polymers: temperature (based on *N*-  
21 isopropylacrylamide, NIPAAAM) or pH (based on vinylpyridine, VP). The key to achieve the polymer shell  
22 growth on individual cubic-IONPs was the choice of viscous solvents as media in which to perform the  
23 polymerization. The thermo-responsive coated cubic-IONPs still preserved their good magnetic  
24 properties with the very high SAR values.<sup>22</sup> The temperature responsive cubic-IONPs were used as drug  
25 carriers for chemotherapy. Doxorubicin (doxo) molecules were loaded at room temperature and after  
26 drug encapsulation released under an alternating magnetic field (AMF) by exploiting the heating ability  
27 of the cubic-IONPs. Under an AMF exposure, the temperature of the thermo-responsive cubic-IONPs  
28 increased and the accompanying coil-globule transition of the polymer led to expulsion of the drug from  
29 the temperature responsive cubic-IONPs. Such nanostructures may then act as drug carriers, providing a  
30 synergistic therapeutic effect in cancer treatment *via* hyperthermia and chemotherapy with triggered  
31 drug release, while not displaying side effects presented by standard chemotherapy.<sup>15</sup> In this work, we  
32 demonstrate that by conducting the surface functionalization of the cubic-IONPs in a viscous medium  
33 we can overcome the challenges associated with the functionalization of such strongly interacting  
34 nanoparticles and yield hybrid nanomaterials with a potentially wide range of applications. The parallel  
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3 comparison of the implementation of the same procedures to superparamagnetic spherical-IONPs  
4 allows us to highlight the challenges we have encountered when using cubic-IONPs.  
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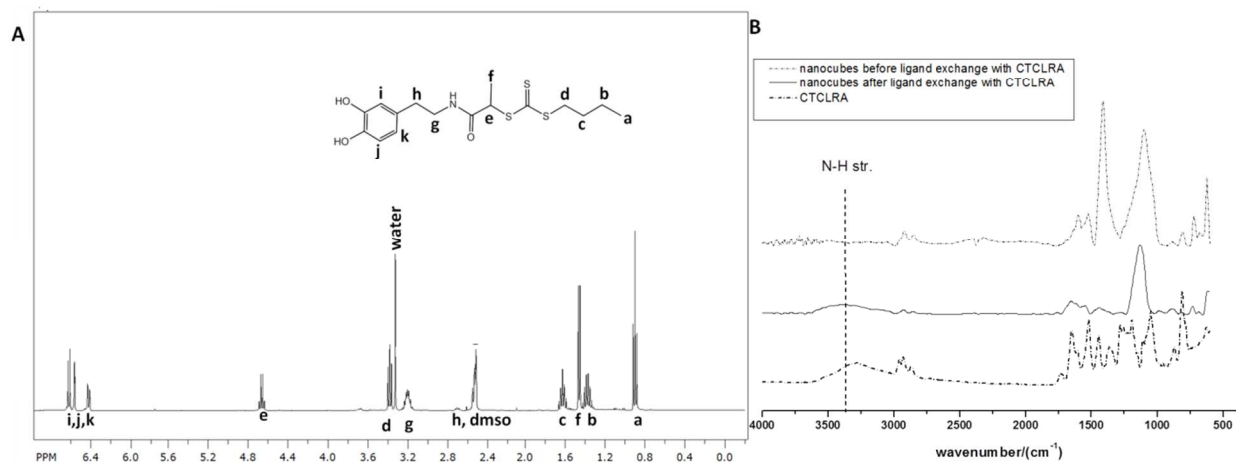
## 10 **2. Results and discussion**

### 11 **2.1 The ligand exchange protocol for the preparation of RAFT functionalized nanocubes**

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13 To functionalize the cubic-IONPs with the responsive polymer, we opted for the *grafting from* approach  
14 using RAFT polymerization taking into account the high grafting densities that arise from using this  
15 grafting approach and the versatility of the polymerization technique. Following procedures similar to  
16 those employed in literature for the functionalization of superparamagnetic IONPs, we first synthesized a  
17 catechol bearing RAFT agent (CTCLRA) to act as an anchor for the polymer chains on the surface as the  
18 catechol moiety is known to bind strongly to iron oxide surface.<sup>4, 25</sup> The CTCLRA was obtained in good  
19 yield (87%) by a two-step reaction between dopamine and succinimide-activated RAFT agent and the  
20 structure was confirmed by <sup>1</sup>H NMR (Figure 1) (for the characterization of the intermediate compound  
21 see Figure S1 of the supporting information, SI). Subsequently, the oleic acid capped cubic-IONPs in  
22 CHCl<sub>3</sub> were exchanged with the CTCLRA using a large excess of RAFT agent (the initial mixing ratio was  
23 fixed at 16 RAFT agent molecules/nm<sup>2</sup>) in tetrahydrofuran (THF). It is worth mentioning that we have  
24 also observed that the ligand exchange process with the RAFT agent, the CTCLRA, could also be  
25 conducted by adding the RAFT agent dissolved in THF directly to the crude reaction mixture obtained at  
26 the end of the synthesis procedure of the nanocubes (usually dissolved in squalane/dibenzyl ether).<sup>22</sup> At  
27 the end of the synthesis of the cubic-IONPs, the reaction mixture was allowed to cool to 60 °C and  
28 without dismantling the apparatus, under argon, the RAFT agent solution in THF was added and the  
29 ligand exchange process was left to proceed overnight at room temperature. The CTCLRA-coated  
30 nanocubes could be then recovered by centrifugation and be completely resuspended in THF. Also, the  
31 CTCLRA exchanged nanocubes were insoluble in hexane which was a good solvent for the starting  
32 nanoparticles thus indicating the change in the coating at their surface.  
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51 The success of the ligand exchange protocol was probed by FTIR and elemental analysis. After CTCLRA  
52 exchange, the amide N-H stretch at 3400 cm<sup>-1</sup> in the spectrum of the CTCLRA coated nanoparticles  
53 reveal the presence of the RAFT agent at the nanoparticle surface (this peak was absent in the spectrum  
54 of the starting nanoparticles) (Figure 1). Furthermore, the sulfur element which was absent in the as-  
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synthesized nanocubes (based on ICP) was abundantly detected via elemental analysis only on CTCLRA coated IONPs after ligand exchange, indicating the successful attachment of the sulfur bearing CTCLRA. Based on the sulfur and iron content measured by ICP, the grafting density was estimated to be 4 molecules/nm<sup>2</sup>.

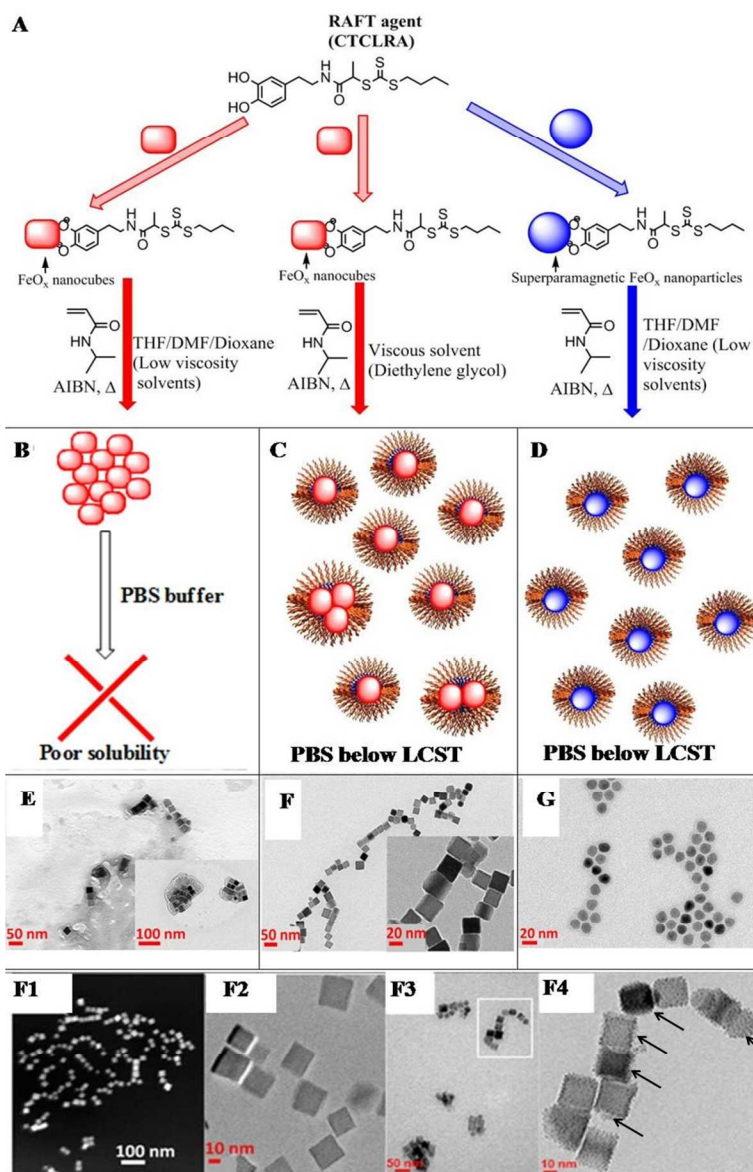


**Figure 1:** (A) <sup>1</sup>H NMR spectrum of catechol functionalized RAFT agent (CTCLRA) in DMSO-*d*<sub>6</sub> and (B) FTIR spectra of CTCLRA and nanocubes before and after ligand exchange with CTCLRA.

## 2.2 Polymerization reaction of thermo-responsive shell on the RAFT-functionalized nanocubes

After functionalizing the cubic-IONPs with CTCLRA, we adopted an approach similar to that employed by Ohno *et al.*<sup>26</sup> to grow polymer on superparamagnetic nanoparticles for the functionalization of our strongly interacting cubic-IONPs with poly(*N*-isopropylacrylamide) (PNIPAAm) which is a non-toxic polymer with a lower critical solution temperature (LCST) of about 32 °C in water.<sup>27</sup> Although the transition temperature of PNIPAAm is lower than 37 °C, copolymerizing NIPAAm with hydrophilic/hydrophobic monomers allows tuning of the LCST. However, under the polymerization conditions employed for superparamagnetic nanoparticles and in THF (see the section of the material and methods) the reaction did not give the desired colloiddally stable nanohybrids and polymer coated nanocubes precipitated out of solution within the first few minutes of polymerization. Functionalization under continuous stirring on a magnetic stirrer was not possible since the cubic-IONPs are strongly attracted to magnetic surfaces and thus they do not stay dispersed but instead they "rush" to the magnet and clump up at the bottom of the reaction vessel. Functionalization was however possible

when the reaction was carried out in an ultrasonication bath under continuous sonication. The samples obtained under continuous sonication appeared mostly as clusters of nanocubes as observed by Transmission Electron Microscope (TEM) analysis (Figure 2E) rather than being singly coated cubic-IONPs as observed upon functionalization of spherical and superparamagnetic IONPs by Ohno *et. al* (see also Figure 2G).<sup>26</sup> suggesting that in our case, particle aggregation took place under these conditions.



