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# Bioconjugation studies of EGF-R targeting ligand on dendronized iron oxide nanoparticles to target head & neck cancer cells

5 **Barbara Freis<sup>1,2</sup>, María De Los Ángeles Ramírez<sup>1</sup>, Sonia Furgiuele<sup>3</sup>, Fabrice Journe<sup>3</sup>, Clémence Cheignon<sup>4</sup>,  
Loïc J. Charbonnière<sup>4</sup>, Céline Henoumont<sup>2</sup>, Celine Kiefer<sup>1</sup>, Damien Mertz<sup>1</sup>, Christine Affolter-  
Zbaraszczuk<sup>5</sup>, Florent Meyer<sup>5</sup>, Sven Saussez<sup>3</sup>, Sophie Laurent<sup>2\*</sup>, Mariana Tasso<sup>1,6</sup>, Sylvie Bégin-Colin<sup>1\*</sup>**

10 (1) Université de Strasbourg, CNRS, Institut de Physique et Chimie des Matériaux, UMR CNRS-UdS 7504, 23 Rue du Loess, BP 43, 67034  
Strasbourg, France.

(2) Laboratoire de NMR et d'imagerie moléculaire, Université de Mons, Avenue Maistriau 19, 7000 Mons, Belgique.

(3) Department of Human Anatomy and Experimental Oncology, Faculty of Medicine, Research Institute for Health Sciences and  
Technology, University of Mons (UMONS), Avenue du Champ de Mars, 8, 7000 Mons, Belgium

(4) Université de Strasbourg, CNRS, Institut Pluridisciplinaire Hubert Curien, UMR 7178, 25, rue Becquerel, 67087 Strasbourg, France.

15 (5) Inserm U1121, Centre de recherche en biomédecine de Strasbourg, 1 rue Eugène Boeckel, CS 60026, 67084 Strasbourg Cedex, France.

(6) Instituto de Investigaciones Físicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas,  
Universidad Nacional de La Plata - CONICET, Diagonal 113 y 64, 1900 La Plata, Argentina.

## 20 **Abstract**

A major challenge in nanomedicine is designing nanoplatforms (NPFs) to target abnormal cells to ensure early diagnosis and targeted therapy. Among developed NPFs, iron oxide nanoparticles (IONPs) are good MRI contrast agents and can be used for therapy by hyperthermia and as radio-  
25 sensitizing agents. Active targeting is a promising method for selective IONPs accumulation in cancer tissues and is generally performed by using targeting ligands (TL). We developed here a coupling strategy for linking a ligand targeting the epidermal growth factor receptor (EGFR) at the surface of dendronized IONPs. Several parameters were tuned to ensure a high coupling yield and to quantify the amount of TL per nanoparticle. We faced important issues during bioconjugation and the reagents and  
30 the pH were shown to be crucial parameters to consider. We succeeded in optimizing the conjugation conditions of the selected TL at the surface of IONPs and in developing an indirect method for their quantification. Head and neck cancer cell lines (FaDu and 93-VU) that overexpress the epidermal growth factor receptor (EGFR) were selected to validate two selected coupling strategies. The presence of the TL at the surface of NPFs was shown to be effective to enhance their internalization  
35 during the *in vitro* experiments.

## 1. Introduction

In nanomedicine, two main mechanisms are reported for the uptake of theranostic NPFs in tumor sites. These mechanisms are known as “passive targeting” and “active targeting”. Briefly, passive targeting occurs because the NPFs, due to their size, can pass easily through the abnormal vasculature of tumors, which generally present irregular fenestrations and poor lymphatic drainage compared to healthy tissues<sup>1-3</sup>. Through this process, certain NPFs tend to accumulate in the tumor site. This mechanism was proposed in the 1980s under the name of Enhanced Permeability and Retention effect (EPR effect)<sup>4</sup>. However, as the tumor microenvironment is so important for the EPR effect, some of the effectiveness of therapies made possible by this effect not only depends on the features of the tumor but also on the capacity of the NPFs to accumulate in high amount in these places.

Because of this limitation, a lot of research was done in order to develop active targeting. The purpose of such active mechanism is also to selectively reach abnormal tissues and to treat only them, avoiding the uptake of nanoparticles by healthy tissues. In this sense, the active targeting consists firstly in finding a cell receptor overexpressed in the type of cancer of interest<sup>2,3,5-8</sup>. Once the suitable overexpressed receptor is defined, a specific molecule, often named targeting ligand (TL) selected to interact with the cell receptor, can be used to deliver the nanoparticles directly to this site. Among the most used receptors are, e.g., the folate receptor, which can be targeted with folic acid on the surface of NPFs<sup>9</sup>; or the Human Epidermal Growth Factor Receptor 2 (HER2+) which is recognized by the monoclonal antibody Trastuzumab<sup>10</sup>. Peptides, nano-bodies, affibodies, proteins, small organic molecules or nucleic acids<sup>2,3,11-15</sup> constitute examples of short TL. The large antibodies or the smaller versions of them, the fragment antigen-binding (Fabs), are also common TL<sup>16</sup>.

These targeting ligands are thus generally coupled to drug or at the surface of functionalized nanoparticles. Iron oxide nanoparticles (IONPs) are among the most developed and promising NPFs at the moment for their use as contrast agent for magnetic resonance imaging (MRI) and for hyperthermia therapies.<sup>2,3,5-8,17,18</sup> They are usually accumulated in tumors without TL via intratumoral injection or passive targeting as a result of the EPR effect. Another way to increase their accumulation is to perform a magnetic targeting, in which a magnetic field gradient guides IONPs towards the tumor. The main drawback of this technique is the requirement of a magnet that should be positioned easily near the tumor, thus limiting this strategy to specific cases. Active targeting studies have been also conducted but *Wilhem et. al.* observed that just 0.7% (median) of the administered nanoparticle dose (with or without TL) was found to be delivered to solid tumors after reviewing the literature from the previous 10 years<sup>18</sup>. Similar low percentages of internalized IONPs were reported also by *Alphandery*<sup>17</sup>. Therefore, one challenge today is the design of functionalized IONPs bearing TL able to specifically target diseased organs without a too strong accumulation in the reticulo endothelial system. Among the characteristics that can be tuned in such IONPs to ensure their high accumulation,

75 we can mention the kind of the organic coating, the size and nature of the TL, its hydrophobicity/hydrophilicity balance, the final mean hydrodynamic size and the surface charge, as the most relevant. In our case, we have developed IONPs with a mean diameter of 10 nm coated with polyethylene glycol (PEG)-based dendron molecules (dendronized IONPs: DNPs), which were demonstrated in several *in vitro* and *in vivo* studies to ensure antifouling properties to dendronized IONPs<sup>19-27</sup>. Thus, these DNPs, which display a favourable *in vivo* biodistribution and bioelimination profile, are well adapted for investigating affinity targeting. Coupled to melanin TL, DNPs were shown to selectively target melanin granules or hypoxia states after i.v. injection<sup>28,29</sup>. Even if specific targeting was evidenced in these studies, the internalized amount in tumors stayed low and the amount of TL was not optimized. Therefore, these DNPs displaying a particularly good biodistribution, without a significant uptake in healthy tissues and showing potential for active targeting, are useful NPFs to answer some important questions related to the targeting process.

85 The choice of TL is challenging as it must target overexpressed receptors at the surface of cancer cells. Moreover, the size of the TL must be suitable to the mean size of IONPs. Furthermore, the NPF-immobilized TL needs to conserve its recognition capacity towards its target, a fact that imposes *sine qua non* conditions to the chosen bioconjugation approach to avoid the involvement of the active site in the coupling. In the case of short TL, a major concern is to avoid abrupt changes in the 3D conformation of the molecule (upon conjugation to the NPFs) that may alter its interactions with the cellular receptors. Lastly, the TL must remain fully exposed at the surface of the IONPs and not buried inside the polymer ligand.

95 In this work, our aim is to target head and neck cancer (HNC) cells with functionalized DNPs. The chosen cancer model for this work was HNC, using the FaDu cell line derived from a hypopharyngeal carcinoma and the 93-VU line from an oral cavity carcinoma. Both cell lines have elevated levels of the epidermal growth factor receptor (EGFR).<sup>30</sup> This receptor, which belongs to the HER/ErbB family of receptors tyrosine kinases (RTKs), is overexpressed in 90% of head and neck squamous cell carcinoma (HNSCC)<sup>31-33</sup>. It has been demonstrated that EGFR has a strong role for tumorigenesis, the higher its expression, the more resistance to therapies and the poorer outcome for patients are evidenced<sup>31</sup>. Some therapies were developed to inhibit the EGFR with the use of anti-EGFR antibodies, like Cetuximab, which, up to now is the only approved targeted drug for HNSCC.<sup>34</sup> However, its efficacy remains limited due to unresolved resistance mechanism of the tumor.<sup>35</sup> As an alternative to antibodies, peptides appear as a good substitute, they have high penetration into solid tumor tissues and limited immunogenic potential<sup>36,37</sup>. Additionally, peptides are small, simple to manufacture and easy to adapt to several bioconjugation approaches by the addition of specific amino acid residues, such as lysines, or terminal tags, such as the Histidine tag. During the conjugation on DNPs bearing a peripheral carboxylate group, the amount of peptide can be tuned to cover a desired surface of the DNPs. Peptides with small molecular weight and high EGFR affinity have been the

110 focus of research for active targeting agents combined with drug delivery systems. The dodecapeptide  
GE11 (YHWYGYTPQNVI) has been shown to have good affinity for EGFR<sup>38</sup>. Many analogs of  
GE11 peptide were synthesized to test their binding affinity to triple negative breast cancer line  
overexpressing EGFR and among 29 of them, Hossein-Nejad-Ariani *et al.* demonstrated that the  
peptide analogue 22 (YHWYGYTPENVI) (P22) shows several fold higher uptake compared to the  
115 lead peptide GE11 when evaluated in solution and in *in vitro* assays<sup>39</sup>. This fact makes P22 an  
interesting candidate to target other types of cancers with overexpression of EGFR.

