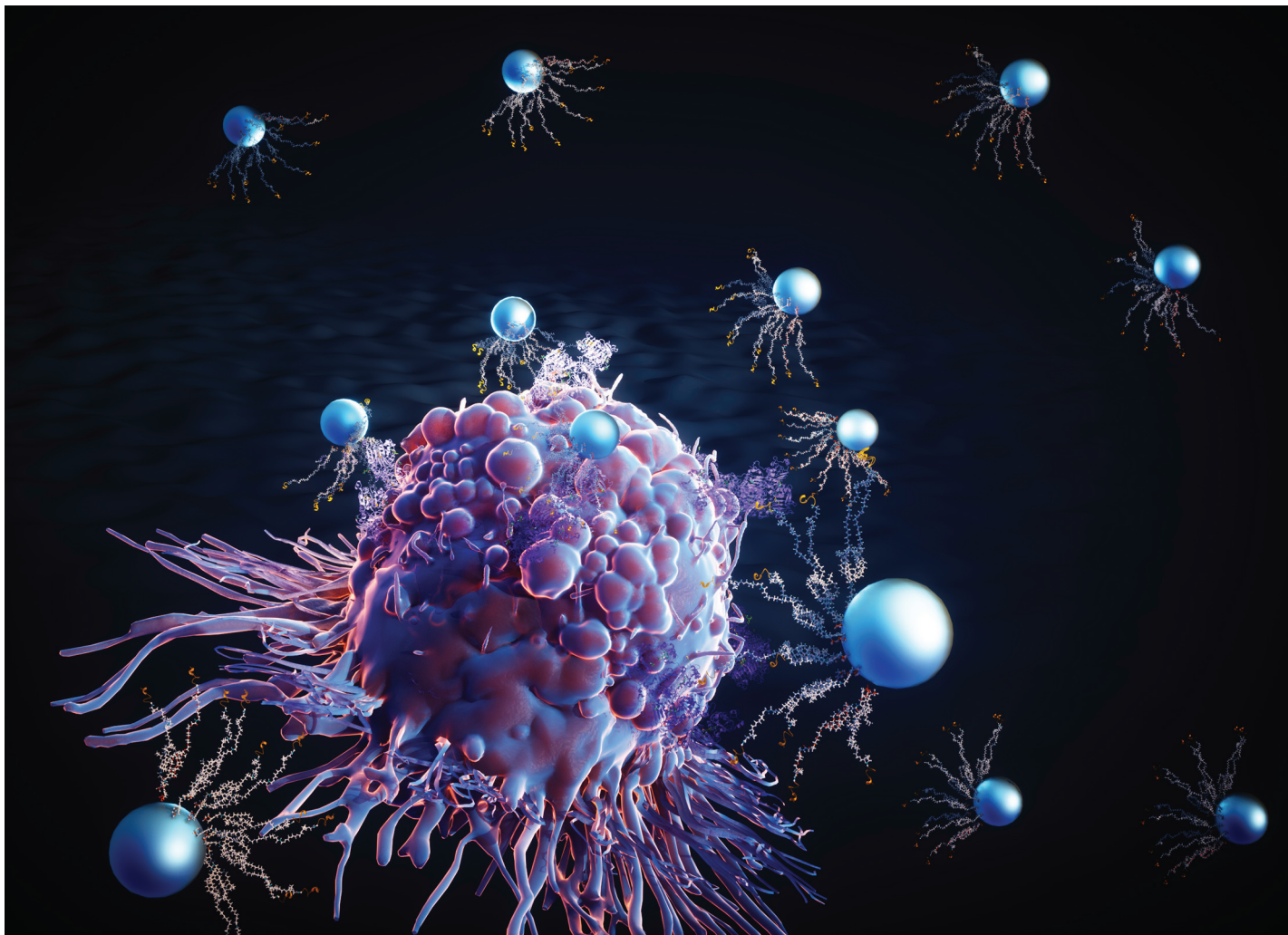


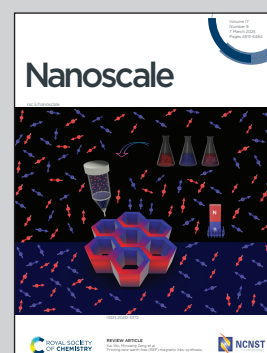


Showcasing collaborative research between Professor Sylvie Bégin-Colin's and Dr. Alexandre Detappe's groups located respectively at the Laboratories of Hybrid Nanomaterials Engineering (IPCMS), Université de Strasbourg, and Institut de Cancérologie Strasbourg Europe (ICANS) in Strasbourg, France.

Spacer engineering in nanoparticle-peptide conjugates boosts targeting specificity for tumor-associated antigens

Despite significant advancements in nanoparticle biofunctionalization strategies, their ability to effectively target and accumulate within tumors remains a major challenge. Optimizing the length and structure of the molecular linkers connecting nanoparticles to their targeting ligands significantly enhances the complex's binding affinity to tumor-associated antigens on cell surfaces. This approach holds promise for improving the precision and efficacy of nanoparticle-based cancer therapies.

As featured in:



See Alexandre Detappe, Sylvie Bégin-Colin, Sébastien Harlepp *et al.*, *Nanoscale*, 2025, 17, 5021.


Cite this: *Nanoscale*, 2025, **17**, 5021

Spacer engineering in nanoparticle–peptide conjugates boosts targeting specificity for tumor-associated antigens†

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Developing and synthesizing nano-objects capable of enabling early targeted diagnosis and ensuring effective tumor treatment represents a significant challenge in the theranostic field. Among various nano-particles (NPs), iron oxide nanoparticles (IONPs) have made significant contributions to advancing this field. However, a key challenge lies in achieving selective recognition of specific cell types. In oncology, the primary goal is to develop innovative strategies to enhance NP uptake by tumors, primarily through active targeting. This involves adding targeting ligands (TL) to the NP surface to facilitate tumor accumulation and increase retention within the tumor microenvironment. Despite