**Figure 2:**(A) Scheme showing the synthetic approach used to prepare thermo-responsive IONPs, (B-D) representation of the nanohybrids upon dispersion in aqueous solution, (E) TEM image of thermo-responsive cubic-IONPs obtained in THF under continuous sonication (scale bar 50 nm), (F) of thermo-responsive cubic-IONPs obtained in viscous solvent (diethylene glycol) (scale bar 50 nm) and (G) of



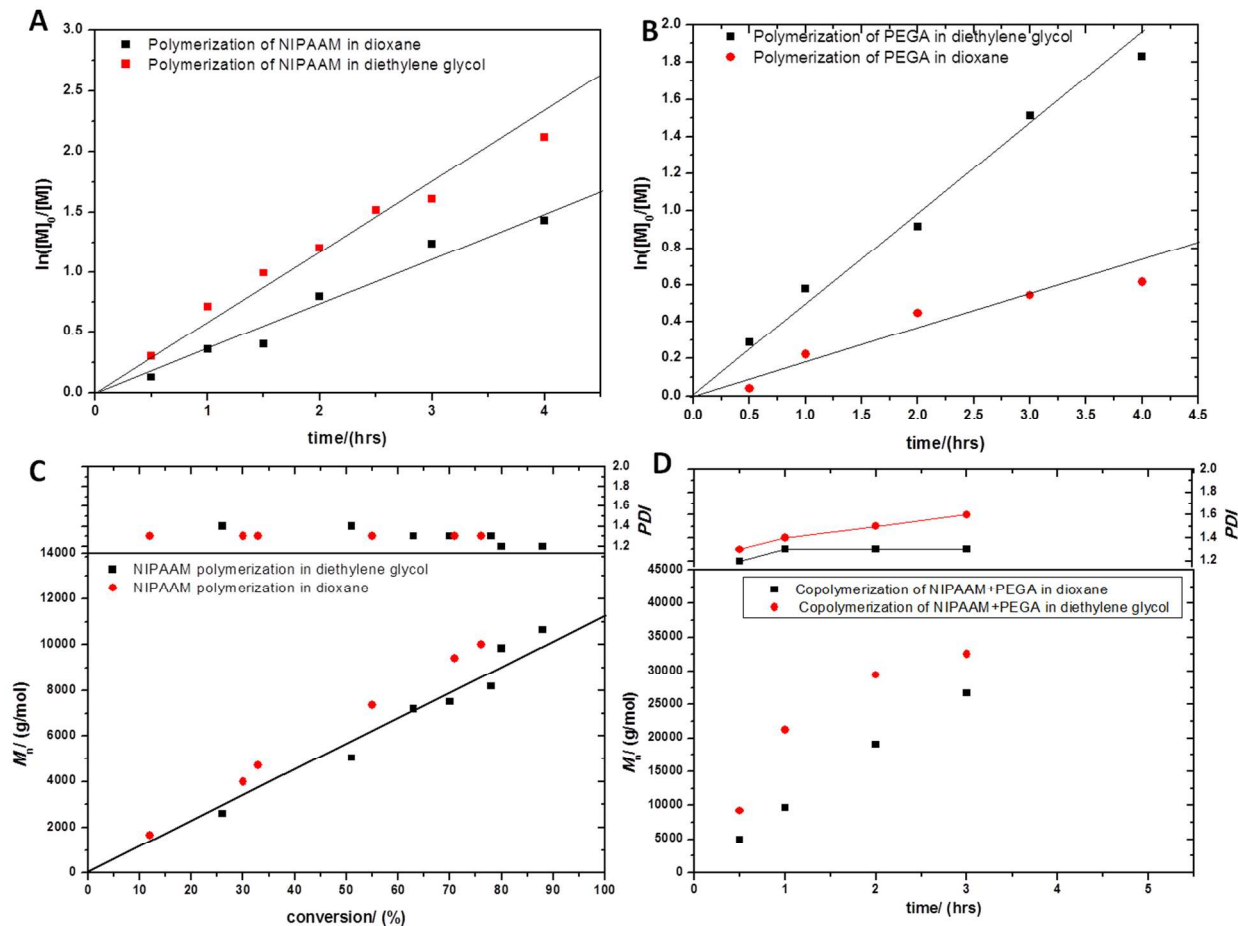
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3 thermo-responsive spherical-IONPs (scale bar 20 nm). (F1-F4) cryo-EM images of cubic-IONPs obtained  
4 from polymerizations done in diethylene glycol (DEG). (F1) cryo-STEM image showing the chain-like  
5 assembly of nanocubes in the vitrified solution (scale bar 100 nm), (F2) cryo-TEM image showing single  
6 nanocubes suspended in the vitreous solution (scale bar 10 nm), (F3) cryo-TEM image showing  
7 nanocubes spontaneously arranged in chain-like assembly and small clusters (scale bar 10 nm). (F4)  
8 detail of (F3) boxed region (scale bar 10 nm). Note that most nanocubes are singly coated by a thin and  
9 wrinkled polymer layer (arrows) *ca.* 1.5 nm thick.  
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19 This was corroborated by dynamic light scattering (DLS) analysis of the nanocubes which showed  
20 multimodal distributions including particles with sizes close to one micron (SI, FigureS2). Furthermore,  
21 the possibility of polymer chain scission during ultrasonication for such long periods also exists.<sup>28-29</sup>  
22 Nevertheless, the use of the ultrasonication bath presented the disadvantage of poor temperature  
23 control which led to poor reproducibility of results. To overcome these challenges, we envisioned that,  
24 in a “frozen” system, the nanocubes mobility could be reduced and hence their chance to aggregate. As  
25 a consequence, the nanocubes would stay longer in solution and polymerization could have higher  
26 chance to occur at the surface of individual nanocubes. We thus dispersed the CTCLRA functionalised  
27 cubic-IONPs in different solvents having different viscosity (either polyethylene glycol with an average  
28 molecular weight of 200 g/mol (PEG200), or PEG400 or diethylene glycol (DEG), or THF, or  
29 dimethylformamide (DMF) or 1,4-dioxane). We placed these solutions in a heated water-bath at 60 °C to  
30 determine if the nanocubes would remain in solution or they would just precipitate as observed during  
31 the polymerization. We observed that the nanocubes in THF, DMF and 1,4-dioxane precipitated out of  
32 solution in less than half an hour whilst they remained dispersed after 6 or 24 hours in the more viscous  
33 solvents (PEG200, or PEG400 or DEG). Viscous solvents act by impairing the movement of the cubic-  
34 IONPs and indeed will allow sufficient time for polymer functionalization. Moreover, such solvents have  
35 been advocated to be optimal media LRP.<sup>30-31</sup>  
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49 Encouraged by this observation, we first studied the RAFT (co)polymerizations of NIPAAM and *N*-  
50 isopropyl acrylamide mixed with polyethylene glycolmethylether acrylate (NIPAAM/PEGA) in DEG and in  
51 1,4-dioxane, mediated by the succinimide bearing RAFT agent(SUCRA). In this experiment no  
52 nanoparticles were present. DEG was found to offer good solubility for the polymerization reagents and  
53 also its lower viscosity (compared to PEG400 and PEG200) would ensure delayed plateauing of the molar  
54 mass ( $M_n$ ). The polymerization of NIPAAM in either solvent proceeded in a controlled manner as  
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3 evidenced by the linear increase in molar mass with conversion as well as the low polydispersity indices  
4 (*PDIs*) (1.1-1.3) (Figure 3). Similarly, the molar masses for the co-polymerizations of NIPAAM and PEGA  
5 were observed to increase with time and the obtained polymers also had low *PDIs* although the  $M_n$   
6 *versus* conversion plot could not be obtained due to peak overlap in the NMR spectra. Both the NIPAAM  
7 and NIPAAM/PEGA (co)polymerizations were observed to proceed faster in the viscous solvent than  
8 those in 1,4-dioxane which is in accordance with the results published by Perrier and co-workers on LRP  
9 in viscous solvents.<sup>30,b</sup>

16 Having now set a new solvent of the reaction, we then tried to perform the ligand exchange procedure  
17 with CTCLRA in the viscous solvent, by first dispersing the as-synthesized decanoic acid capped cubic-  
18 IONPs in viscous solvent under ultrasonication prior to adding the CTCLRA in excess (30 molecules/nm<sup>2</sup>).  
19 Success of the ligand exchange process was confirmed by FTIR as in the previous case and also by TGA  
20 (Figures S3 and S4 of the SI). We however observed no difference in surface coverage obtained using the  
21 different methods as by sulfur/iron content by ICP the final coverage ratio was found to be 4  
22 molecules/nm<sup>2</sup> (see experimental section for the formula used). This suggests that the ligand exchange  
23 process was not severely impacted on by the strong interaction of the cubic IONPs. This can be  
24 explained by taking into account that in both solvent mixtures (THF/CHCl<sub>3</sub> or dioxane or DEG) there is an  
25 ultrasonication step which allows the nanocubes to become well dispersed exposing most of their  
26 surfaces for ligand exchange to take place and if the exchange process is occurring at similar rates in  
27 both cases, the surface coverage will therefore be more or less the same. Moreover, during  
28 ultrasonication heat is produced and at higher temperature the nanocubes have low tendency to  
29 interact with each other as the magnetic remanence is decreased or cancelled. After a cleaning step,  
30 functionalization of CTCLRA-cubic-IONPs (19 nm) with NIPAAM was undertaken in DEG in presence of  
31 free SUCRA to control the polymerization (see materials and methods). After removal of free polymer  
32 from the reaction mixture, the polymer coated cubic-IONPs were observed to be soluble in water or  
33 phosphate buffered saline (PBS) (pH 7.4) at room temperature.  
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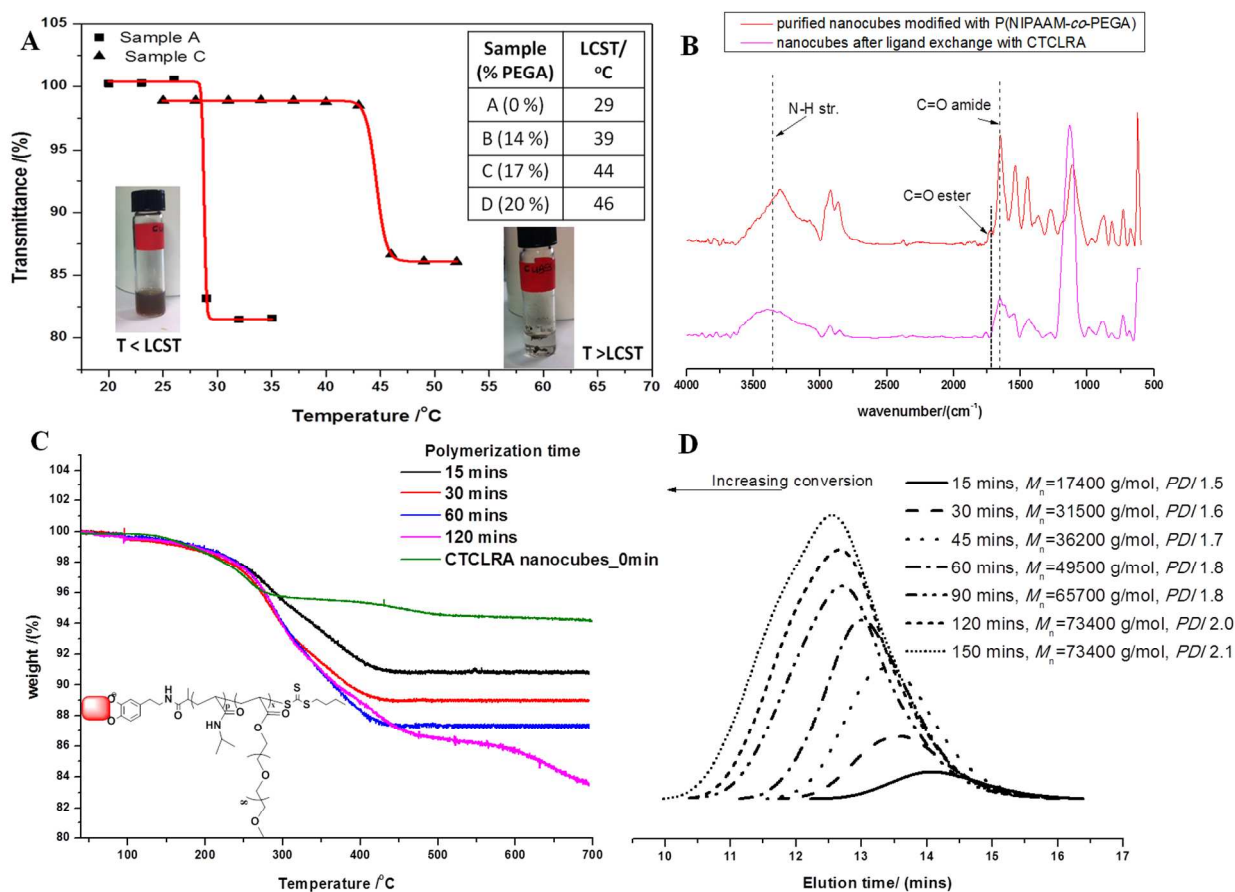