As the EGFR inhibition strategy was not efficient enough, the objective here is to specifically  
internalize DNPs bearing the targeting ligand in HNC cell lines by taking advantage of their EGFR  
overexpression and to use the therapeutic properties of IONPs based on magnetic hyperthermia or  
120 photothermia<sup>25,40,41</sup>

There is a wide spectrum of bioconjugation reactions<sup>42</sup> used to attach TL at the surface of  
functionalized IONPs and; globally they can be divided into two types: covalent conjugations or non-  
covalent conjugations. To the first group, belong the carbodiimide chemistry<sup>43</sup>, Michael addition<sup>44,45</sup>,  
click Chemistry<sup>46-48</sup>, or Diels-Alder cycloaddition, among others<sup>3,49</sup>. In non-covalent conjugation, the  
125 most common are carried out by electrostatic interactions, metal affinity coordination or biotin-avidin  
interaction<sup>50</sup>. As DNPs possess carboxylate functions at their periphery, the coupling of P22 on DNPs  
surface was achieved using carbodiimidation reactions between a free carboxylate group present at the  
extremity of the dendron molecules, and a primary amine of P22, resulting in the formation of an  
amide bond.

130 Therefore, in this work, we have optimized the coupling reaction of P22 at the DNPs surface and  
studied the impact of the coupling strategies on the amount of coupled TL and on the cell  
internalisation yield of DNPs bearing TL. During these investigations, we confirmed the generally  
acknowledged importance that should be conferred to the purity and storage conditions of the reagents  
involved in this reaction (particularly EDC and sulfo-NHS), as well as the significant impact of  
135 carefully controlling the pH during the different steps of the coupling.

We have taken care also in validating the presence of TL at the surface of IONPs and in quantifying it.  
Commonly, the presence of TL at the surface of IONPs may be checked by FTIR spectroscopy or  
thermogravimetric analysis if the amount of TL is high enough, but most often, it is confirmed by the  
observation of an increase in the hydrodynamic size or a change of the zeta potential values. Hence,  
140 the most challenging step is the quantification of the amount of TL at the surface of IONPs<sup>2,6,14,51</sup>. This  
quantification can be done by an indirect way, analysing the amount of uncoupled TL, or directly by  
fluorescence spectroscopy, for example, when a fluorescent TL is used. The presence of the P22 at the  
surface of the DNPs was verified by zeta potential changes and by the increase in hydrodynamic size.  
The P22 coupled amount was quantified by an indirect method based on the quantification of the  
145 unbound peptide by HPLC-UV (between 40% and 50% coverage of dendrons). The specific

internalization of these optimized DNPs bearing the P22 targeting ligand by the two HNC cell lines was then studied and discussed.

## 2. Experimental

### 150 2.1 Materials

Iron stearate ( $\text{FeSt}_3$ ) was purchased from TCI (purity min 60% of stearic acid, 5.8 to 7% of iron and maximum 10% of free acid),  $\text{FeSt}_2$  was home made. Acetone (purity 99.8%), chloroform (purity 99%) and THF (purity 99.5%) were purchased from Carlo Erba. Dioctyl ether (OE) (purity 99%), HEPES buffer (purity 99.5%), N-hydroxy sulfosuccinimide (sulfo-NHS, purity 98%), sodium carbonate ( $\text{Na}_2\text{CO}_3$ , purity 99.5%) and thiazolyl blue tetrazolium bromide (MTT, purity 98%) were purchased  
155 from Sigma Aldrich. Peptide 22 (P22) (purity 98.7%) was purchased from Polypeptide. Sodium hydrogen carbonate ( $\text{NaHCO}_3$ , purity 99.5%) was purchased from Bioreagent. 1-Ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl, purity 99%) was purchased from Carl Roth. Oleic acid (OA, purity 99%) was purchased from Alfa Aesar. Dimethyl sulfoxide (DMSO,  
160 purity 99%) was purchased from Fisher Scientific. The dendron D1-2P was provided by Superbranche. All reagents for cell culture: cell medium (DMEM (Dulbecco's Modified Eagle's Medium)) High Glucose with Glutamine and Sodium Pyruvate), PBS 1X, Penicillin/Streptomycin 100X (P/S), trypsin-EDTA and Foetal Bovine Serum (FBS) were purchased from Dominique Dutscher. Cell lines FaDu and 93-VU were donated by the Department of Human Anatomy and Experimental Oncology from the  
165 University of Mons (FaDu provided from ATCC (ATCC® HTB43™) and 93VU-147T from the University Medical Centre of Amsterdam (Dr. de Winter).

### 2.2 Synthesis of 10 nm IONPs

Iron oxide nanoparticles were synthesized by thermal decomposition of an iron stearate precursor<sup>52</sup> in  
170 an organic solvent with a high boiling point and a surfactant<sup>53-55</sup>. 2.2 mmol of  $\text{FeSt}_3$  (1.99 g) or  $\text{FeSt}_2$  (1.44 g), 4.4 mmol of OA (1.24g) were mixed with OE (16.2 g, 20 mL) in a two-neck round-bottom-flask of 100 mL. The mixture was first heated to 120°C (heating device temperature: 130°C) for 60 min to dissolve the reagents in OE. The condenser was then connected to the flask and the mixture was heated up to 290°C (heating device temperature: 320°C) with a 5°C/min ramp. The mixture was  
175 refluxed at 290°C for 120 min. The obtained black NPs suspension was cooled down to 100°C to proceed to the washing steps.

### 2.3 Washing

10 mL of chloroform were added to the IONPs suspension at a temperature around 100°C and the  
180 suspension was introduced in a flask containing 400 mL of acetone. This mixture was heated at 60°C for 1 h under mechanical stirring using a thermal bath. The IONPs were collected with a magnet and

the supernatant was discarded. The collected IONPs were re-dispersed in 50 mL of chloroform. 400 mL of acetone were added again to proceed to a second washing step: the mixture was once more heated at 60°C for 45 min under mechanical stirring. At the end, the IONPs were collected with a magnet and the supernatant was discarded. Finally, the IONPs were re-suspended in 40 mL of THF for their storage or further utilization.

## 2.4 Dendronization of IONPs

The ligand exchange between OA and the dendron (D1-2P) was performed in THF<sup>23</sup>. Briefly, 5 mL of IONPs@OA at 1 mg Fe/mL were stirred for 24 h with 7 mg of dendron. To maximize the ligand exchange and remove most of oleic acid molecules<sup>56</sup>, the suspension was then purified by ultrafiltration. 5 mg of dendron were added to the mixture for another 24 h of stirring. The suspension was mixed with hexane (volume ratio 1/3:2/3) to precipitate the DNPs. The suspension was centrifuged at 8000 rpm 5 min and the supernatant was discarded. Finally, the DNPs were collected and dispersed in deionized water. The water suspension was purified by ultrafiltration 3 times.

## 2.5 Coupling reaction

The DNPs suspension was initially buffered-exchanged with HEPES 0.1 M, pH 6.5 in a centrifugal filter unit (Amicon®, MW cut off: 30 kDa; 10 min at 8000 rpm), the filtrate was suspended in the appropriate volume to reach a final concentration of 5 mg Fe/mL in HEPES. The suspension was mixed with EDC (20 molar excess compared to carboxylate groups present on dendron molecules) during 10 min and sulfo-NHS (20 molar excess compared to carboxylate groups present on the dendron molecule) was added during additional 20 min. Upon the sulfo-NHS addition, the volume was increased to reach a final iron concentration of 0.5 mg/mL in HEPES buffer. The activated DNPs were rinsed with 0.1 M carbonate buffer, pH 9.2, in a centrifugal filter unit (10 min at 8000 rpm) to eliminate excess EDC, sulfo-NHS and other possible reaction intermediates. The filtrate was rapidly recovered and mixed with various P22 molar equivalents (compared to dendron molecule) in carbonate buffer. The volume of the reaction was completed with carbonate buffer to reach a final iron concentration of 0.5 mg/mL. The reaction was let overnight at room temperature under magnetic stirring. At the end, the supernatant was recovered by centrifugation (10 min at 8000 rpm) in centrifugal filter units (Amicon®, MW cut off: 30 kDa). Three washes with milliQ water were done and the washes recovered for unbound P22 quantification.