biofunctionalization strategies, overall tumor uptake remains modest at only 5–7% of the injected dose per gram. In this work, we demonstrate the effect of spacing between the NPs and the TL to improve their availability and thus the tumor uptake of the complex. This proof-of-concept study targets the epidermal growth factor receptor (EGFR) using a peptide as a targeting ligand. Specifically, we characterized the PEG-peptide coupled to dendronized IONPs, including the density of grafted TL. These nano-objects underwent *in vitro* evaluation to assess their ability to specifically target and be internalized by tumor cells. Therapeutically, compared to non-functionalized NPs, the presence of the TL with a PEG linker enhanced targeting efficacy and increased internalization, leading to improved photothermal efficacy.

Received 2nd September 2024,
Accepted 27th December 2024

DOI: 10.1039/d4nr02931c

rs.c.li/nanoscale

Introduction

Precision therapy relies on the intricacies of delivering therapeutics to the tumor microenvironment (TME), where nanoparticles (NPs) play a pivotal role as transport vectors. Upon their administration into the bloodstream, these NPs dynamically acquire a distinctive biological identity by interacting with various organs, which underscores the complexity of their journey towards the targeted site. The success of a tumor targeting strategy requires these NPs to adeptly navigate through tumors while concurrently minimizing off-target distribution in healthy organs. As a result, their transport dynamics are influenced by their physicochemical properties, size, shape, and surface chemistry.^{1,2} The optimization of tumor uptake involves strategic functionalization,^{3–5} wherein monoclonal antibodies (mAbs) and peptides have emerged as key players. Surprisingly, despite the implementation of biofunctionalization strategies, the pharmacokinetic (PK) parameters of these NPs exhibit minimal changes when compared to their unmodified counterparts.^{6,7} This suggests that the biofunctionaliza-