**Figure 3:** (A) Kinetic plots for the polymerization of NIPAAM in dioxane (■) and in DEG (■) at 60 °C. [NIPAAM]:[SUCRA]: [AIBN] was 100:1:0.2. (B) Kinetic plots for the polymerization of PEGA in dioxane (●) and in DEG (■) at 60 °C. [PEGA]:[SUCRA]: [AIBN] was 100:1:0.2. (C)  $M_n$  vs conversion and  $PDI$  vs conversion graphs for the polymerization of NIPAAM in dioxane (●) and in DEG (■) at 60 °C [NIPAAM]:[SUCRA]: [AIBN] was 100:1:0.2. The straight line represents the theoretical  $M_n$ . (D)  $M_n$  vs time and  $PDI$  vs time plot for the copolymerization of NIPAAM and PEGA in dioxane (■) and in DEG (●) at 60 °C. [NIPAAM]:[PEGA]: [SUCRA]: [AIBN] was 100:100:1:0.2.

The thermo-responsive nanocubes obtained using this procedure have a typical diameter of *ca.* 200 nm by DLS in size and showed no sign of large clusters formation as evidenced from TEM/cryo-TEM (Figure 2, F1-F4). Moreover, under cryo-TEM (Figure 2, F1-F4), it is clearly evident the quality of the monodispersed samples and the spontaneous formation of chain-like assembly of the nanocubes in the hydrated solution which might explain the high DLS values measured. Based on cryo-TEM images however, the presence of few grouping of nanoparticles as the one shown in Figure 2, F4 (see SI, Figure

S5) were also observed suggesting the coexistence of a mixture of nanoparticles within the solution with a large proportion of single nanoparticles and some small aggregated particles.

Turbidimetric analysis confirmed the thermo-responsiveness of these hybrid nanoparticles and the LCST was *ca.*29 °C which is in accordance with literature values (Figure 4). Indeed, when the nanoparticle solutions were heated above 29 °C, precipitation of the particles was observed (polymer shell underwent a coil to globule transition and thus nanoparticles collapsed into macroscopical aggregates) while upon cooling below 29 °C, their solubilization occurred as the polymer became again water soluble leading to hydration of the polymer shells (Figure 4).



**Figure 4:** (A) Turbidimetric analysis of PNIPAAm (sample A) and PNIPAAm-co-PEGA functionalized cubic-IONPs (sample C). The inserted table shows the variation of LCST with change in copolymerization feed composition of NIPAAm and PEGA obtained for the nanocubes. The inserted picture shows the thermo-responsive behavior of the polymer functionalized nanocubes. The vial on the left below the LCST of

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3 PNIPAAm (inset) shows a brown-black solution of well dispersed nanoparticles whilst the vial on the  
4 right (inset) above the LCST shows an almost colourless solution with most of the nanoparticles floating  
5 in macroscopic aggregates on top of the water and at the bottom of the glass vial. (B) FTIR spectra of  
6 iron oxide nanocubes functionalised with CTCLRA before and after polymerization with PNIPAAm/PEGA  
7 in diethylene glycol. (C) Thermogravimetric analysis of PNIPAAm-co-PEGA functionalized cubic-IONPs  
8 (structure in inset) obtained at different polymerization times and (D) SEC chromatograms obtained by  
9 analysis of free polymers during the functionalization of iron oxide nanocubes via RAFT polymerization  
10 at different times.  
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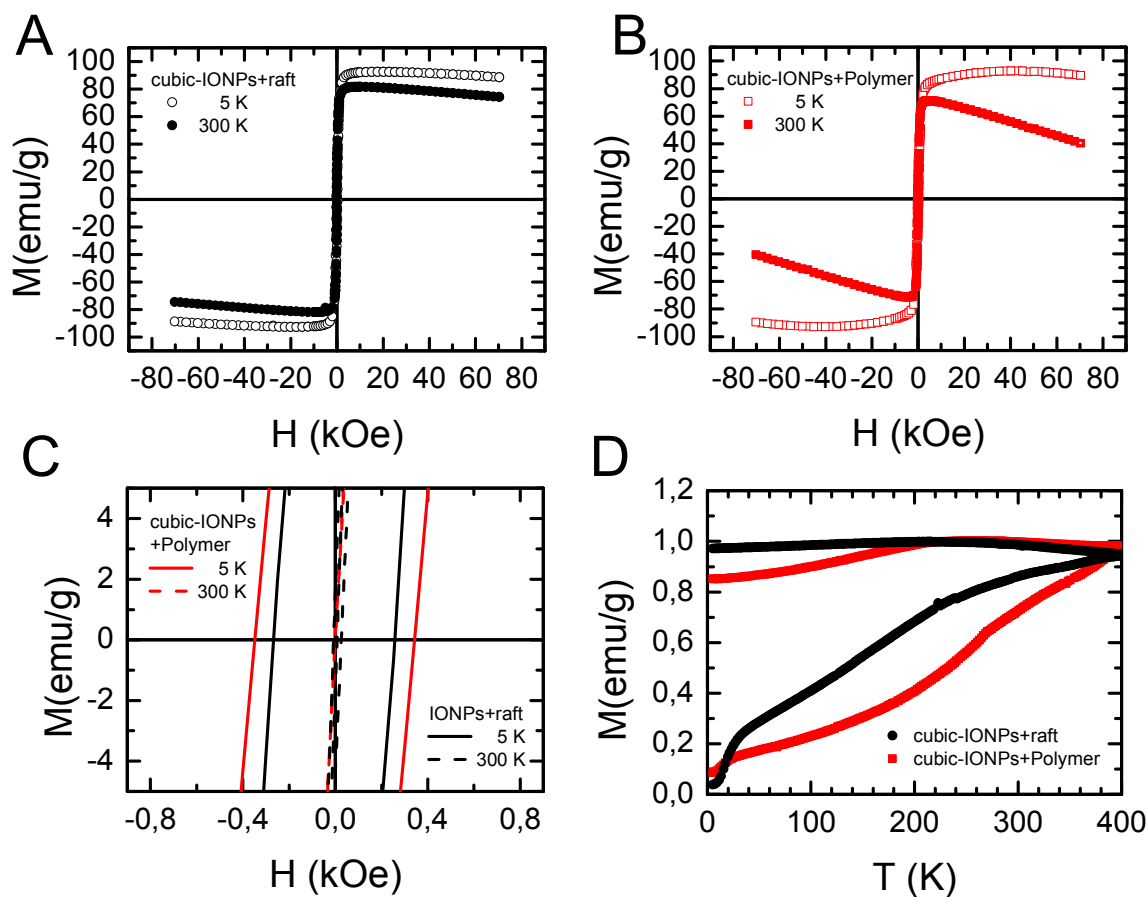
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18 As we sought to prepare thermo-responsive IONPs for cancer treatment with thermal response  
19 significant for hyperthermia (41-47 °C),<sup>15,32</sup> we targeted coil-globule response temperatures above body  
20 temperature (37 °C) and maintaining this behavior in physiological conditions (saline buffer solutions at  
21 155 mM of NaCl). Copolymerization of NIPAAm with hydrophilic monomers increases the LCST and thus,  
22 we chose to copolymerize NIPAAm with polyethylene glycol methylether acrylate (PEGA). In addition to  
23 offering hydrophilicity, polyethylene glycol also has the advantages of being non-toxic ( $M_n > 400$  Da but  
24 below 40 kDa) as well as reducing *in vivo* protein adsorption and thus improving biodistribution although  
25 few drawbacks PEG dose and PEG molecular weight related should not be neglected (*i.e.* immunogenic  
26 issues due to complement activation and antibody response).<sup>33</sup> Varying in the feed, the initial mole  
27 percentage of hydrophilic monomer (PEGA) between 0 and 35% with respect to that of NIPAAm we  
28 were able to tune the LCST to a temperature above 37 °C thus ensuring cargo (drug) encapsulation and  
29 water solubility at temperatures below this value and conversely cargo release at temperatures  
30 recommended for hyperthermia above 40 °C (Figure 4).  
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41 Thus, using our functionalization procedure, the LCST of the hybrid cubic-IONPs could be easily modified  
42 by copolymerizing NIPAAm with PEGA (Figure 4A). Below LCST the nanocubes were completely soluble  
43 while above the LCST, the nanocubes collapsed (inset images of figure 4A) and this behavior was totally  
44 reversible by switching the temperature. The copolymerization with a monomer feed composition of  
45 80% mol NIPAAm and 20% mol PEGA was studied over time and found to give hybrid nanostructures  
46 with an LCST of 45 °C. Analysis of the thermo-responsive cubic-IONPs by TGA revealed an increase in %  
47 weight loss with increasing polymerization time which corresponds with gradual molar mass increase  
48 observed in LRP (Figure 4C). Analysis of the free polymers by size exclusion chromatography (SEC)  
49 indicated that the molar mass increased with time although the *PDI* values were relatively high (1.5-1.7)  
50 (Figure 4D). These *PDI* values were however lower than those obtained when the polymerizations were  
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conducted using oleic acid capped cubic-IONPs in the absence of free RAFT agent (2.5-4.3) suggesting the *grafting from* polymerizations done using CTCLRA coated nanocubes was controlled albeit with less efficiency. Finally, by FTIR, the amide (C=O and N-H) and ester (C=O) peaks indicate the presence of the thermo-responsive polymer shell present at the nanocube surface (Figure 4B).

The thermo-responsive cubic-IONPs also exhibited magnetization properties (at low temperature, 5K) similar to those of the as synthesized or PEG water transferred cubic-IONPs reported previously (Figure 5).<sup>22</sup> Noteworthy, at 300 K and high magnetic fields (above 5 kOe) the magnetization signal for the polymer coated nanocubes system decreases while increasing the magnetic field (Figure 5B). This is due to the diamagnetic contribution of the polymer which becomes relevant at high temperatures and fields.<sup>34</sup> As a result, and depending on the amount of polymer (and possible solvent entrapped on/in it) a negative susceptibility at high magnetic fields and at high temperatures is observed. For the thermo-responsive nanocubes, this further corroborates the presence of a polymer, the thermo-responsive shell, at the surface of nanocubes.