## 2.6 Characterization techniques

### 2.6.1 TEM

The IONPs were characterized by transmission electron microscopy (TEM) with a JEOL 2100 microscope operating at 200 kV (point resolution 0.18 nm). The size distribution of NPs was estimated from the size measurements of more than 300 nanoparticles using ImageJ software.

## 220 2.6.2 X-Ray Diffraction

The X-ray diffraction (XRD) patterns were collected at room temperature with a Bruker D8 Discover diffractometer in Bragg Brentano geometry equipped with a monochromatic copper radiation source ( $K\alpha_1 = 0.154056$  nm) and an energy resolved Lynx-Eye XE-T detector in the  $25\text{--}65^\circ$  ( $2\theta$ ) range with a scan step of  $0.03^\circ$ . High purity silicon powder ( $a = 0.543082$  nm) was used as an internal standard.

225 The diffraction patterns were refined by LeBail's<sup>57</sup> method using the Fullprof software<sup>58</sup>. The background, modeled as a linear function based on 20 experimental points, was refined, as well as the zero shift. The peaks were modeled with the modified Thompson-Cox-Hasting (TCH) pseudo-Voigt profile function.

## 230 2.6.3 FTIR analysis

The ligand exchanged was confirmed with standard infrared spectra recorded between  $4000$  and  $400$   $\text{cm}^{-1}$  with a Fourier Transform infrared (FTIR) spectrometer, Spectrum 100 from Perkin Elmer. Samples were ground and diluted in a non-absorbent KBr matrix before their analysis.

## 235 2.6.4 Dynamic Light Scattering and Zeta Potential

Dynamic Light Scattering measurements were performed on a MALVERN (nano ZetaSizer) equipment to assess the colloidal stability of the IONPs suspension in water and to determine their mean hydrodynamic diameter ( $D_h$ ) after ligand exchange. The zeta potential was also recorded using the same equipment. The coupling of the TL was confirmed by an increase of  $D_h$  and a change in the  
240 zeta potential.

## 2.6.5 High Resolution Magic-Angle Spinning NMR (HRMAS NMR)

HRMAS NMR experiments were conducted on a Bruker FT-NMR Avance 500 equipped with an 11.75 T superconducting ultra-shield magnet. All the experiments were performed at a spinning rate of  
245 5 kHz with a  $50$   $\mu\text{L}$  zirconium rotor. This technique was used to confirm the presence of the TL at the surface of the IONPs.

## 2.6.6 Nuclear magnetic resonance dispersion (NMRD) profiles

Proton NMRD profiles were recorded using a Stellar Fast Field Cycling relaxometer (Mede, Italy). The  
250 system operates over a range of magnetic fields extending from  $0.25$  mT to  $0.94$  T ( $0.01\text{--}40$  MHz) at  $37^\circ\text{C}$ .  $T_1$  and  $T_2$  measurements were performed on a Bruker Minispec mq60 (Karlsruhe, Germany) working at a Larmor frequency of  $60$  MHz ( $1.41$  T) at  $37^\circ\text{C}$ .

## 2.6.7 High Performance Liquid Chromatography-UV spectroscopy (HPLC-UV)



255 HPLC-UV spectrophotometry analysis was performed on an Agilent 1100 system equipped with a  
Diode-Array Detector (DAD). 10  $\mu$ L of samples (supernatant and washes obtained after the coupling  
reaction for P22 quantification), were injected onto the column (Zorbax Extend-C18, 150 x 4.6 mm,  
3.5  $\mu$ m, Agilent) at room temperature. Ultrapure water, HPLC grade solvents and additives were used  
for the analysis. The elution gradient was carried out with trifluoroacetic acid (TFA) 0.1% (mobile  
260 phase A) and acetonitrile acidified with TFA 0.1% (mobile phase B) at a flow-rate of 1 mL/min. The  
mobile phase gradient was programmed with the following time course: 10% mobile phase B at 0 min,  
linear increase to 60% B at 15 min, held 1 min, linear decrease to 10% B at 17 min and held 7 min.  
The DAD was used as detector, with simultaneous detections at  $220 \pm 5$  nm and  $280 \pm 5$  nm.  
Quantification was realized by integration of the peak of the chromatogram corresponding to the  
265 analyte of interest compared to the integration calculated from a calibration curve of a standard  
solution of P22 (Figure S1).

### 2.7 Cell lines

FaDu (hypopharyngeal carcinoma) and 93-VU (oral cavity carcinoma) were cultured in DMEM  
270 medium supplemented with 10% FBS, 1% P/S and 2% L-Glutamine (200mM). They were cultured in  
an incubator at 37°C with 5% CO<sub>2</sub> under fully humidified conditions.

### 2.8 Cell viability assay

Cell viability assays were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-  
275 diphenyltetrazoliumbromide] assay. FaDu or 93-VU cells were seeded in 96 well plates at a density of  
 $10^4$  cells/well. After 24 h, the medium was taken out and DNPs in fresh medium were added at  
different concentrations (from 0  $\mu$ g/mL to 400  $\mu$ g/mL Fe) together with a negative control with 100  
 $\mu$ L DMSO. The cells were growth 24 h at 37 °C and 5% CO<sub>2</sub> to ~80% confluence. After 24 h the  
medium was retrieved, and the cells were washed twice with PBS and incubated with 100  $\mu$ L of a  
280 MTT solution (0.5 mg/mL in cell medium). The plates were incubated for 3 h at 37°C and 5% CO<sub>2</sub>,  
then the MTT solution was taken out and 100  $\mu$ L of DMSO were added to each plate to dissolve the  
crystals under orbital shaking. The absorbance was read in a microplate reader (Xenius XC  
spectrophotometer (SAFAS, Monaco)) at 570 nm. The cytotoxicity was expressed as a percentage of  
cell viability compared to the control cells that were not exposed to the DNPs.

285

### 2.9 Iron uptake

The internalization study of DNPs and DNPs@P22 into cells at a concentration of 100  $\mu$ g/mL was  
measured using the well-known Prussian Blue reaction developed by *Boutry et al*<sup>59</sup>. FaDu or 93-VU  
cells were seeded in a 6-well plate and incubated 24 h at 37 °C and 5% CO<sub>2</sub> for cells' attachment to  
290 reach 80% confluence in 24 hrs. The next day, the medium was replaced with fresh medium  
containing 100  $\mu$ g/mL of suspension with DNPs or DNPs@P22 for 24 h. Then, cells were trypsinized,

recovered gently from the wells in a 1:2 volume ratio of trypsin to cell medium and then counted in a hemacytometer. After cell counting, cells were pelleted by centrifugation and washed three times with PBS. The final cell pellet was lysed with 5 N HCl for 24 h at 37°C. HCl also digested the cell-internalized DNPs leading to Fe<sup>3+</sup>. The digested solution was finally put in contact with potassium ferrocyanide trihydrate, a salt which will react with Fe<sup>3+</sup> to form a colored complex: the Prussian Blue dye. Furthermore, 100µL of suspension of DNPs at various concentrations were digested in the same conditions as the cell pellets to build a calibration curve (0 to 0.3 µmol/mL Fe). (Figure S2). All suspensions were let under orbital stirring for 15 min, and the absorbance was read in a microplate reader (Xenius XC spectrophotometer (SAFAS, Monaco)) at 630 nm. In this way, the amount of iron internalized per cell could be determined.

### 3. Results

#### 3.1 Characterization of IONPs

IONPs were synthesized by the thermal decomposition method using a standard established protocol<sup>53-55</sup> and were characterized structurally. TEM images gave information on the shape and size distribution of IONPs (Figure 1 A), showing that they are spherical with a monomodal size distribution and a mean diameter of  $12.8 \pm 1.8$  nm (Figure 1B). X-Ray Diffraction pattern (Figure 1C) displays the characteristic XRD peaks of an iron oxide spinel structure and the lattice parameter calculated from this XRD pattern thanks to profile matching on FullProf (Figure S3) is of  $8.37 \text{ \AA}$ , which is, as expected, intermediate between those of the magnetite and maghemite phases ( $8.395 \text{ \AA}$  and  $8.347 \text{ \AA}$ , respectively)<sup>53</sup>.