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4nr02931c>



Here, we aimed to use a biofunctionalized dendronized iron oxide NPs (DNPs) with EGFR-targeted agents presenting a compelling alternative to potentially address the challenge of drug delivery while mitigating MDR mechanisms, offering an effective solution in cancer therapeutics. The use of these DNPs additionally provides theranostic properties due to the intrinsic superparamagnetic properties of iron oxide.^{19–22} Indeed, spherical DNPs with a mean size of 18 nm (saturation magnetization (M_s) of 89 emu g⁻¹) were shown to display SAR values of 147 W g_{Fe}⁻¹ under laser irradiation for photothermal therapy (808 nm, 0.3 W cm⁻²) and around 100 W g_{Fe}⁻¹ under magnetic hyperthermia (100 kHz, 32 kA m⁻¹).²³ In another study, dendronized 22 nm nanospheres and 19 nm nanocubes presented promising theranostic properties (respectively, $r_2 = 416$ s⁻¹ mM⁻¹, SAR_{MH} = 580 W g⁻¹, SAR_{PTT} = 800 W g⁻¹; and $r_2 = 407$ s⁻¹ mM⁻¹, SAR_{MH} = 899 W g⁻¹, SAR_{PTT} = 300 W g⁻¹).¹ DNPs with a mean size of 12 nm and a M_s value of 56 emu g⁻¹ did not heat at 300 G and 395 kHz (requiring a higher frequency of 536 kHz, which is outside the clinical limit, to generate heat) but displayed high heating power under photothermal conditions.¹ Recently, we demonstrated that DNPs with

This study aims to address this critical challenge by improving the availability of P22 and avoiding its folding into PEG chains. By adjusting the distance between NPs and peptides,

of P22 and PEG@P22 grafted onto the surface of DNPs, we performed indirect quantification by high performance liquid chromatography coupled with UV spectroscopy (HPLC-UV). After synthesis, the supernatant was weighed to ascertain the combined mass of unbound P22 and PEG@P22 using the HPLC standard curve, specifically by evaluating the area under the peaks at 280 nm (ESI Fig. 1J†). The binding efficiency was subsequently determined by subtracting the predetermined quantity of unbound PEG@P22 or P22 from the total initial amount. The results revealed 33% and 37.5% of PEG@P22 or P22, respectively, were covalently linked to the dendrons. Thus, on average, each DNP is coated with a first layer of approx. 700 carboxylate groups on the dendrons and a second layer consisting of 230 PEG@P22 or 260 P22 molecules attached to the dendrons. This results in an increase in the mean hydrodynamic size up to 25.8 ± 8.3 and 17.9 ± 5.1 nm after the coupling of PEG@P22 and P22 respectively (ESI Fig. 1I†). The larger increase in the mean hydrodynamic diameter with DNPs@PEG@P22 compared with DNPs and DNPs@P22 strongly suggests that P22 is positioned outside the DNPs rather than being buried within the dendron PEG chains.

The addition of a PEG spacer increased the specificity of EGFR targeting *in vitro*

After successfully functionalizing DNPs with P22, both with and without a PEG spacer in between, we assessed whether the addition of the PEG chain on the dendrons of the DNPs affected the specificity of P22 in binding to EGFR. Previously conducted viability assays with DNP@P22 demonstrated no cytotoxicity up to 100 $\mu\text{g Fe per mL}$ in head and neck cancer cell lines.³⁴ Similarly, we did not observe any signs of cytotoxicity at this concentration in the breast cancer cell lines used in this study. Subsequently, we first sought to validate that the addition of P22 led to improved uptake in EGFR+ cells (MDA-MB-231) (ESI Fig. 2A and B†). These cells were incubated at 37 °C overnight with either DNPs, DNP@P22, or DNP@PEG@P22. Following incubation, the cells underwent three successive washes with PBS, and we quantified their uptake of iron using the Prussian blue staining.³⁴ As expected,