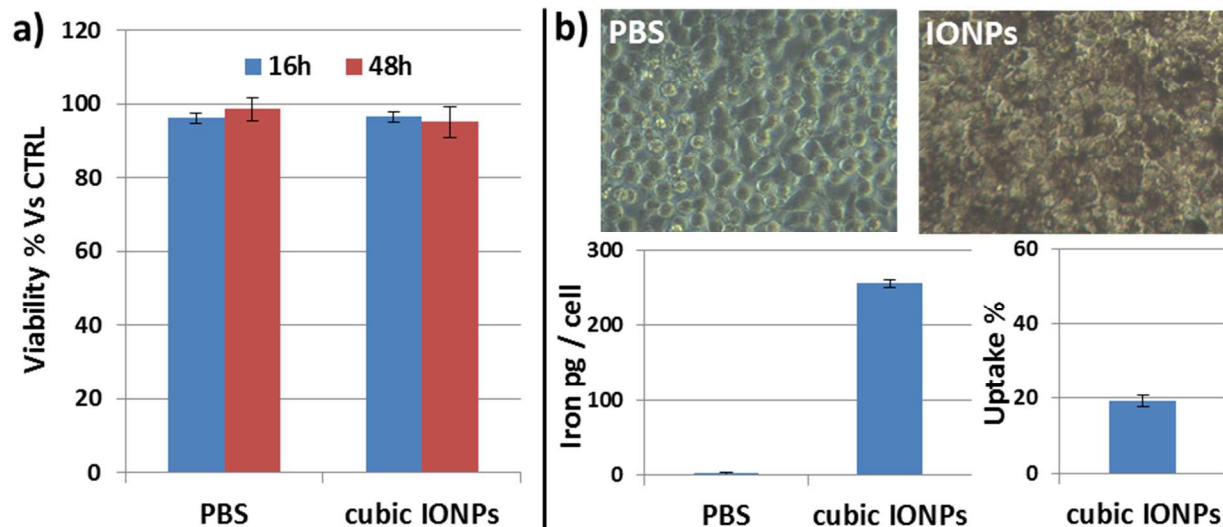


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3 **Figure 5:** (A) Hysteresis loops at 5 K (open black circles○) and 300 K (black circles●) for 19 nm iron oxide  
4 nanocubes- functionalized with CTCRLA, the raft agent and (B) the same nanoparticles after having  
5 grown the thermo-responsive polymer 5 K (open red square□) and 300 K (red square■). Notice how the  
6 diamagnetic signal contribution derived from the polymer increases while increasing temperature. (C)  
7 Low field detail of the magnetization hysteresis loops at 5 K (solid line) and 300 K (dashed line) for  
8 CTCRLA raft functionalized (black line) and thermo-responsive polymer coated (red line) 19 nm iron  
9 oxide nanocubes. (D) Temperature dependence of the zero field cooling and field cooling (cooling field  
10  $H_{FC} = 100$  Oe) magnetization curves measured at 100 Oe, as a function of particle size for CTCRLA raft  
11 functionalized (black circles●) and thermo-responsive polymer coated (red square■) 19 nm iron oxide  
12 nanocubes. For both samples the blocking temperature ( $T_B$ ) is above room temperature.

### 23 24 **2.3 Cytotoxicity assay**

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26 Besides tuning the phase transition temperature for feasible applications at body temperature, for any  
27 material to be applicable as a drug delivery vehicle, it is imperative that the material is non-cytotoxic.  
28 We studied the effect of our polymer coated (PNIPAAAM-co-PEGA) nanocubes on Kb cells by conducting  
29 cell viability and cell uptake studies. Cell viability studies showed no difference in % viability between Kb  
30 cells incubated for 16 hours with polymer coated nanocubes at high concentration (1 g/L of iron) and Kb  
31 cells treated only with the corresponding amount of PBS, used as control; in a parallel experiment under  
32 the same conditions, cells that were rinsed from the excess of IONP and kept for additional 32 hours in  
33 incubation, also showed very high viability(in this case the total exposure time of cells to the cubic-  
34 IONPs is of 48 h), indicating the nanohybrids were not cytotoxic (Figure 6A). On the other hand, as  
35 confirmed by measuring the iron amount by ICP, the uptake of the thermo-responsive coated  
36 nanocubes is slightly higher than that of similar nanocubes coated by only PEG or enwrapped in an  
37 amphiphilic polymer and as previously found by us<sup>22</sup> (250 picomol/cells versus 200 and 150 respectively)  
38 which does corresponds roughly to 20% of the administered dose (Figure 6B). It is important to note  
39 that the high amount of magnetic material incubated for 16 hours in the cell dish formed a dark layer  
40 on top of the adherent cells. This was clearly visible under optical phase contrast microscopy on cell  
41 plate (after having rinsed the excess of nanoparticle in solution), and is due to the high level of IONPs  
42 internalized by the cells or deposited at the cell surface (Figure 6B, right upper panel). However despite  
43 the high amount of iron associated to the cells, the Kb cells kept their normal morphology and the  
44 nanocubes did not appear to be toxic (Figure 6B, upper panel). Worthily, despite the very high  
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concentration of nanoparticles administered, the stability of the IONPs in cell medium was not compromised, leading to very few aggregates that do not cause any toxic response (figure S11 in the ESI).



**Figure 6:** Cell study on Kb cells: (A) % of viability assessed by trypan blue assay after 16 and 48 h of incubation with thermo-responsive cubic-IONPs (1 g/L of iron) with respect to a cell control sample (CTRL) to which a corresponding volume of PBS (no nanoparticles) was administered; (B) Uptake analysis: the phase contrast images above show how the cells appear before (left) and after (right) 16 h of incubation with thermo-responsive-cubic-IONPs (1 g/L of iron); the panels below show the amount of iron per cell measured by ICP elemental analysis for two cell samples treated under the same conditions but one exposed to the IONPs and the other to just buffer (left), and the percentage of iron uptake with respect to the extracellular iron (1300 pg iron per cell) added to the cell media (right).

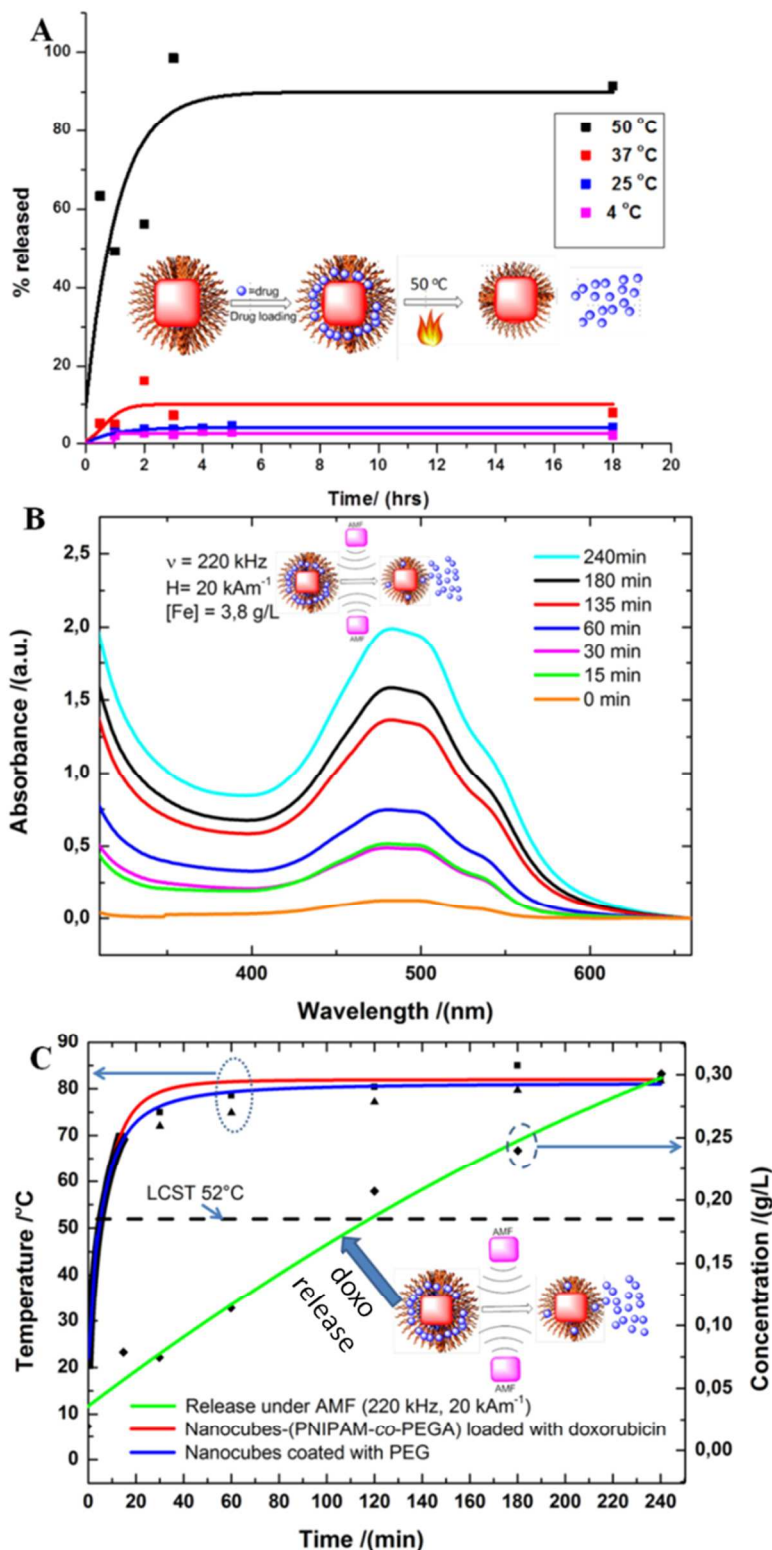
#### 2.4 Drug loading and release experiments

Cubic-IONPs (LCST of sample, 47 °C) were loaded with doxo at room temperature and in order to demonstrate the temperature dependence of doxo release, the drug release was assessed at 4 °C, 25 °C, 37 °C and at 50 °C (above LCST of copolymer). For the loading, thermo-responsive coated cubic-IONPs (50 ppm Fe) were mixed with doxo (150 µg in 2 mL) and after a 24 hour incubation period, the excess (free) doxo was removed by at least five washing steps in which the drug loaded nanocubes were



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3 collected by means of a magnet leaving in solution the excess of doxo which was measured and then  
4 discarded. The drug loading achieved was 44.75% with respect to the initial doxo amount, , as measured  
5 by difference between the initial doxo absorbance and the absorbance of the supernatants (which  
6 contains the doxo not loaded into the polymer shell) found in the 5 washing steps. This also means that  
7 67.125  $\mu\text{g}$  of doxo were associated per 0.1 mg of iron. The release of the drug from the dispersion of  
8 doxo loaded nanoparticles (200 ppm in Fe) was found to be only 8% at 37 $^{\circ}\text{C}$  over 24 hours and even less  
9 at 25  $^{\circ}\text{C}$  and 4  $^{\circ}\text{C}$ . However, more than 90% of the drug was released in less than 5 hours at 50  $^{\circ}\text{C}$  clearly  
10 suggesting that the release was heat triggered (Figure 7A). In these cases a water bath was used to  
11 increase the temperature and after thermal treatment the thermo-responsive-IONPs were collected to  
12 the magnet and the supernatant analyzed for the doxo quantification by absorption. We also extended  
13 our study to demonstrate the “on-demand” and remotely triggered drug release by conducting drug  
14 release under an external activation, the AMF. We therefore prepared another sample (LCST, 52  $^{\circ}\text{C}$ ) of  
15 drug loaded cubic-IONPs as described above but on a larger scale (6 mg Fe diluted to 50 ppm) and the  
16 drug loading achieved in this case was 23% (2.2 mg of doxo). Doxo release was observed to take place  
17 from drug loaded thermo-responsive cubic-IONPs when the concentration of nanocubes in the solution  
18 was fixed at 3.8 g/L (of iron) and under an AMF (220 kHz, 20  $\text{kAm}^{-1}$ ) whilst negligible release was  
19 observed for a similar sample not subjected to an AMF (Figure 7B and SI, Figure S6) and kept at 25  $^{\circ}\text{C}$ .  
20 About 25% of the loaded drug was released over 240 minutes (Table S1).”