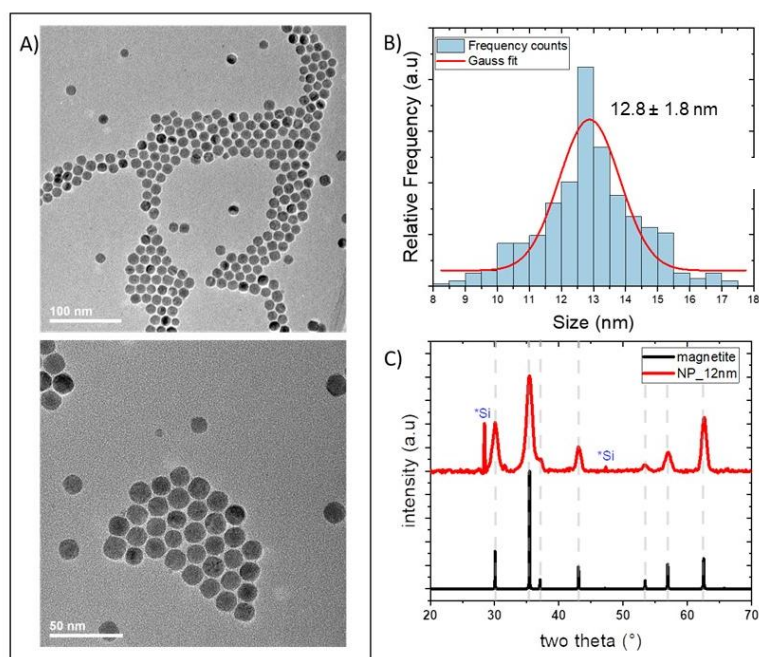


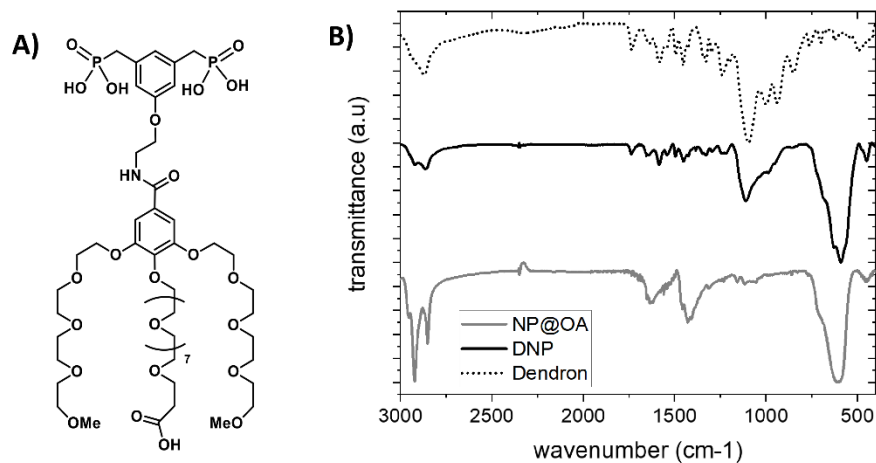
Figure 1 : A) TEM images of IONPs, B) Size distribution determined from TEM images and C) bulk magnetite and IONPs XRD pattern (\*Si (silicon) is an internal reference).

### 3.2 Coating of IONPs with dendron molecules (dendronization)

After the synthesis, IONPs remain coated with hydrophobic oleic acid ligands. Dendronization is thus an essential step to obtain stable colloidal suspensions of IONPs in water, this procedure occurs by a ligand exchange process<sup>23</sup>. The chemical formula of the dendron molecule is given in Figure 2A: the dendron bears two phosphonate groups at one side for a strong anchoring of the dendron at the surface of IONPs<sup>23,27,56</sup> and three PEG chains at the opposite side with the central one being longer and ended with a carboxylate function. This free carboxylate group is used to couple the TL, fluorophores, or molecules of interest at the surface of DNPs.

FTIR analysis has confirmed the dendronization of IONPS. In Figure 2B, the comparison of IR spectra of the dendron, of IONPs coated with oleic acid and of DNPs showed the presence, in the DNPs IR spectrum, of the bands of PEG (C-O-C bond) chains of dendron around 1100 cm<sup>-1</sup> and a modification of the phosphonate bands in the wavenumber range 1100-800 cm<sup>-1</sup> (proving the anchoring of dendrons through the phosphonate groups)<sup>23</sup>. This confirmed the efficacy of the ligand exchange process. The presence of the dendron and of the low remaining amount of oleic acid was also demonstrated by HRMAS as can be seen in Figure S4 showing HRMAS spectra of IONPs, DNPs and the dendron molecules. **Indeed, the presence of two phosphonate groups used to anchor the dendron at the surface of IONPs has been shown effective to ensure a low amount of remaining oleic acid<sup>56</sup>.**

As the final objective is to use these DNPs in *in vitro* or *in vivo* studies, it is crucial to control this ligand exchange to preserve a good hydrodynamic size distribution with a mean hydrodynamic size smaller than 100 nm. The hydrodynamic size distribution after the dendronization step is monomodal with a mean hydrodynamic size of 18.7 nm. The surface density deduced from elemental analysis is about of ca. 1.4 dendron/nm<sup>2</sup> in agreement with the expected value<sup>60</sup> which corresponds to ~700 dendron molecules per DNP.



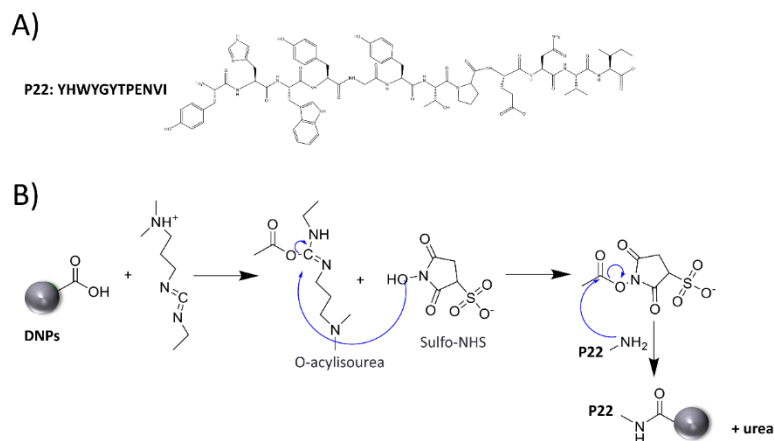
340

Figure 2 A) Scheme of the dendron molecule, B) FTIR spectra of the dendron (black dotted spectrum), of IONPs coated with oleic acid (grey spectrum) and of DNPs (black spectrum).

### 3.3 Coupling reaction of TL on the peripheral carboxylate groups of dendronized IONPs

345

A carbodiimidation reaction was done to conjugate the TL (P22 molecule: Figure 3A) at the DNP surface. The typical reaction proceeds in two steps as shown in Figure 3B.



350

Figure 3 A) Chemical structure of P22 B) Carbodiimide reaction: first step: activation of the carboxylate groups at the DNP surface. Second step: amide bond formation between the active carboxylates and primary amine groups of the P22.

Several protocols were carried out with the aim of finding the optimal conditions to do the coupling reaction between the TL (P22) and the dendron molecules at the surface of the DNPs. Overall, five

355 different protocols were carried out (protocols A to E) and the suspensions obtained are called  
 respectively: DNP@P22-A, DNP@P22-B, DNP@P22-C, DNP@P22-D, DNP@P22-E. They are  
 described in Table 1 for 0.6 molar equivalents of P22. During the optimization of the coupling  
 protocol, we noticed how relevant it is to pay attention to the stability of the EDC over time. We saw  
 that after 3 months of opening the bottle several times, the EDC started to hydrolyze with ambient  
 360 water. After that, we bought a new EDC and stored it at -20°C in an inert atmosphere. The opening of  
 the bottle is also a crucial point: the bottle must be back at ambient temperature before opening it to  
 avoid rapid hydrolysis of the compound.

Table 1: From A to E, five different conditions evaluated for the carbodiimidation reaction between  
 365 the carboxylate group of the dendron and the P22 TL.

	<b>DNPs + P22 A</b>	<b>DNPs + P22 B</b>	<b>DNPs + P22 C</b>	<b>DNPs + P22 D</b>	<b>DNPs + P22 E</b>
<b>Coupling conditions</b>	EDC only (molar ratio compared to dendron 20:1)	EDC only (molar ratio compared to dendron 20:1)	EDC only (molar ratio compared to dendron 20:1)	EDC + sulfo- NHS (molar ratio compared to dendron 20:1)	EDC + sulfo- NHS (molar ratio compared to dendron 20:1)
	In milliQ water	In milliQ water	In milliQ water	In milliQ water	HEPES and carbonate buffer
	No pH control	No pH control	pH controlled with NaOH (rise to 9 after P22 addition)	pH controlled with NaOH (rise to 9 after P22 addition)	pH controlled with buffers
	No washing after EDC addition	Addition of a washing step after EDC activation	Addition of a washing step after EDC activation	Washing step after EDC + s- NHS activation	Washing step after EDC + s- NHS activation

At the beginning, both (activation and coupling) steps were conducted in water without precise pH  
 control (e.g., protocols A and B), but we did not see clearly neither the appearance of characteristics  
 peaks by FTIR spectroscopy, nor high changes in hydrodynamic size ( $D_h$ ) by DLS or zeta potential  
 370 value variations. Nevertheless, with the protocol A by comparison with protocol B, slight variations  
 are observed suggesting some P22 coupling (Table 3). Indeed, as the P22 molecule is not a big  
 molecule, it is not so easy to confirm its presence by DLS measurements (Figure 4). For the one pot  
 protocol A, some aggregates were sometimes observed (higher  $D_h$  in Figure 4), **that we suspected to  
 be due to** a copolymerization of P22 as no wash was performed between the two steps. Indeed, EDC

375 was not removed before adding P22, it may also activate the carboxylate groups present on the peptide and therefore lead to polymerization between peptides molecules. In fact, P22 bears carboxylate and amine functions, which can interact between them if they are also activated as allowed by the protocol A.