minimal internalization was observed with EGFR-MCF-7 cells, but statistically significant differences were observed between the different NPs (Fig. 2A) with MDA-MB-231 cells. As expected, the enhancement in internalization between DNPs and DNP@P22 was quite low (p -value = 0.17, one-way ANOVA test), whereas a 10-fold increase in uptake was observed were DNPs were coupled with PEG@P22 (p -value = 0.003, one way ANOVA test). This increased uptake resulted in higher cytotoxicity at high concentrations in these EGFR+ cells compared to both DNPs@P22 and DNPs alone. Importantly, no differences were observed between these constructs in EGFR- cells (MCF7). This cell internalization study strongly suggests that the PEG spacer facilitates a suitable conformation of P22 at the surface of DNPs, thereby promoting its interactions with EGFR+ MDA-MB-231 cells (Fig. 2B).

With the observed enhancement in NP uptake prompted by the presence of the PEG linker surrounding P22, our research shifted focused to elucidating the cellular effects of these DNP@PEG@P22s. Considering the low cell internalization of DNPs@P22 and our objective to specifically study the interaction of P22 with cells while minimizing artefacts from any potentially exposed P22 at the surface of DNPs@P22, we narrowed this study to DNPs and DNPs@PEG@P22. We first confirmed the internalization of DNP@PEG@P22 by confocal microscopy. Specifically, our analysis focused on visualizing the adherence patterns of untargeted DNPs and targeted DNP@PEG@P22 on both MDA-MB-231 cells and MCF-7 cells (Fig. 3A and B). As anticipated, untargeted DNPs did not bind to MCF-7 cells, even when coupled with the PEG@P22. However, an increase in binding was observed with MDA-MB-231 cells when PEG@P22 was added (Fig. 3A). A competition binding study was then conducted on MDA-MB-231 cells using fluorescently labeled cetuximab, a clinically approved monoclonal antibody targeting EGFR. Interestingly, we observed that DNP@PEG@P22 demonstrated comparable binding activity to cetuximab on MDA-MB-231 cells, confirming that both cetuximab and P22 exhibited non-competitive interaction. This suggests that the two agents target distinct domains of the EGFR, allowing the possibility to use the

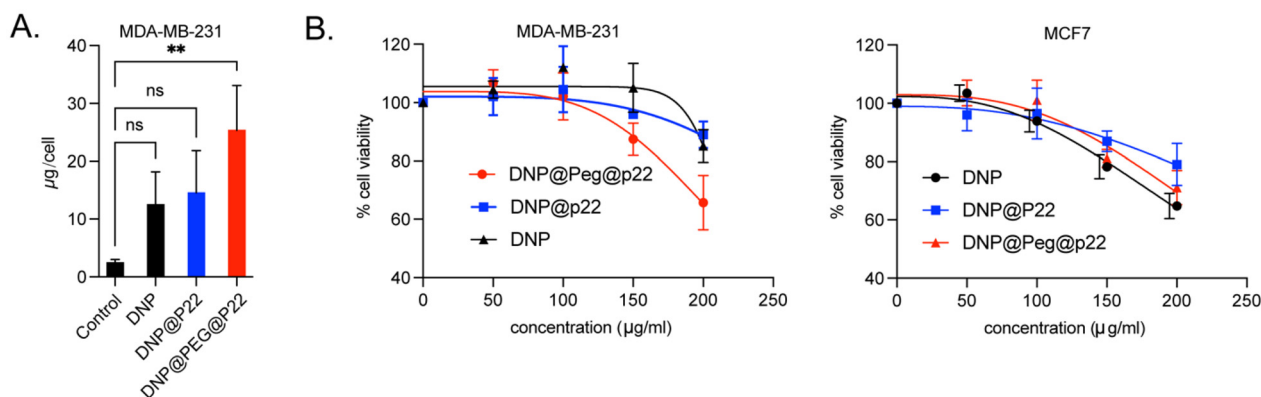


Fig. 2 A. Fe uptake ($\mu\text{g per cell}$) in MDA-MB-231 for the different types of nanoparticles. B. Alamar blue cell viability assay for MDA-MB-231 and MCF-7 cell lines using different DNPs. Fe concentrations varied from 0 to 200 $\mu\text{g mL}^{-1}$.