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35 During the AMF exposure at this nanocube concentration, the temperature of the solution reached 80  
36  $^{\circ}\text{C}$  in the first 15 minutes which is clearly above the LCST of 52  $^{\circ}\text{C}$  of the thermo-responsive sample  
37 employed. Remarkably, the temperature profile of the PNIPAAAM-*co*-PEGA cubic-IONPs is comparable to  
38 that of the same batch of cubic-IONPs brought in water by a chatecol-PEG using a ligand exchange  
39 procedure developed by our group (Figure 7C).<sup>22</sup> It is worth mentioning that when we used the water-  
40 bath, we could achieve the release even at nanocube concentration as low as 0.2 g/L of iron.  
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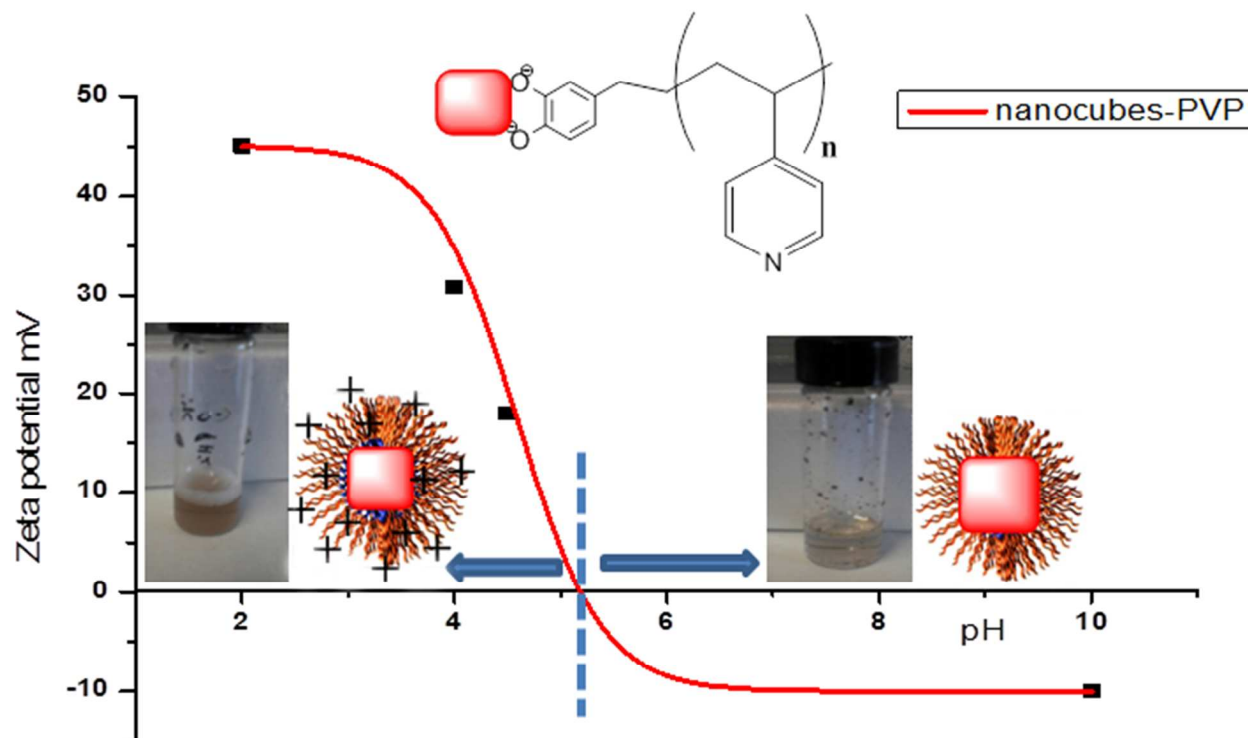
**Figure 7:** (A) Drug release from thermo-responsive PNIPAAm-co-PEGA cubic-IONPs in a water bath at various temperatures (50 °C ■, 37 °C ■, 25 °C ■ and 4 °C ■), (B) Absorption spectra obtained at

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3 different times showing release of doxo under AMF from PNIPAAm-co-PEGa cubic-IONPs and (C)  
4 Comparison of the temperature profiles of PNIPAAm-co-PEGa functionalized cubic-IONPs (red line, (■))  
5 and PEG functionalized cubic-IONPs (blue line, (▲)) at 3.8 g/L [Fe](left axis) and the concentration of  
6 doxo release (right axis, green line (◆)) over time.  
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11 However, under AMF, the temperature of a 0.2 g/L of iron solution did not increase and remained  
12 constant at 25 °C and under those conditions no drug release was observed. This suggests that the  
13 polymer shrinking with consequent drug release is a collective property which involves inter-particle  
14 interaction rather than a single particle shrinking process. In other words, to activate the shrinking of the  
15 nanosystem, a macroscopical temperature increase rather than local heating effects plays a major role.  
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## 23 **2.5 Implementation of the polymerization procedure on nanocubes with pH-responsive monomer** 24 **units** 25

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27 As a further extension of this work, to demonstrate the versatility of our synthetic approach, we also  
28 prepared nanocubes functionalized with a pH-responsive polymer shell, poly(vinyl pyridine) (PVP) by  
29 simply choosing a different monomer, 4-vinylpyridine (VP) and keeping all the other conditions as  
30 employed for the thermo-responsive shell polymerization. The pH-responsive polymer functionalized  
31 nanocubes were water soluble below pH 5 due to protonation of the pyridine groups of the polymer  
32 (positive zeta potential) while they precipitated out of water above pH 5 due to neutralization of the  
33 positive charge (zeta potential  $\leq 0$ ) (Figure 8). Copolymerization of VP (50 %) with PEGa (50 %) gave pH-  
34 responsive cubic-IONPs that were water soluble even above pH 5 indicating the water solubilizing effect  
35 of PEG (ESI, Tables S2 and S3, FigureS7).  
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**Figure 8:** Zeta potential as a function of pH of PVP functionalized cubic-IONPs. The protonation of PVP below pH 5 allows the stabilization of the nanocube in solution by electrostatic repulsions. The vial on the left below the  $pK_a$  of PVP (inset) shows a brown solution of well dispersed nanoparticles whilst the vial on the right (inset) above the  $pK_a$  shows an almost colourless solution with most of the nanoparticles stuck on the wall of the glass vial.

## 2.6 Control polymerization experiments using superparamagnetic and spherical IONPs

To confirm that the complication in functionalization of the cubic-IONPs resulted from their strongly interacting character, we functionalized spherical superparamagnetic IONPs synthesized by following the protocol reported by Colvin and coworkers.<sup>35</sup> Due to their superparamagnetic behavior at room temperature, these spherical-IONPs have no tendency to aggregate. The oleic acid capped spherical-IONPs were easily exchanged with the CTCLRA (see materials and method section) and on the CTCLRA functionalized nanoparticles polymerization with NIPAAMor PNIPAAAM-co-PEGA could be undertaken with no difference either in dioxane or in THF affording water or PBS buffer (pH 7.4) dispersible thermo-responsive spherical-IONPs. In this case, no precipitation or aggregation was observed over entire

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3 duration of the polymerization which is similar to the observations made in the work by Onho *et al.*<sup>26</sup> in  
4 which non-aggregating superparamagnetic IONPs were successfully functionalized *via* surface mediated  
5 RAFT polymerization. Analysis of the typical coated nanoparticles obtained by TEM also showed a thin  
6 shell of polymer around the nanoparticles and the complete absence of clusters (Figure 2G). In addition,  
7 an increase in size after polymerization was observed by DLS and thermo-responsiveness of the  
8 nano hybrids was ascertained by turbidimetric analysis (SI, Figure S9-S10). This direct comparison just  
9 confirms that cubic-IONPs, which are strongly interacting nanoparticles, are more challenging to  
10 functionalize with respect to superparamagnetic IONPs which do not interact at all with each other.  
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### 20 21 **3. Conclusion**

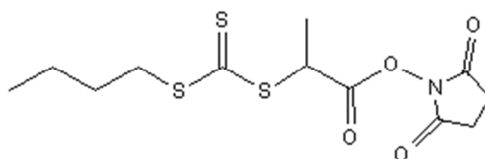
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23 In summary, we have developed a facile procedure to functionalize strongly interacting magnetic  
24 nanoparticles *via* RAFT polymerization with stimuli-responsive polymers and generate smart  
25 nanomaterials that have potential application in the biomedical field. Using our procedure, we  
26 successfully functionalized iron oxide nanocubes with a thermo-responsive and also with a pH-  
27 responsive polymer to obtain responsive biocompatible nano hybrids. The exploitation of the thermo-  
28 responsive hybrids was demonstrated by loading and release of an anti-cancer drug (doxo) into the  
29 nano hybrids which could be release, on demand, under an AMF due to the heat generated by the  
30 nanocubes. Such materials may provide a synergistic therapeutic effect in cancer treatment *via*  
31 hyperthermia and chemotherapy with triggered drug release while not displaying the side effects of  
32 standard chemotherapy.  
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### 44 **4. Experimental Section**

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47 **4.1 Materials.** N-isopropylacrylamide (NIPAAM) (Aldrich, 99%) and N-isopropylmethacrylamide  
48 (NIPMAM) (Aldrich, 99%) were purified by re-crystallization from hexane three times and dried under  
49 vacuum at room temperature before use. Poly(ethylene glycol) methyl ether acrylate (average  $M_n$  480)  
50 (Aldrich) and 4-vinyl pyridine (Aldrich) were destabilized by passing the monomer through a short  
51 column packed with inhibitor remover (Aldrich). Azobis(isobutyronitrile) (AIBN) was purified by  
52 recrystallization from methanol twice and then it was left to dry under reduced pressure in a desiccator.  
53 1,4 Dioxane (Sigma Aldrich, 99%) and tetrahydrofuran (THF) were distilled and stored under nitrogen  
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3 prior to use. The trithiocarbonate RAFT agent, 2-(butylthiocarbonothioylthio) propanoic acid (BTTPA)  
4 was synthesized in accordance with a previously published protocol.<sup>36</sup> Doxorubicin hydrochloride and all  
5 other solvents were purchased from Sigma Aldrich at the highest purity available and used as received.  
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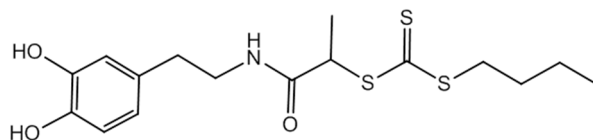
#### 4.2 Synthesis of succinimide RAFT agent (SUCRA).