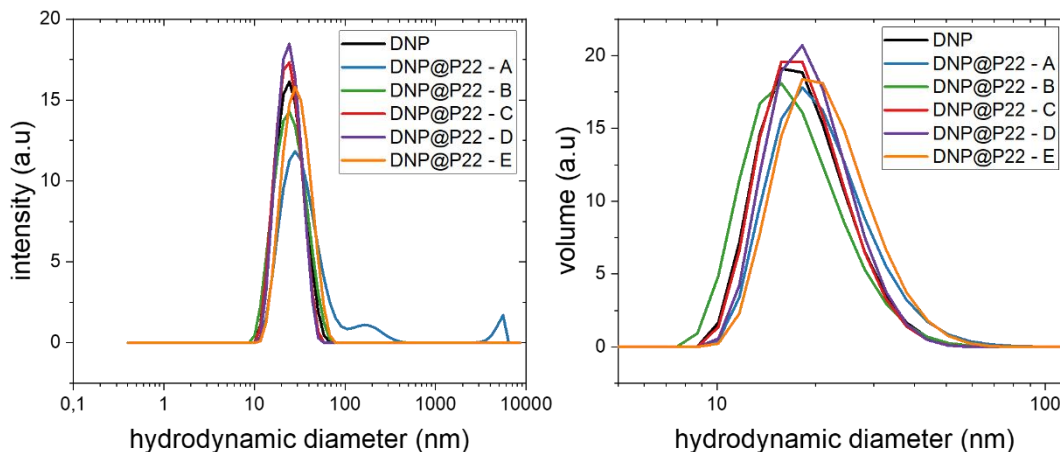
The consideration of the reported pH used for some EDC/NHS reactions in published papers (Table 2) evidenced strong discrepancy in the pH values. The pH was either in the range 5-6 or 8-10 for both activation or coupling reactions. In addition, the coupling was reported at room temperature or assisted by microwave. After considering carefully, the two steps of the reaction, discussing with organic chemists, going back to the main literature sources and after performing several trials, we finally established that the activation and coupling reactions had to be performed at different pH ranges 5-6 and 8-10 respectively.

Table 2. Reported pH range for the carbodiimidation reaction according to the literature

Reference	Solvent	Molecules	EDC	NHS	sNHS	pH	time	Temp	pH	time	Temp
Arriorta, 2018 – Biointerfaces <sup>61</sup>	Sodium tetraborate 50 mM	cRGD + Iron oxide NPs	Yes	-	-	9	2 h	RT		2h	35 C
Han, 2022 – Biomaterials <sup>62</sup>	water	cRGD + NPs	Yes	Yes	-	no info	4 h	RT	no info	24 h	RT
Wang, 2019 – Acta Biomaterialia <sup>63</sup>	Dry DMSO	cRGD + NPs	Yes	Yes	-	-	24 h	RT			
Hermanson, 2008 - Bioconjugate techniques 2 <sup>nd</sup> ed <sup>64</sup>	MES 0.1 M	Proteins + amines	Yes	-	-	4.7	2 h	RT			
Richard, 2017 – Biochimica et biophysica acta <sup>65</sup>	-	cRGD + IONPs	Yes	Yes	-	4	no info	no info	9	3 cycles 5'	65 C MW
Bolley, 2013 – Nanoscale <sup>66</sup>	-	cRGD + IONPs	Yes	Yes	-	4	no info	no info	9	3 cycles 3'	65 C MW
Bolley, 2013 – Langmuir <sup>67</sup>	water	cRGD + IONPs	Yes	Yes		4	2 h	RT	9	overnight	RT
Panahifar, 2013 – Applied Materials Interfaces <sup>68</sup>	PBS 50mM	IONP + Alendronate	Yes	Yes		7	30 min + (2 h washing by dialysis)	RT	7	3 h	RT
Walter, 2015 – J.Mater Chem B <sup>21</sup>	water	IONP + Alexa dye	Yes			No info	30min			1 h after addition of dye	
Perton, 2019 – Applied Materials <sup>69</sup>	water	Stelate mesoporous silica + quantum dots	Yes	Yes		7	One pot (4hrs)				
Sun ; 2006 – Molecular imaging <sup>70</sup>	MES 0.1 M / DMSO		Yes	Yes	-	6	3 h				
Bartczak, 2011 – Langmuir <sup>71</sup>	Sodium borate	peptide + Gold NPs	Yes	-	Yes	9	24 h	RT	One pot		
Jia, 2021 – Food Chemistry <sup>72</sup>	PBS 0.01 M	NPs + polylysine	Yes	Yes	-	no info	1 h	RT	no info	12 h	45 C
Morade, 2021 – Int Journal of Biological Molecules <sup>73</sup>	MES 0.1 M	NPs + Ab	Yes	Yes	-	6.2	2 h	RT	6.2	4h	no info

Han, 2020 - Journal of Materials Chemistry <sup>74</sup>	PBS 0.01 M	SPIONs + dye	Yes	-	Yes	7.2	10 min	no info	7.2	3 h	25 C
Saha, 2020 – MDPI <sup>75</sup>	PBS 0.01 M	NPs + Ab	Yes	Yes	-	no info	90 min	RT	no info	2h 30'	RT
Wen, 2019 - Colloids and surfaces, Biointerfaces <sup>76</sup>	DMF	CDs + IONPs	Yes	Yes	-	-	1 h	RT		24 h	RT
Bordeianu, 2018 - Molecular Pharmaceutics <sup>28</sup>	water	NPs + NH <sub>2</sub> -DOTA	Yes	-	-	6.5	30 min	0 C	6.5	2 h	RT
Totaro, 2016 - Bioconjugate Chemistry <sup>77</sup>	MOPS 50 mM + NaCl 50 mM	Peptides	Yes	-	Yes	7.1	20 h	RT	On e pot		
Lee, 2008 - Journal of Nuclear Medicine <sup>78</sup>	no info	NPs+DOTA +linker	Yes	-	Yes	5.5	30 min		8.5	1 h	4 C
Hermanson, 2008 - Bioconjugate techniques 2nd ed <sup>64</sup>	MES 50 mM	NPs -COOH + R-NH <sub>2</sub>	Yes	-	Yes	6	2-4 h	RT	On e pot		
Hermanson, 2008 - Bioconjugate techniques 2nd ed <sup>64</sup>	MES 50 mM	NPs -COOH + R-NH <sub>2</sub>	Yes	-	Yes	6	15 min	RT	6/9	2-4 h	RT





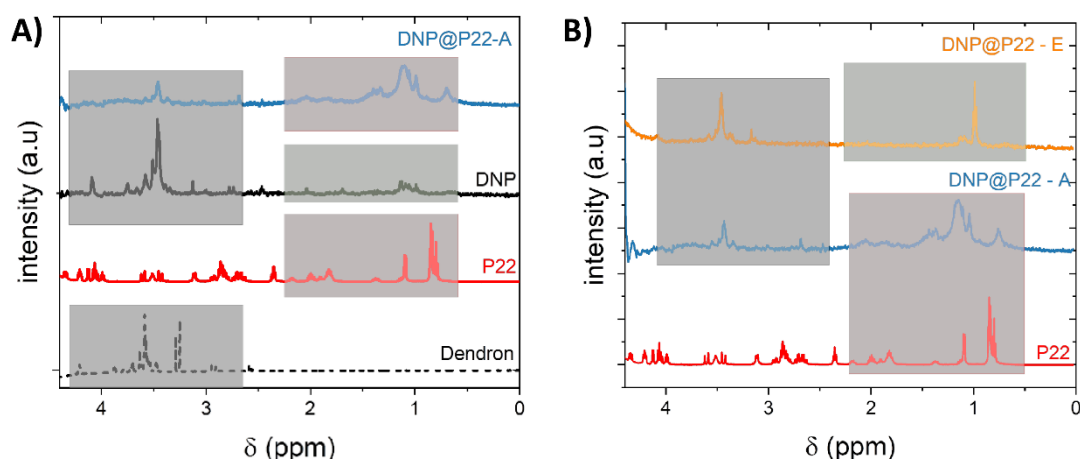
390 *Figure 4: Mean hydrodynamic size distribution measured by DLS after the coupling reactions for the different protocols with 0.6 molar equivalent of P22 compared to the carboxylate groups present on the dendron molecule. A) Intensity distribution of the  $D_h$ , B) Volume distribution of the  $D_h$*

Among all the protocols in which we carefully considered the pH conditions (C to E, Table 1), protocol E is the one leading to higher variations in  $D_h$  and zeta potential (Table 3). Protocol D results in higher variations in  $D_h$  and zeta potential than protocol C, showing that sulfo-NHS was an important reagent for our coupling conditions. Nevertheless, one can see that the variations with protocol D are close to those of protocol A. In any case, protocol E displayed the highest change in zeta potential value toward negative values, together with a meaningful change in the mean  $D_h$ . Basically, working in buffer solutions (protocol E) instead of water (protocols A, B, C and D), we were able to better control the pH conditions in both steps. An acidic pH of 6.5 for the carboxylate activation, with the addition of EDC first, alone for 10 min and in concentrated conditions (both for EDC and for the DNPs), followed by the addition of sulfo-NHS for 20 additional minutes were the optimal conditions for the first step. As expected, the absence of EDC washing out prior the addition of the peptide can be linked to a potential undesired copolymerization of the peptide. The overall time for the activation step was kept low (30 min), and the temperature at 24°C. After thorough washing of unreacted EDC, sulfo-NHS and reaction products, with a buffer at higher pH than for step 1, samples were quickly recovered in the least possible volume and exposed to the peptide. For this second step, we increased the pH until 9.2 where we are close to the pKa of primary amines.