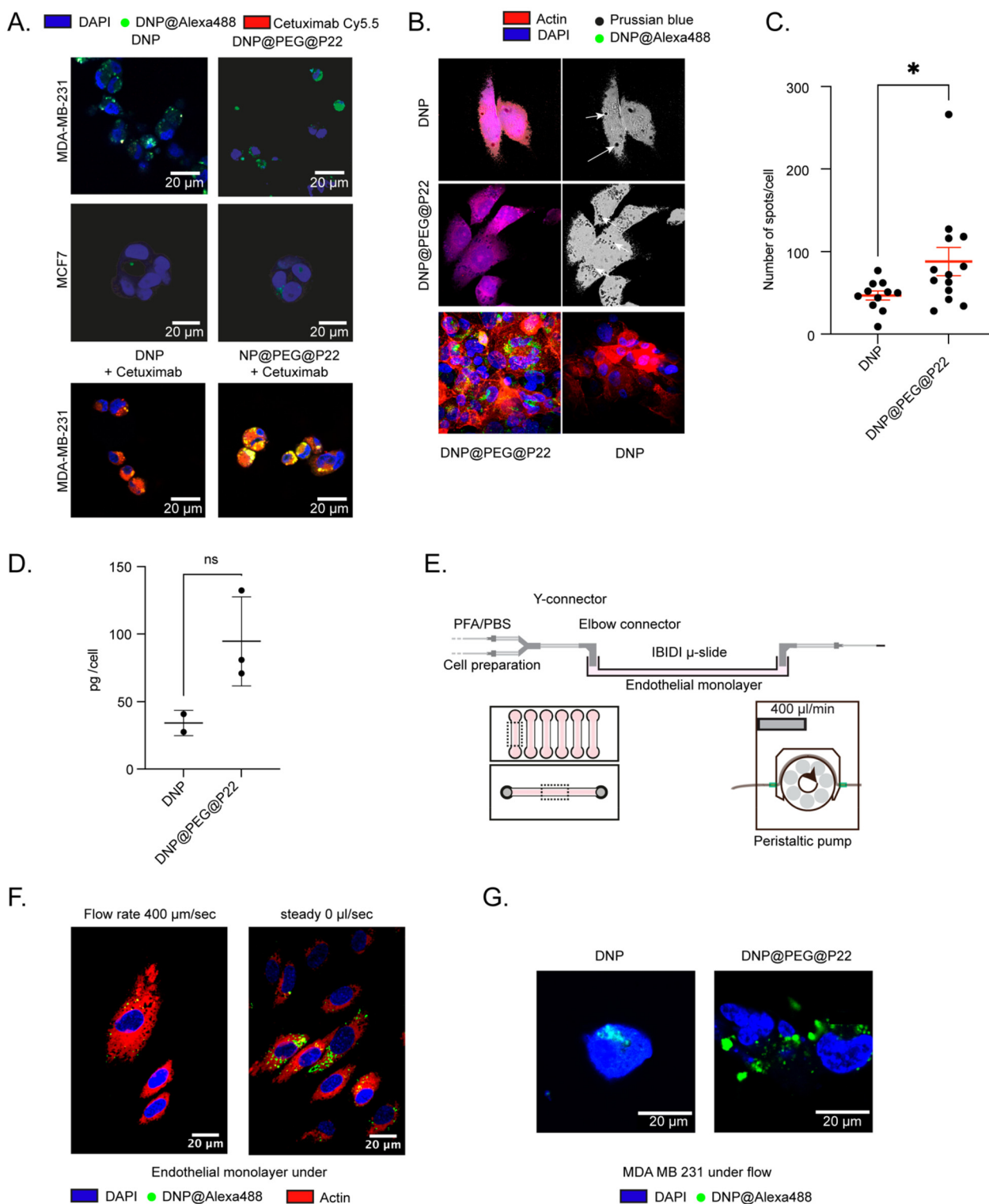


Fig. 3 DNPs@PEG@P22 affinity to different cell lines with or without hydrodynamic flow. **A.** Fluorescence confocal images of MDA-MB-231 and MCF7 cells. Nuclei are labelled with DAPI, NPs labelled with Alexa-488 and Cy5.5-cetuximab labels EGFR proteins. **B.** Confocal images of MDA-MB-231 cells treated with DNP or DNP@PEG@P22. Nuclei are labeled with DAPI, actin is labeled with Alexa Fluor™ 532 Phalloidin, and the NPs are labeled with Prussian blue. Arrows indicate the DNPs stained with Prussian blue. **C.** Graphical representation of the number of dots counted in different cells. **D.** ICP-MS quantification on MDA-MB-231 cells for the different synthesized NPs (pg per cell). **E.** Schematic representation of the microfluidic system developed to perform experiments under flow conditions. **F.** Confocal images obtained on endothelial cells incubated with Alexa488-DNP@PEG@P22 under flow conditions or in steady conditions. **G.** Confocal images obtained on MDA-MB-231 cells incubated with Alexa488-DNP or Alexa488-DNP@PEG@P22 under flow conditions.