Scheme 1: SUCRA.

To a 500 mL round bottom flask was added BTTPA (10.00 g, 42.00 mmol), *N*-hydroxysuccinimide (7.25 g, 63.02 mmol), DMAP (0.51 g, 4.20 mmol) and dry THF (200 mL). The reaction vessel was placed in an ice bath and the contents were stirred for 15 minutes followed by dropwise addition of DIC (10.60 g, 84.00 mmol) in THF(50 mL). The reaction was kept at 0 °C for 4 hours before it was allowed to gradually warm up to room temperature and then left to stir for 24 hours. At the end of the reaction, the reaction mixture was filtered *via* gravitational filtration to remove a white precipitate. Solvent from the collected filtrate was removed *in vacuo* to yield a yellow residue which was taken up in dichloromethane and transferred to a separating funnel. The organic layer was washed with water (3×) and brine (1×) then dried over MgSO<sub>4</sub>. After filtration to remove MgSO<sub>4</sub>, removal of solvent *in vacuo* afforded the crude product which was purified via flash chromatography (SiO<sub>2</sub>, Initially, Hexane 95%: Ethyl acetate 5% for approximately 5-6 column volumes (elutes yellow coloured impurities, product does not move) then Hexane 65%: Ethyl acetate 35% (elutes product, R<sub>f</sub> 0.65). Removal of solvent *in vacuo* gave the product as a viscous yellow oil (12.30 g, 87%). For the <sup>1</sup>H NMR characterization see Figure S1 (SI) ( 0.93 (t, *J*=7.3 Hz, 3H), 1.43 (sextet, *J*=7.5 Hz, 2H), 1.75 (d, *J*=7.4 Hz, 3H), 1.69 (m, 2H), 2.83 (s, 4H), 3.38 (t, *J*=7.4 Hz, 2H), 5.15 (q, *J*=7.4 Hz, 1H)

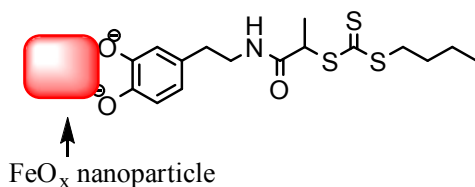
#### 4.3 Synthesis of catechol functionalised RAFT agent (CTCLRA).



Scheme 2: CTCLRA.

Dopamine hydrochloride (6.25 g, 32.98 mmol) and SUCRA (10.00 g, 29.85 mmol) were dissolved in dry methanol (300 mL) followed by addition of triethylamine (3.63 g, 35.82 mmol) under argon flow. The reaction mixture was left to stir for 72 hours and after methanol was removed *in vacuo*. The remaining residue was taken up in dichloromethane and the organic layer was washed (2×) with aqueous HCl solution (0.1 M), water (3×), then with brine (1×) followed by drying over  $\text{MgSO}_4$ . The drying agent was removed by filtration and the solvent was removed *in vacuo* to yield a yellow solid which was suspended and stirred in hot hexane followed by filtration (3×) to afford the pure product as a yellow solid. Yield (6.80 g, 61 %).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ): 0.90 (t,  $J=7.4$  Hz, 3H), 1.38 (sextet,  $J=7.6$  Hz, 2H), 1.46 (d,  $J=7.1$  Hz, 3H), 1.63 (m, 2H), 2.51 (m, 2H) overlaps with solvent, 3.19 (m, 2H), 3.38 (t,  $J=7.4$  Hz, 2H), 4.70 (q,  $J=7.06$  Hz, 1H), 6.40 (dd,  $J_a=8.0$ ,  $J_b=2.1$ , Hz, 1H), 6.57 (d,  $J=2.1$  Hz, 1H), 6.63 (d,  $J_a=8.0$  Hz, 1H).

#### 4.4 Functionalization of iron oxide nanocubes (cubic-IONPs) with CTCLRA in low viscosity solvent (Method 1).



Scheme 3: Cubic-IONPs functionalized with CTCLRA.

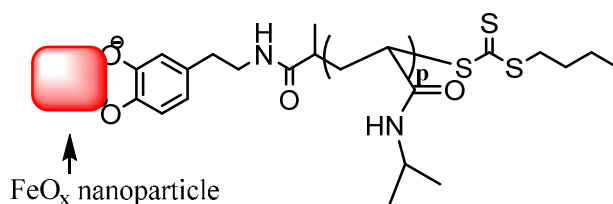
The stock solution of iron oxide nanocubes (see below) was placed in the sonication bath for fifteen minutes after which the desired volume (see below) was transferred to a glass vial followed by ultrasonication at  $0^\circ\text{C}$  in an ice bath for one minute. To a solution of CTCLRA (7 mmol in 50 mL THF) was added to the nanocubes (5 mL in chloroform,  $0.14\ \mu\text{M}$ ,  $[\text{Fe}]$  2.0 mg/mL,  $18\pm 2$  nm edge length as by TEM) and then triethylamine (1 mL in 50 mL THF) and the resulting colloidal solution was ultrasonicated for 1 minute at  $0^\circ\text{C}$  in an ice bath then transferred to a sonication bath with sonication on full power (30 W) to keep the cubes suspended in solution. After sonication for two hours, the vial was transferred to the

shaker and left to shake vigorously overnight. The nanoparticles were recovered by centrifugation at 3500 rpm for 10 minutes and the collected nanoparticles were re-dispersed in THF. Hexane was added to near the point of particle precipitation (the amount of hexane added was such that no particle or CTCLRA precipitation was observed but further addition of hexane, few mL, would result in particle precipitation). The solution was centrifuged to recover the nanoparticles and the supernatant solution was decanted. This process was repeated until the characteristic yellow color of the RAFT agent in the supernatant disappeared after which the nanoparticles were washed two more times. The functionalized nanoparticles were insoluble in hexane which is a good solvent for the starting nanoparticles thus indicating the change in the coating at their surface.

ICP (starting nanoparticles),  $[\text{Fe}] = 2 \text{ g/L}$ ,  $[\text{S}] = 0$  ICP (CTCLRA functionalized nanoparticles),  $[\text{Fe}] = 1.98 \text{ g/L}$ ,  $[\text{S}] = 0.1 \text{ g/L}$  (calculated based on same volume of starting and CTA functionalized nanoparticles).

FTIR: C=S  $1060 \text{ cm}^{-1}$ , overlaps with nanocubes peak. N-H,  $3400 \text{ cm}^{-1}$ . Initiator grafting density (molecules/ $\text{nm}^2$ ) was calculated using the formula  $(C_{\text{Sulfur}}/96)/(C_{\text{NPs}} \times 6S_{\text{TEM}}^2)$ .  $C_{\text{Sulfur}}$  is the concentration of sulfur from ICP in g/L,  $S_{\text{TEM}}$  is the average edge size of the nanoparticles from TEM in nanometers and  $C_{\text{NPs}}$  is the concentration of nanoparticles (mol nanoparticles/L).

#### 4.5 Functionalization of cubic IONPs with thermo-responsive polymer (PNIPAAM) in low viscosity solvents without sonication (Example procedure).



**Scheme 4:** Cubic-IONPs functionalized with thermo-responsive polymer (PNIPAAM).

To a glass vial was added NIPAAM (4.000 g, 35.4 mmol), SUCRA (0.011 g, 32.8  $\mu\text{mol}$ ), solvent (dioxane or THF) (4 g), and CTCLRA coated iron oxide nanocubes dispersed in THF (1 mg Fe, from 0.6 $\mu\text{M}$  stock solution of NPs). The solution was vortexed for a few seconds and the vial was sealed with a rubber septum followed by degassing with nitrogen gas for 30 mins. AIBN (25  $\mu\text{L}$ , 3.28  $\mu\text{mol}$ ) was then added from a stock solution in dioxane via a microliter syringe and degassing was continued for a further 10 minutes. The reaction contents were mixed for a few seconds and the vial was placed in a thermostated



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3 water bath at 60 °C for 3 h. The reaction was stopped by placing the vial in an ice bath and opening it to  
4 the atmosphere. A sample for analysis by SEC was obtained by taking an aliquot of the reaction mixture  
5 (ca. 100 mg in around 100 μL of the sample) which was diluted with THF and placed on a magnet to  
6 remove the nanoparticles prior to collection of the supernatant for analysis. The remainder of the  
7 reaction mixture was precipitated into cold diethyl ether and the collected precipitate was dissolved in a  
8 small amount of THF and reprecipitated in cold diethyl ether. This process was repeated one more time  
9 and the precipitate was then dissolved in THF/diethyl ether (1:2 in volume) and centrifuged at 5000 rpm  
10 for 10 minutes. The supernatant was decanted into cold hexane where precipitation of free polymer  
11 could be observed. The precipitated nanoparticles were resuspended in THF/diethyl ether (1:2) and  
12 centrifuged again at 5000 rpm for 10 minutes and this approach was repeated 3 times with the  
13 supernatant being poured into cold hexane. The supernatant from the last wash was concentrated on  
14 the rotary evaporator and analysed by SEC to confirm the absence of free polymer. In this case (no  
15 continuous sonication), the cubic-IONPs precipitated during the early stages of polymerization (ca. 10  
16 mins).