405

To favour a high coupling yield, the peptide molar ratio to the carboxylate groups in the dendron was varied and the system was stirred overnight at room temperature. We have tested three different P22 amounts: 0.25, 0.6 and an excess with 1.2 molar equivalents of P22 with respect to the number of activated carboxylates present on the DNPs. Most experiments have been performed with the molar ratio 0.6.

410



415 *Figure 5: A) comparison of HRMAS spectra of (from top to bottom): DNP@P22-A (blue), DNP (black), P22 (red) and dendron (black dots), B) Comparison of HRMAS spectra of (from top to bottom): DNP@P22 – E (orange), DNP@P22 – A (blue) and P22 (red).*

In the HRMAS spectra, the presence of the dendron on DNPs/DNPs@P22 is evidenced for both  
 420 conditions (protocols A and E). The signal of P22 is clearer for protocol E compared to protocol A and that can be due to the copolymerization taking place with protocol A. Nevertheless, by HRMAS, it was not possible to unambiguously confirm the presence or quantification of the P22.

Table 3. pH conditions for each protocol, mean hydrodynamic size ( $D_h$ ) and zeta potential value at pH  
 425 6.5 after the coupling step performed with 0.6 molar equivalents of P22 as a function of each protocol detailed in Table 1 and percentage of the P22 coupling determined by HPLC-UV (ND: not determined, \*: water, <sup>b</sup>: buffer).

Samples	DNPs	A	B	C	D	E
pH 1 <sup>st</sup> step		no pH	no pH	6-7*	6-7*	6.5 <sup>b</sup>
2 <sup>nd</sup> step		control*	control*	9*	9*	9.2 <sup>b</sup>
$D_h$ (nm)	18.7	20.6	17.4	18.7	20.1	21.7
$\Delta D_h$	-	1.9	-	0	1.4	3
Zeta potential $\zeta$ value (mV)	-22	-29	-22	-22	-35	-42
$\Delta\zeta$	-	-7	0	0	-13	-30
% of coupling		ND	40 %	50 %	25 %	45 %

The NMRD profile corresponds to the measurement of the proton relaxivity  $r_1$  as a function of the  
 430 proton Larmor frequency within IONPs suspension. The NMRD profiles of superparamagnetic IONPs have a characteristic shape from which the fitting can extract different structural and magnetic parameters of the IONPs such as saturation magnetization ( $M_s$ ), or NMRD radius. These two parameters depend on how close the water protons to the magnetic core of IONPs can be. The coating of the IONP can affect this distance between proton and magnetic core and thus the NMRD profile.

435 The denser the coating is, the further the protons will be from the core. Thus, smaller  $M_s$  values and  
 bigger radius will be determined from NMRD measurements. Here, the radius determined by NMRD  
 profile is quite close to the radius determined after TEM images analysis (Table 4). Indeed, the  
 dendron with its three branched PEG allows a lot of mobility and the protons can easily access toward  
 the core of the NPs<sup>21</sup>.

440 Adding the P22 at the surface of DNPs could hinder this mobility and thus decrease both the  $M_s$  and  
 the radius determined by NMRD. As seen in Table 4 and Figure 6, this effect is more visible for the  
 protocol A, where we suspect that a copolymerization happens between the P22 molecules. Indeed, the  
 measured  $M_s$  is only 37.5 emu/g compared to 46.8 emu/g before coupling. As for the radius, it goes  
 from 6.9 nm for DNPs to 7.9 nm when the peptide is coupled with protocol A. This strong difference  
 445 tends to validate the copolymerization theory, which is happening with this protocol. The effect is less  
 marked for the protocol E, where the radius determined by the NMRD profile fitting shows only an  
 increase of 0.4 nm after the coupling of P22 and the  $M_s$  is also less impacted by the presence of P22  
 (47.8 emu/g compared to 49.9 emu/g before coupling). These results seem to agree with the fact that  
 with protocol A, copolymerized P22 are on the DNPs surface whereas for protocol E, P22 is coupled  
 450 without this copolymerization phenomenon and in a smaller quantity.

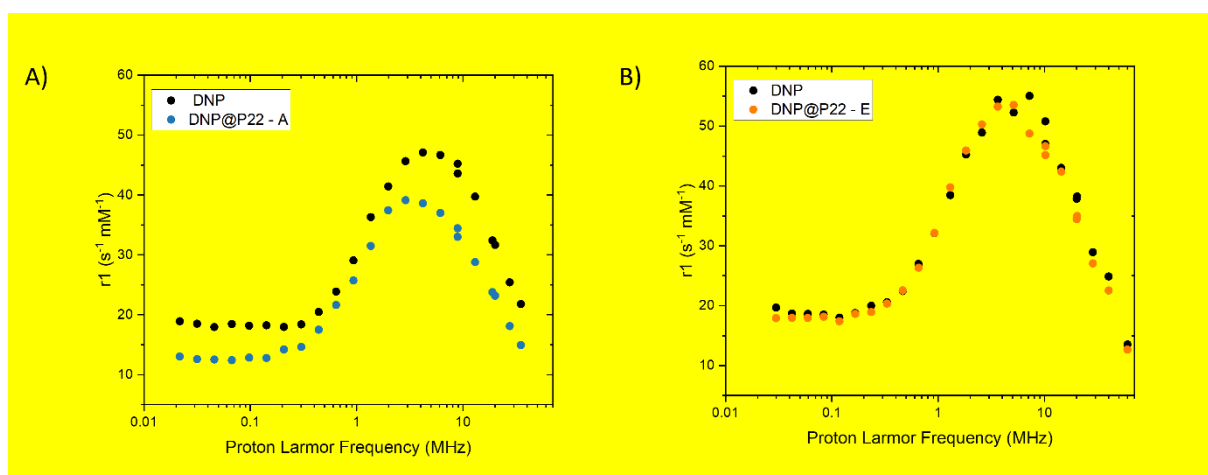


Figure 6: A) NMRD profiles of DNP (black) and DNP@P22-A (blue), B) NMRD profiles of DNP (black) and DNP@P22 -E (orange)

455 Table 4: TEM radius, NMRD radius and  $M_s$  determined after NMRD profile fitting for four suspensions: two of DNP, DNP@P22 – A and DNP@P22 -E

	DNP before protocol A	DNP@P22 - A	DNP before protocol E	DNP@P22 - E
Radius (TEM) (nm)	6.2	/	6.4	/
Radius (NMRD) (nm)	6.9	7.9	6.8	7.1

$M_s$ (NMRD) (emu/g)	46.8	37.5	49.9	47.8
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### 3.4 P22 quantification by HPLC-UV

Concerning peptide quantification methods, we faced many difficulties. A direct quantification can be done in specific situations: for example, if the attached molecule is fluorescent, it can be measured by fluorescence microscopy. Another technique could be elemental analysis, but in our case, it is not useful because we have the same elements (C, N, O) in the dendron and in the P22. Lastly, as IONPs are magnetic, NMR is not an appropriate technique.

As an alternative, we decided to develop an indirect quantification method. Briefly, knowing the initial amount of P22 in the coupling reaction, the unbounded P22 was quantified by HPLC-UV. To determine the amount of unbound P22, the supernatant (S) and the three consequent washes (W1, W2, and W3) were analyzed by HPLC-UV (Figure S5). The separation of the DNPs@P22 from the free P22 was done using Amicon filter tubes with a cut-off of 30 kDa and centrifugation at 8000 rpm for 5 min (Figure 5). A calibration curve for P22 was obtained in milliQ water (Figure S1). Quantification was carried out by peak integration of the chromatogram peak corresponding to the P22 at an elution time of 10.2 min.

The supernatant and the washes are weighted, so, knowing the mass and the concentration by HPLC-UV, the  $\mu\text{g}$  of unbounded P22 were obtained. The difference between the initial mass and the mass found by HPLC measurements gave the number of molecules of P22 bound to the dendron at the DNPs surface. This quantification was possible for protocols B to E but not for the A one. Indeed, with protocol A, the HPLC chromatogram does not display a single peak as with all other protocols, but several peaks possibly due to the polymerization of P22 (Figure S6).