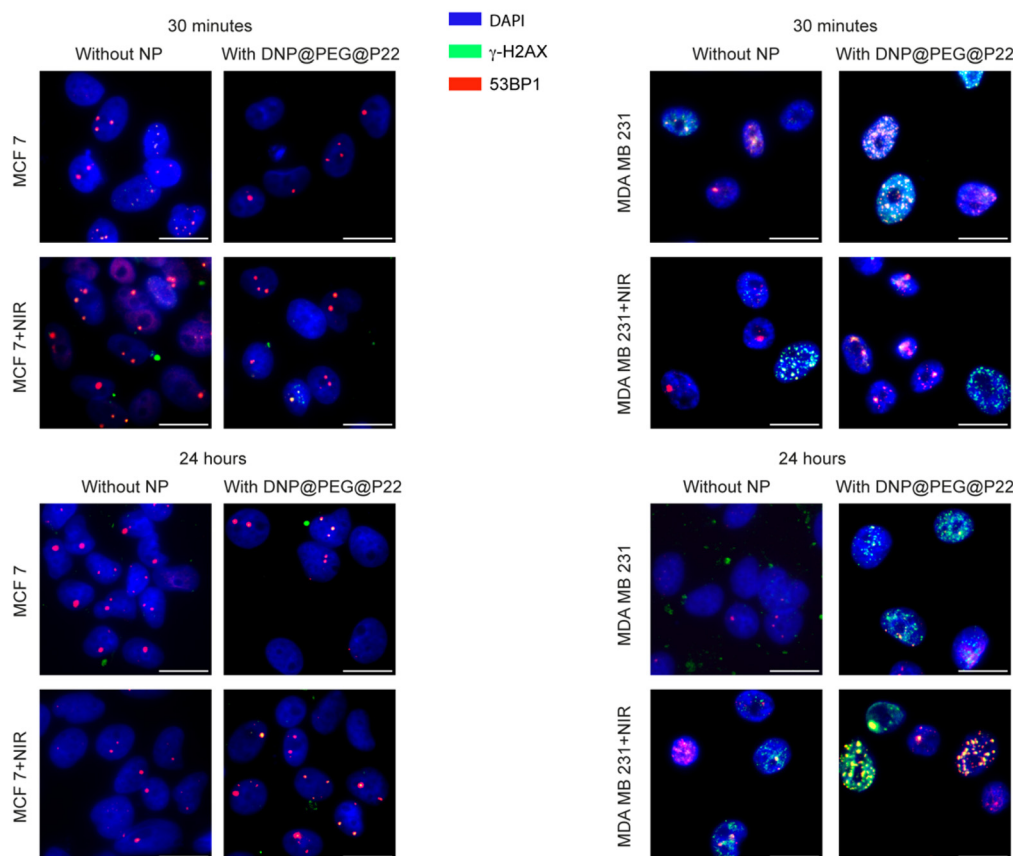


former as either a companion biomarker for the latter or in a therapeutic combination.

To further characterize the cellular uptake of DNP@PEG@P22, we initially employed a classical 2D culture system. MDA-MB-231 cells were incubated with DNPs and DNP@PEG@P22 formulations at a concentration of $100 \mu\text{g mL}^{-1}$ for 24 hours. Following incubation and washing steps, Prussian blue staining was performed and subsequently

imaged and quantified to determine the number of DNPs per cell, taking advantage of the specificity of this staining method toward the iron core of the DNPs.^{1,46,47} Subsequently, imaging was conducted on fluorescently labeled DNPs to discern differences in internalization between the DNPs and DNP@PEG@P22 formulations (Fig. 3B). While some non-specific internalization of passively targeted DNPs could not be entirely avoided, the specific interaction with the

A.



B.