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28 **4.6 Functionalization of cubic IONPs with thermo-responsive polymers (NIPAAM) in low viscosity**  
29 **solvents under continuous sonication.** The procedure followed is similar to that detailed above (when  
30 no sonication was employed) with the only difference being that instead of conducting the reaction in a  
31 thermostated water bath, the reaction vessel was placed in a sonication bath with sonication set at full  
32 power (30 W) and the temperature set at 60 °C for the entire duration of the polymerization. Worthily,  
33 in this case (under continuous sonication), the cubic-IONPs did not precipitate as observed in the  
34 previous case when no sonication was employed during the polymerization. However, though the  
35 temperature was set at 60 °C, it fluctuated significantly (60-85 °C) during the polymerization and it was  
36 very difficult to keep it within a small range of fluctuation ( $\pm 2^\circ\text{C}$ ) leading to poor reproducibility of  
37 results.  
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47 **4.7 Functionalization of iron oxide nanocubes with CTCLRA after initially dispersing the IONPs in**  
48 **viscous solvent (Method 2).** A  $\text{CHCl}_3$  solution of decanoic acid capped iron oxide nanocubes (5 mL, 0.14  
49 μM, [Fe] 2.0 mg/mL,  $18 \pm 2$  nm edge length (TEM)) was added to a glass vial containing diethylene glycol  
50 (20 mL) which was sealed with a suba seal and degassed with nitrogen to evaporate off most of the  
51  $\text{CHCl}_3$ . The resulting solution was vortexed and cooled to 0 °C in an ice bath before it was subjected to  
52 ultrasonication for 20 minutes. To the resulting solution of nanocubes was added a solution of CTCLRA  
53 (7 mmol in 50 mL THF) and triethylamine (1 mL in 50 mL THF) and the colloidal solution was  
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3 ultrasonicated for a total of 20 minutes at 0 °C in an ice bath (ultrasonication cycle, 30 seconds on , 30  
4 seconds off) then left on a shaker overnight. The nanoparticle solution was then diluted with THF (1:3)  
5 and centrifuged at 4000 rpm for 10 minutes to recover the nanoparticles. The collected nanoparticles  
6 were redispersed in THF (20 mL) before hexane was added to near the point of particle precipitation  
7 (the amount of hexane added was such that no particle or RAFT agent precipitation was observed but  
8 further addition of hexane (few mL) would result in particle precipitation). The solution was centrifuged  
9 at 4000 rpm for 10 minutes to recover the nanoparticles and the supernatant solution was decanted.  
10 This process was repeated with THF/hexane until the characteristic yellow color of the RAFT agent in the  
11 supernatant disappeared after which the nanoparticles were washed three more times. The  
12 functionalized nanoparticles were insoluble in hexane which is a good solvent for the starting  
13 nanoparticles. ICP (starting nanoparticles), [Fe] = 2 g/L, [S] = 0. ICP (CTCLRA  
14 functionalisednanoparticles) ), [Fe] = 1.98 g/L, [S] = 0.1 g/L (calculated based on same volume of  
15 starting and CTCLRA functionalized nanoparticles). FTIR: C=S 1060 cm<sup>-1</sup>, overlaps with nanocubes peak.  
16 N-H, 3400 cm<sup>-1</sup>. Initiator grafting density (molecules/nm<sup>2</sup>) was calculated using the formula  
17  $(C_{\text{Sulfur}}/96)/(C_{\text{NPs}} \times 6S_{\text{TEM}}^2)$ .  $C_{\text{Sulfur}}$  is the concentration of sulfur from ICP in g/L,  $S_{\text{TEM}}$  is the average size of  
18 the nanoparticles from TEM in nanometers and  $C_{\text{NPs}}$  is the concentration of nanoparticles (mol  
19 nanoparticles/L).  
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#### 34 **4.8 Polymerization of NIPAAM, PEGA and NIPAAM/PEGA copolymerization (Example procedure).**

35 SUCRA (0.130g,0.35 mmol) and AIBN (0.006g, 0.04 mmol) were weighed separately into glass vialsand  
36 dissolved in dioxane (1.0 mL) to make chain transfer agent and initiator stock solutions respectively.The  
37 desired amounts of RAFT agent and initiator were then added from the stock solutions to a pre-weighed  
38 amount of monomer(s) in a flask containing a stir bar. The solvent was then added to obtain a  
39 homogeneous solution with a monomer content of 20 % (w/v). The reaction vessel was degassed and  
40 transferred to a water bath maintained at 60 °C. Polymerization was allowed to proceed for the desired  
41 period and the reaction was stopped by quenching in liquid nitrogen and opening the reaction to the  
42 atmosphere. Conversions were determined by <sup>1</sup>H NMR.  
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51 **4.9 Functionalization of iron oxide nanocubes with thermo-responsive polymer in viscous solvents.** To  
52 a glass vial was added NIPAAM (2.5 g, 21.9 mmol), SUCRA (2.0 mg, 5.4 μmol), solvent (diethylene glycol)  
53 (2 mL), and CTCLRA functionalized iron oxide nanocubes (0.25 mg Fe, from 0.6 μM of iron stock solution)  
54 in THF. The solution was vortexed for a few seconds then ultrasonicated at 0 °C in an ice bath for one  
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3 minute. The vial was sealed with a rubber septum, degassed with nitrogen gas for 30 minutes, and AIBN  
4 was then added from a stock solution in dioxane (25  $\mu\text{L}$ , 3.28  $\mu\text{mol}$ ) via a microliter syringe. Degassing  
5 was continued for a further 10 minutes and the reaction contents were vortexed for a few seconds and  
6 the vial was placed in a thermostated water bath at 60  $^{\circ}\text{C}$  for 2 h. The reaction was stopped by placing  
7 the vial in an ice bath and opening it to the atmosphere. A sample for analysis by SEC was obtained by  
8 taking an aliquot of the reaction mixture (ca. 100 mg) which was diluted with THF (5 mL) and placed on a  
9 magnet to remove the nanoparticles prior to collection of the supernatant for analysis. The reaction  
10 mixture was precipitated into cold diethyl ether and the collected precipitate was dissolved in a small  
11 amount of THF and reprecipitated in cold diethyl ether. This process was repeated one more time and  
12 the precipitate was then dissolved in THF/diethyl ether (1:2) and centrifuged at 3500 rpm for 10  
13 minutes. If the supernatant was decanted into hexane precipitation of free polymer could be observed.  
14 The precipitated nanoparticles were resuspended in THF/diethyl ether (1:2) and centrifuged at 3500  
15 rpm for 10 minutes and this approach was repeated 3 times with the supernatant being poured into  
16 hexane. The supernatant from the last wash was concentrated on the rotary evaporator dryness and  
17 analyzed by SEC to detect the presence of free polymer. If free polymer was detected, the nanoparticles  
18 were washed one more time and the supernatant analysed again by SEC. After the last wash, the  
19 nanoparticles were dispersed in 5 mL of THF and 1 mL of this solution was transferred to a glass vial  
20 which was placed on a magnet to precipitate the particles and the supernatant was filtered,  
21 concentrated to 0.3 mL by evaporation using a slow flow of nitrogen gas and subjected to analysis by  
22 SEC to verify the absence of free polymer in the sample (DRI/UV 306 nm).  
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39 **4.10 Cell studies: Viability and Uptake Assay.**  $5 \times 10^5$  Kb cells were seeded into 12 wells multiwell plates  
40 24 h prior the treatment started. A dispersion of PNIPAAm-co-PEGA functionalized IONPs in PBS at a  
41 concentration of 2.1 g/L of iron was diluted in 10% FBS supplemented RPMI, in order to reach a  
42 concentration of 1 g/L of iron. As controls, cells under the same conditions were left untreated or also  
43 were incubated with the same ratio PBS/medium. After 16 h of incubation, the cells were rinsed 5 times  
44 with PBS, and an aliquot of cell dishes were re-incubated with fresh medium for further 32 hours and  
45 then analyzed, while other cell wells were directly analyzed under the microscope. For the viability  
46 trypan blue assay, after cell trypsinization and washing steps for 3 more times by centrifugation in 10%  
47 FBS supplemented RPMI, all the washing solutions were centrifuged in order to recovery the dead cells  
48 eventually detached present in the media. Then, all the cells were mixed together and resuspended in  
49 500  $\mu\text{L}$  of medium. 10  $\mu\text{L}$  of this cell suspension was mixed with 10  $\mu\text{L}$  of trypan blue solution, incubated  
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3 for 20 min and analyzed in the Burkner chamber to count the number of dead and alive cells. 100  $\mu\text{L}$  of  
4 the cell solution, corresponding to  $125,7 \times 10^3$  cells were dissolved in aqua regia and the iron contents  
5 were estimated by ICP-AES measurements. All measures have been performed in triplicate.  
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#### 8 9 10 **4.11 Drug loading and release**

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12 **(a) Drug loading.** To polymer functionalized nanoparticles (2 mL, 50 ppm of Fe) in PBS buffer (pH 7.4)  
13 was added doxorubicin hydrochloride (150  $\mu\text{g}$ ). The solution was placed on an orbital shaker for 24  
14 hours (1000 rpm) to load the drug within the polymer layer. The unloaded drug was removed by placing  
15 the solutions on a magnet whereupon the loaded nanoparticles sedimented and the free drug remained  
16 in solution and was drawn off using a micropipette. Fresh PBS buffer was then added to the vial with the  
17 nanoparticles in order to re-suspend them and more unloaded drug was then removed as previously  
18 described. The process was repeated (usually three to four times) until no absorption signal due to doxo  
19 in the supernatant was observed via UV spectroscopy ( $\lambda_{\text{max}}$  485 nm). For the sample used to  
20 demonstrate drug release under AMF, the amount of doxo loaded for 6 mg of iron was 2,2 mg which  
21 corresponds to 23% of doxo loading.  
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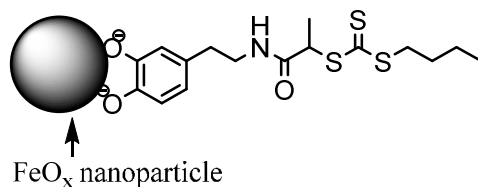
#### 29 30 **4.12 Drug release:**

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33 **(i) Drug release in thermostated water bath.** A colloidal solution of the drug loaded hybrid  
34 nanoparticles (2 mL, 200 ppm Fe) was placed in a thermostated water bath at a desired temperature  
35 (main text, figure 3A) and at desired intervals, the samples were placed on a magnet for 5 minutes to  
36 attract the hybrid nanoparticles and the supernatant was analyzed by UV spectroscopy to determine the  
37 amount released using the Beer-Lambert-Law. After each absorption measurement, the supernatant  
38 was transferred back into the original vial and the nanoparticles were completely re-dissolved and then  
39 incubated again in the water bath.  
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46 **(ii) Drug release under an alternating magnetic field.** Two samples of FeO-(PNIPAAm-co-PEGA)  
47 nanocubes loaded with doxo with an iron concentration of 3.8 g/L (1 mL) in a glass vials were placed on  
48 a magnet (0.3T) to remove any traces of unbound/leaked doxorubicin. The collected nanoparticles were  
49 re-suspended in 1 mL of fresh PBS buffer (pH 7.4) and then one sample was placed centrally in the  
50 magnetic coil of the instrument followed by application of an alternating magnetic field whilst the other  
51 sample was left at room temperature. The AMF was initially applied for 15 minutes (220 kHz, 20 kAm<sup>-1</sup>)  
52 with the temperature measuring probe dipped in solution. The sample was then taken out of the  
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3 instrument and placed on the magnet for 5 minutes to collect the nanoparticles at the bottom of the  
4 vessel. The record of the UV-VIS absorption spectra on the supernatant allows for the measurement of  
5 the amount of drug released. After analysis of the supernatant, it was returned back into the vial  
6 containing the thermo-responsive coated nanoparticles and completely re-mixed before another cycle  
7 of AMF was applied for the desired period. At the next time point, the separation was reiterated and the  
8 amount released was again measured by UV-vis spectroscopy. An aliquot of the doxo loaded sample was  
9 kept at room temperature and treated in the same way (but not subjected to an AMF) for comparison.  
10 No unspecific release was measured on this sample (see SI).  
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#### 21 **4.13 Functionalization of superparamagnetic spherical nanoparticles (spherical-IONPs) with catechol** 22 **bearing RAFT agent (CTCLRA).** 23