The P22 coupling yield was estimated for protocols B to E. For all protocols, the coupling is around 40% except for protocol D where only 25% coupling is noticed. This lower coupling amount could be explained by the more difficult control of pH by adding HCl and NaOH to water, which may have perturbed the reaction. However, it is important to say that these coupling yields may have been overestimated as some peptide could be retained in the filters of the Amicon tubes. The amount of P22 bound to the filter is hard to quantify accurately. Otherwise, it allows a fair comparison between the different samples. The comparison of the  $D_h$  and zeta potential value and percentage of P22 coupling (Table 3) shows that, for protocol B, a coupling of 40% is obtained when no variation in  $D_h$  and zeta potential value is observed. By contrast, with the protocol D, a variation in these values is observed (quite similar to that of protocol A) but the coupling percentage is rather low. This can be attributed to the uncertainty of HPLC quantification, with the retention of some P22 in the filter and/or to an interaction of P22 with PEG chains like a “burial” of P22 in PEG chains depending on the protocol.

The highest variations in mean hydrodynamic size and zeta potential values were noticed with the

protocol E. Furthermore, the protocol E showed to have the best reproducibility: it was assessed several times with various molar equivalents of P22 compared to carboxylate groups: 0.25, 0.6 and 1.2 molar equivalents, as it can be observed in Figure 7. With this protocol, 19.50 % of the carboxylate groups at the surface of DNPs are coupled with P22 at 0.25 molar equivalent. When using 0.60 equivalents, 45 % of the carboxylate groups are coupled with P22. When using 1.2 equivalent, 37.5 % are coupled with P22. The amount of coupled P22 increased when the amount of P22 introduced increased but one may notice that the coupling with an excess of P22 does not imply more coverage. Indeed, it seems that the coupling maximum is around 40 %, which may certainly be explained by some steric hindrance at the surface of DNPs.

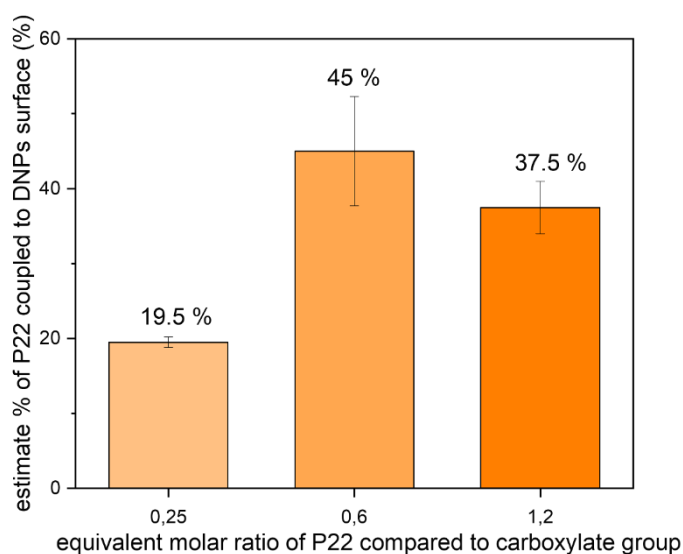


Figure 7 Estimation of P22 coupling after protocol E with (from left to right) 0.25 equivalent of P22, 0.6 equivalent of P22 and 1.2 equivalent of P22, the bars show the mean percentage reached for each condition, with their respective standard deviations. The number of experiments for each condition was  $\geq 3$ .

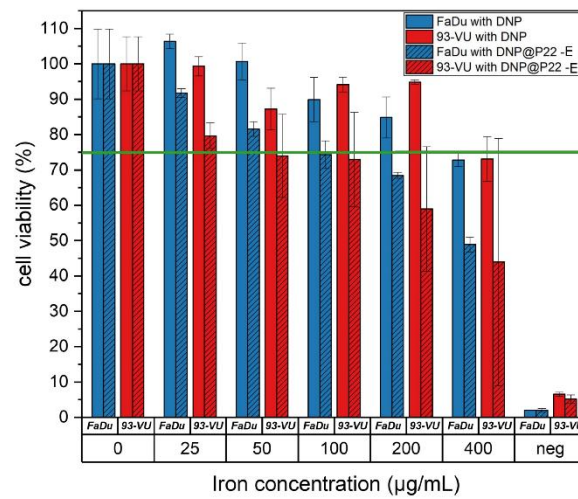
### 3.5 Cell internalization studies

Before investigating the cell internalization of DNPs and DNPs@P22 in *in vitro* studies, we first carried out cytotoxicity tests on a panel of DNPs@P22 made according to different protocols. After that, DNPs and DNPs@P22 were exposed to the cells to evaluate their cell uptake.

#### 3.5.1 Cytotoxicity study

Cytotoxicity of both suspensions (DNPs and DNPs@P22) was evaluated by MTT assay using both cell lines incubated for 24 h with the suspensions at various concentrations (from 0 and 400  $\mu\text{g/mL}$  of iron). DNPs@P22 were prepared with a concentration of 0.6 molar equivalent of P22 using the conditions of the protocol E. In Figure 8, DNPs do not show any cytotoxicity up to 200  $\mu\text{g Fe/mL}$ .

More importantly, the presence of the peptide even if it decreases a bit cell viability, seems to not strongly affect the cells up to 100  $\mu\text{g Fe/mL}$ .

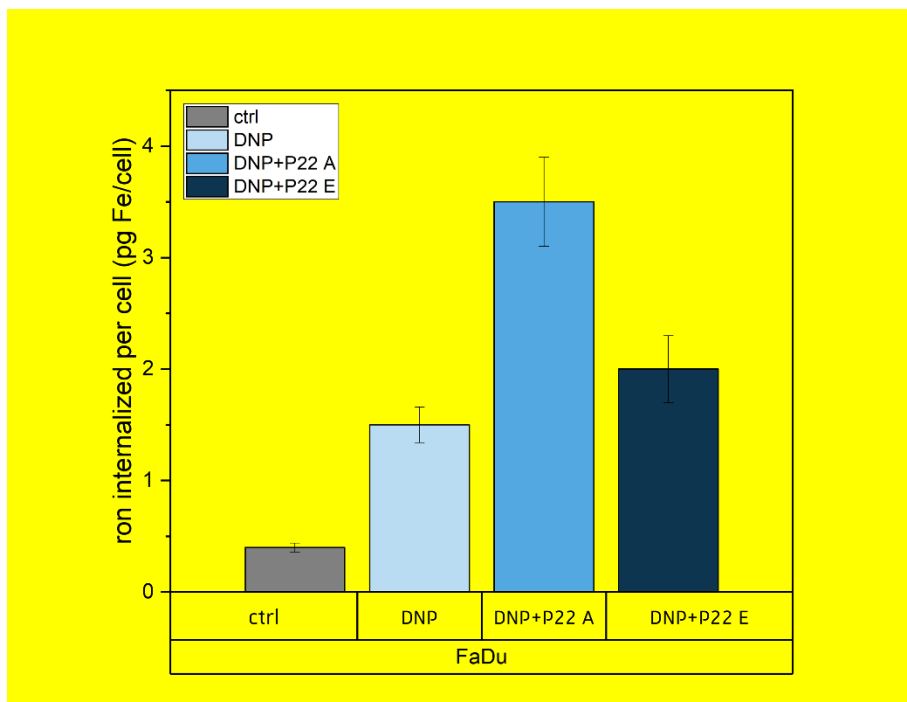


520 *Figure 8 Cell viability of FaDu (blue) and 93-VU (red) cell monolayers as a function of the amount of DNPs and DNPs@P22 – E. neg: negative control with DMSO*

### 3.5.2 Internalization study

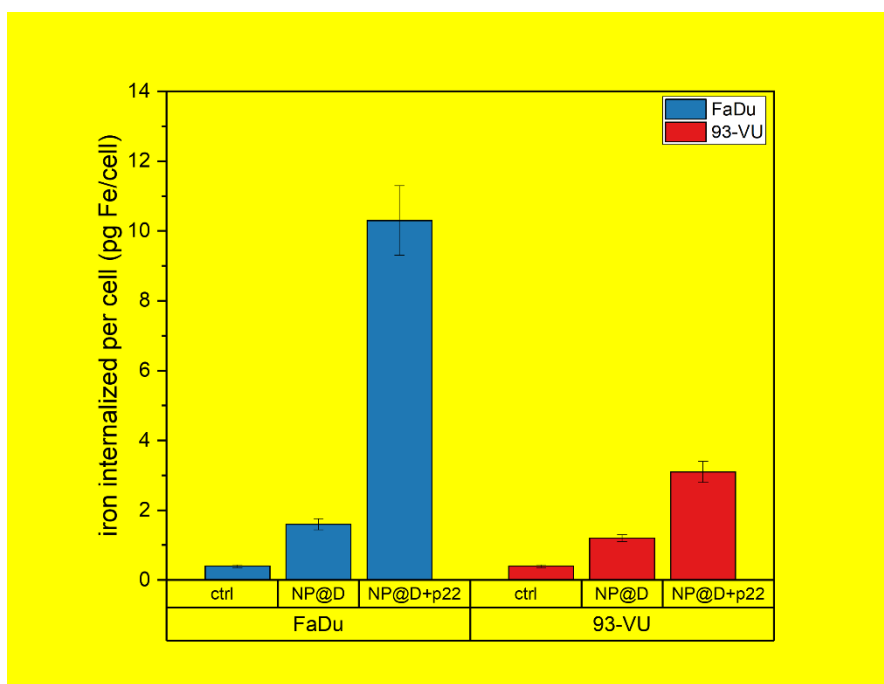
Cell internalization studies have been conducted at Fe concentrations of 100  $\mu\text{g/mL}$ , which are below the concentration inducing cytotoxicity. As a time point, 24 h was the time chosen to study the internalization of DNPs or DNPs@P22 in both cell lines (FaDu and 93-VU).  
525