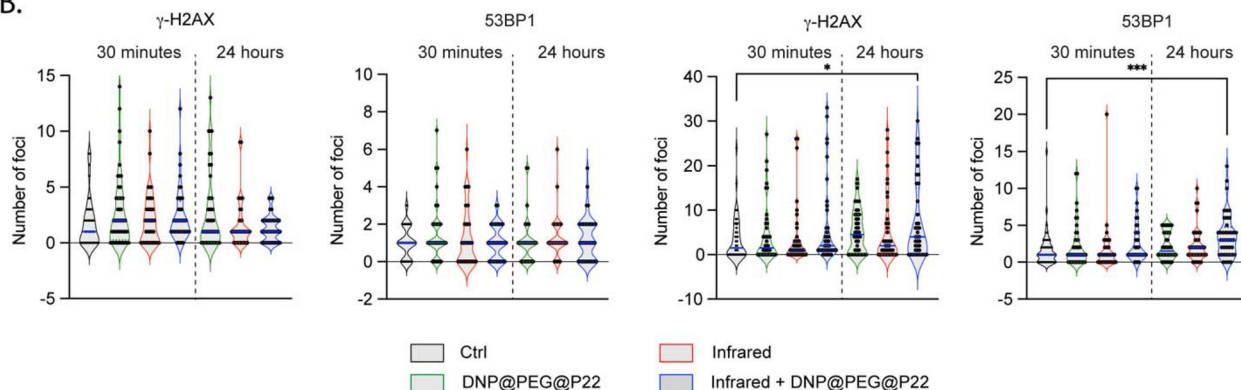


Fig. 4 Cell viability and affinity for the different treatments with and without DNP@PEG@P22. A. Immunofluorescence images of γH2AX (green) and 53BP1 (red) foci in MDA-MB-231 and MCF7 cells at 30 minutes and 24 hours post-treatments. B. Foci quantification under the different treatments.



acetone. This mixture was heated at 60 °C for 1 h under mechanical stirring in a thermal bath. The IONPs@OA were collected with a magnet, and the process was repeated. The final IONPs@OA were collected, and after discarding the supernatant, they were resuspended in 40 mL of THF for storage until further use.

Dendronization of IONPs

This study, which demonstrates the effect of a spacer on NPs cell internalization underscores the importance of the availability of targeting ligands at the NP surfaces to enhance specific internalization marking a critical advancement in nanoparticle-based cancer therapies.