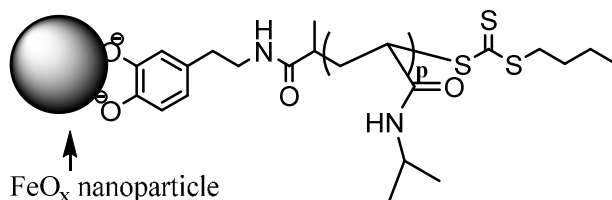


33 **Scheme 5:** Spherical-IONPs functionalized CTCLRA.  
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35 To a toluene solution of oleic acid capped spherical IONPs (4 mL, 0.6  $\mu$ M, [Fe] 1.8 mg/mL, 14 nm  
36 diameter(TEM)) was added a THF solution of CTCLRA agent (24 mmol in 45 mL THF) and then a  
37 triethylamine (3 mL in 10 mL THF) was added. The resulting colloidal solution was left on a shaker  
38 overnight after which the nanoparticles were recovered by centrifugation at 4000 rpm for 10 minutes.  
39 The collected nanoparticles were redispersed in THF and nanoparticles were washed by the excess of  
40 CTCLRA by centrifugation and re-dispersion: before centrifugation hexane was added to the vicinity of  
41 particle precipitation (the amount of hexane added was such that no particle precipitation was observed  
42 but further addition of hexane (few mL) would result in precipitation). The solution was centrifuged at  
43 4000 rpm for 10 minutes to recover the nanoparticles and the supernatant solution was decanted. This  
44 process was repeated until the characteristic yellow color of the RAFT agent in the supernatant  
45 disappeared (3-4 times) after which the nanoparticles were washed three more times. The  
46 functionalized nanoparticles were insoluble in hexane which is a good solvent for the starting  
47 nanoparticles, thus indicating a change at the NP surface. ICP (starting nanoparticles), [Fe]= 1.8 g/L, [S]=  
48 0. ICP (CTCLRA functionalized nanoparticles) [Fe]= 1.79 g/L, [S]= 0.14 g/L (based on same volume of  
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starting and CTA functionalized nanoparticles). FTIR: C=S 1061  $\text{cm}^{-1}$ , N-H 3450  $\text{cm}^{-1}$ . Initiator grafting density (9 molecules/ $\text{nm}^2$ ) was estimated using the formula  $(C_{\text{Sulfur}}/96)/(C_{\text{NPs}} \times 4\pi R_{\text{TEM}}^2)$ .  $C_{\text{sulfur}}$  is the concentration of sulfur from ICP in g/L,  $R_{\text{TEM}}$  is the calculated radius of the nanoparticles from TEM in nanometers and  $C_{\text{NPs}}$  is the concentration of nanoparticles (mol nanoparticles/L).

#### 4.14 Functionalization of superparamagnetic spherical nanoparticles (spherical IONPs) with thermo-responsive polymer.



**Scheme 6:** Spherical-IONPs functionalized with thermo-responsive polymer (PNIPAAm).

To a glass vial was added NIPAAm (4.000 g, 35.4 mmol), SUCRA (0.011 g, 32.8  $\mu\text{mol}$ ), solvent (dioxane or THF) (4 g), and CTCLRA-iron oxide nanoparticles dissolved in THF (1 mg Fe, from 0.6  $\mu\text{M}$  stock solution of NPs). The solution was vortexed for a few seconds and the vial was sealed with a rubber septum followed by degassing with nitrogen gas for 30 mins. AIBN (25  $\mu\text{L}$ , 3.28  $\mu\text{mol}$ ) was then added from a stock solution in dioxane via a microliter syringe and degassing was continued for a further 10 minutes. The reaction contents were mixed for a few seconds and the vial was placed in a thermo-stated water bath at 60  $^{\circ}\text{C}$  for 3 h. The reaction was stopped by placing the vial in an ice bath and opening it to the atmosphere. A sample for analysis by SEC was obtained by taking an aliquot of the reaction mixture (ca. 100 mg in around 100  $\mu\text{L}$  of the sample) which was diluted with THF and placed on a magnet to remove the nanoparticles prior to collection of the supernatant for analysis. The reaction mixture was precipitated into cold diethyl ether and the collected precipitate was dissolved in a small amount of THF and reprecipitated in cold diethyl ether. This process was repeated one more time and the precipitate was then dissolved in THF/diethyl ether (1:2 in volume) and centrifuged at 5000 rpm for 10 minutes. The supernatant was decanted into cold hexane where precipitation of free polymer could be observed. The precipitated nanoparticles were resuspended in THF/diethyl ether (1:2) and centrifuged again at 5000 rpm for 10 minutes and this approach was repeated 3 times with the supernatant being poured into cold hexane. The supernatant from the last wash was concentrated on the rotary evaporator and analyzed by SEC to confirm the absence of free polymer.

## Characterization techniques

**4.15 Nuclear Magnetic Resonance (NMR):** NMR analyses were done using Bruker Ultra Shield Avance spectrometers (400 MHz). For all NMR analyses, unless stated otherwise, deuterated chloroform ( $\text{CDCl}_3$ ) was used as the solvent with tetramethylsilane (TMS) as the internal standard.

**4.16 Size exclusion chromatography (SEC):** SEC was used to determine the molecular weight of the polymers. SEC analyses were carried out at 60°C using an Agilent SEC system equipped with a guard column and two Agilent PolarGel M columns (molecular weight range of 500–2 000 000 g/mol) attached to a differential refractive index (DRI) detector. The flow rate of the system was set at 1 mL/min and the eluent was DMF with 0.3% (w/v) LiBr. The SEC system was calibrated using Agilent narrow molecular weight distribution polystyrene standards.

**4.17 Dynamic Light scattering (DLS):** Particle size measurements were carried out by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer nano series instrument. An equilibration time of 3 minutes was allowed before each measurement and at least three replicate measurements were made for each sample ( $[\text{Fe}] = \text{ca. } 25 \text{ ppm}$ ).

**4.18 Zeta potential measurements:** Zeta potential measurements were carried out using a Malvern Instruments Zetasizer nano series instrument. An equilibration time of 3 minutes was allowed before each measurement and at least five replicate measurements were made for each sample ( $[\text{Fe}] = \text{ca. } 25 \text{ ppm}$ ).

**4.19 Transmission electron microscopy (TEM):** Conventional TEM images were obtained using JEOL JEM 1011 electron microscope, working with an acceleration voltage of 100 kV and equipped with a W thermionic electron source and a 11Mp Orius CCD Camera (Gatan company, USA). Samples were prepared by placing a drop of sample onto a carbon coated copper grid which was then left to dry before imaging. Frozen hydrated samples were prepared by applying a 3  $\mu\text{l}$  aliquot to a previously glow-discharged 200-mesh Quantifoil holey carbon grid (Ted Pella, USA). Before plunging the grid into liquid ethane, the grid was blotted for 1.5 s in a chamber at 4°C and 90% humidity using a FEI Vitrobot Mark IV (FEI company, the Netherlands). The particles were imaged, in both cryo-TEM and cryo-STEM mode, using a Jeol JEM 2200FS (Jeol, Japan) and a Tecnai G2 F20 microscopes (FEI company, the Netherlands), respectively. Both the microscopes were equipped with a Schottky Field Emission electron source, a

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3 US1000 2kx2k Gatan CCD camera and operated at an acceleration voltage of 200 kV. The cryo-TEM  
4 imaging was carried out under low dose condition in order to limit water sublimation from the vitrified  
5 sample and its recrystallization on the specimen surface.  
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10 **4.20 Fourier transform infra-red (FT-IR):** FT-IR spectra were recorded using a with a Bruker  
11 vertex 70v Fourier transform infrared spectrometer. Samples were prepared by placing a drop of sample  
12 onto a silicon wafer and left to dry in a desiccator at room temperature overnight before analysis.  
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16 **4.21 Turbidimetric analysis:** Turbidimetric analyses were done using a Varian Cary 5000 UV-vis  
17 spectrophotometer equipped with Peltier elements for temperature control. Measurements were done  
18 on solutions of ca. 50 ppm (Fe) and before each measurement, the sample was left to equilibrate at the  
19 desired temperature for 3 minutes.  
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24 **4.22 pH measurements:** Measurements were done using a calibrated Crison Basic 20 pH meter.  
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28 **4.23 TGA analysis:** The weight loss of the functionalised nanoparticles was determined by using a TA  
29 Instruments Hi-Res TGA 2950 thermogravimetric analyzer under a nitrogen atmosphere (60 cm<sup>3</sup>/min).  
30 The samples (2-3 mg) of the thermo-responsive coated nanocubes were heated from room temperature  
31 to 50 °C and isothermal for 15 mins then to 700 °C with the heating rate set at 5 °C/min.  
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36 **4.24 Elemental analysis:** Elemental analysis was carried out via Inductively Coupled Plasma (ICP)  
37 Atomic Emission Spectroscopy on a ThermoFisher CAP 6000 series. The samples were prepared by  
38 digesting 25 µL of sample in 2 mL of aqua regia overnight followed by dilution with milliQ water to 25  
39 mL.  
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44 **4.25 Magnetic characterization:** Magnetic characterization was carried out by using a  
45 superconducting quantum interference device (MPMS SQUID) from Quantum Design. Magnetization  
46 curves were measured from -80 to 80 kOe at 5 K and 298 K upon zero field cooling (ZFC). Zero field cool  
47 (ZFC) and field cool (FC) curves were recorded to measure the thermal dependence of the  
48 magnetization. Samples were prepared by drop casting an IONPs solution onto a Teflon tape. After  
49 evaporation, the final powder was enwrapped and measured. The amount of iron was determined by  
50 elemental analysis (ICP).  
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4 **4.26 Drug release under an alternating magnetic field (AMF):** The hyperthermia experiment  
5 was conducted using a NanoscaleBiomagnetics instrument (DM100 series) equipped with a  
6 temperaturemeasuring probe. The AMF was set at 220 kHz, 20kAm<sup>-1</sup>.  
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11 **Supporting Information Available:** <sup>1</sup>NMR characterization of succinimide RAFT agent, DLS analysis  
12 of poly (NIPAAM) functionalized IONPs in standard media, FTIR spectra and thermogravimetric analysis  
13 of nanocube sampels before and after the CTCLRA functionalization, UV-vis spectra of doxo release at  
14 various times in the absence of an AMF (room temperature) from NIPAAM-co-PEGA functionalized  
15 cubes, characterization of nanocubes having pH-responsive shell as PVP or PVP-co-PEGA and  
16 turbidimetric analysis of thermo-responsive spherical-IONP have been added in the supporting  
17 information. This information is available free of charge via the Internet at <http://pubs.acs.org/>.  
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## 25 **Acknowledgments**

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28 The authors are grateful for financial support from the European project Magnifyco (Contract NMP4-SL-  
29 2009-228622) by the Italian AIRC project (contract n. 14527 to TP), EU-ITN network Mag(net)icFun  
30 (PITN-GA-2012-290248) and by the Italian FIRB projects (Nanostructured oxides, contract no.588  
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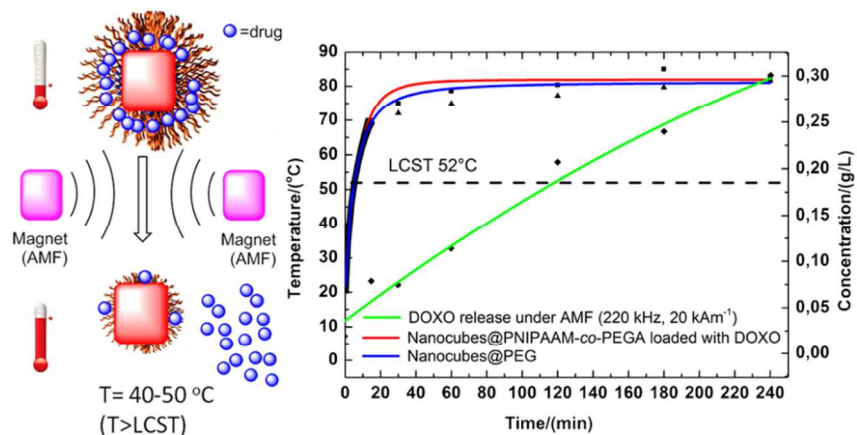
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