With protocols A and E, when the amount of P22 increases up to 0.6 equivalent of P22 (coverage around 45%), internalization was observed but the internalization amount seems to depend on the concentration and also, on the protocol. **Indeed, with the protocol A, a higher internalization is observed in comparison with DNPs without P22.** The comparison between protocols A and E shows that protocol A leads to higher internalization values with 0.6 equivalent of P22 at a concentration of 100  $\mu\text{g/mL}$  of iron (Figure 9). With the optimized protocol E, the highest internalized amounts were obtained with a P22 equivalent of 1.2 and a concentration of 100  $\mu\text{g/mL}$  (Figure 10).  
530



535 *Figure 9 Amount of iron internalized in cells with DNPs and DNPs@P22 prepared with the protocol A (DNP@P22 – A) and protocol E (DNP@P22 – E) with 0.6 molar equivalent of P22 compared to carboxylate group (concentration of suspension: 100 µg Fe/mL). The test was carried out once with triplicate.*

540



*Figure 10 Amount of iron internalized in cells with DNPs and DNPs@P22 prepared with the protocol E and a P22 of 1.2 (coverage 37.5%) (concentration of suspension: 100 µg Fe/mL). The test was carried out once with triplicate.*

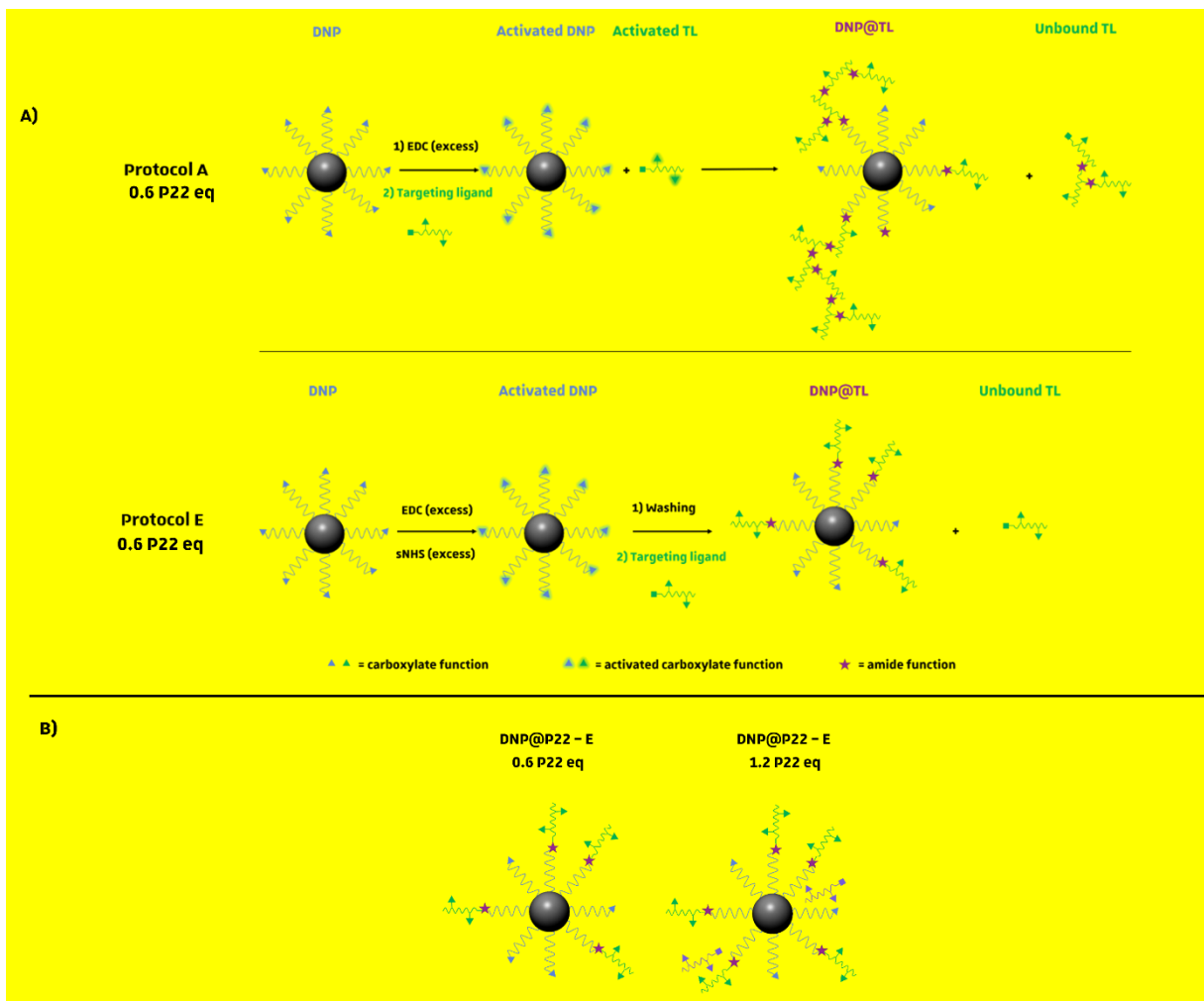
545 The amount of iron internalized in cells using DNPs and DNPs@P22 suspensions prepared with optimized protocol E and P22 equivalent of 1.2 at a concentration of 100  $\mu\text{g}/\text{mL}$  is given in Figure 10. The effect of P22 is clearly visible for both cell lines and especially for the FaDu line as the amount of pg Fe/cell is higher as well as the percentage of iron internalized into cells. Indeed, around 10 pg of iron per cell are found with DNPs bearing the P22 against 1.6 pg for the DNPs. The 93-VU cell line

550 seems less prone to the effect of targeting of the peptide, strong differences both in terms of percentage and iron amount is visible between the two suspensions.

Among all protocols, the protocol A, where a copolymerization of P22 is suspected, and the protocol E, with a P22 equivalent of 1.2 instead of 0.6, which leads however to the same P22 coverage, have conducted to cell internalization. Our hypothesis is that these coupling conditions should favor an

555 interaction between P22 molecules and EGFR in cells. P22 molecules are coupled on DNPs but there are other P22 molecules interacting strongly with them and certainly more available for interacting with cells as depicted on Figure 11. This suggests that it would be important to have a longer linker between targeting ligand and the surface of DNPs to ensure a higher internalization amount as the targeting ligand could be more accessible for the cell receptor.

560





565 *Figure 11 Hypothesis on P22 coverage to explain in vitro internalization results for A) protocols A and E with 0.6 P22 molar equivalent compared to dendron molecules and B) protocol E with either 0.6 and 1.2 P22 molar equivalent compared to dendron molecules*

570 Overall, the *in vitro* studies confirmed the need of P22 targeting ligand to increase DNPs internalization inside cancerous cells that overexpress the EGF receptor. It is in agreement with other previous studies based on iron-oxide nanoparticles coupled with GE11 (original peptide that P22 was derived from) that suggested the strong role of the peptide to increase nanoparticle accumulation inside cells.<sup>79,80</sup>

## 5. Conclusions

575 In this work, we have highlighted the difficulties in coupling and quantifying a peptide grafted on the surface of DNPs. Even if the carbodiimidation reactions are widely used, their conditions are not so trivial as evidenced by the discrepancies observed for the pH conditions of coupling, the different tested temperatures (4°C, room temperature or assisted by microwave), the washing between the steps, and the use of reagents (*e.g.*, with or without sufo-NHS). The stability and/or ageing of EDC was also found to be a crucial point to have efficient and reproducible coupling as well as the careful control of pH during both coupling steps. The different performed studies allowed us to optimize the coupling conditions in order to establish a reproducible protocol leading to a controlled amount of peptide grafted at the surface of DNPs. The amount of P22 has been determined by an indirect method and may slightly be overestimated due to the possible loss of low amounts of P22 during the different washing steps. Nevertheless, the amount of coupled P22 is reproducible. The first *in vitro* studies have confirmed that there is an enhancement in the uptake of DNPs@P22, compared to DNPs without TL. This study suggested, that the TLs need to be highly accessible for cancer cells receptors and not too close from the surface of DNPs to facilitate the nanoparticles internalization.

590

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605

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