Synthesis of 10 nm iron oxide nanoparticles

Coupling reaction

Peptide 22 (P22) and $\text{NH}_2\text{-PEG}(6)\text{-YHWYGYTPENVI}$ (PEG-P22) with a purity of 98.7% were procured from polypeptide SAS, and the coupling process followed the protocol developed by Freis *et al.*⁵⁸ The reaction involved two steps. In the first step, DNPs were suspended in HEPES 0.1 M, pH 6.5, at a concentration of 5 mg Fe per mL. EDC was then added at a 20-fold molar excess compared to the carboxylate groups of the dendron. The reaction was stirred for 10 minutes, followed by the addition of sulfo-NHS (also at a 20-fold molar excess compared to carboxylate groups on dendron molecules) for an additional 20 minutes. The activated DNPs are washed with 0.1 M carbonate buffer, pH 9.2, using a centrifugal filter unit (10 minutes at 8000 rpm) to eliminate excess EDC, sulfo-NHS, and other possible reaction intermediates. The filtrate was promptly recovered and mixed with 0.9 molar equivalents of PEG-P22 (compared to dendron molecules) in carbonate buffer. The reaction proceeded overnight at a final iron concentration of 0.5 mg mL^{-1} before purification with centrifugal filter units (Amicon®, MW cutoff: 30 kDa in cycles of 10 minutes at 8000 rpm). Multiple washes were performed, and each supernatant was retained for indirect quantification (of unbound PEG-P22) by HPLC. For the coupling with Alexa, the same protocol was used.

IONPs were synthesized *via* thermal decomposition of an iron stearate precursor, following a reported procedure.³⁵ In summary, 2.2 mmol of FeSt₃ and 4.4 mmol of OA were mixed with 20 mL of dioctyl ether in a 100 mL two-neck round-bottom flask. The mixture was heated to 120 °C for 60 min until complete reagent dissolution. Subsequently, a condenser was attached, and the mixture was heated to 290 °C at a rate of 5 °C min⁻¹. Refluxing at 290 °C occurred over 120 min, resulting in the formation of black IONPs suspended in OA (IONPs@OA). After cooling to 100 °C, washing steps were initiated by adding 10 mL of chloroform to the IONPs@OA, followed by placement in a flask containing 400 mL of

Transmission electron microscopy (TEM) was employed to characterize the sizes and structures of the IONPs. The analysis was conducted using a JEOL 2100 microscope operating at 200 kV with a point resolution of 0.18 nm. Size distribution estimation was performed using ImageJ software.

X-ray diffraction (XRD) patterns were collected at room temperature using a Bruker D8 Discover diffractometer in

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For the competition study between cetuximab and the P22 peptide, the cetuximab was labeled using reaction between amine groups of the mAb and NHS ester group. The cetuximab was purchased at the ICANS Pharmacy and labeled with Cy5.5 NHS ester (#27020 Lumiprobe). The reagents were incubated in a 1 : 5 molar ratio of cetuximab and dye overnight at 4 °C. Purification was performed using dye removal columns (#22858 ThermoFisher). The labeled mAbs were stored at 4 °C after purification.

Statistical analysis

Data are represented as mean $\pm$ standard deviation. All results were analyzed using GraphPad Prism 9.5.1. Statistical analysis was performed using unpaired non-parametric *t*-test for multiple groups comparison at a specific time point (Mann-Whitney test) or a one-way ANOVA for group comparison. Bonferroni *post hoc* test was performed to compare the different conditions. Significance was determined at the following cutoff points: **P* < 0.05, ***P* < 0.01, and ****p* < 0.001.

Author contributions

M. A. R. and B. F. designed and characterized the nanoparticles, performed the cellular uptake experiments with Prussian blue. J. D. labeled cetuximab with the Cyanine 5.5 and imaged the cells. T. G. performed the Alamar blue experiments. F. R. and O. T. performed and analyzed the ICP-MS experiments. C. C., M. A. R. and B. F. performed the HPLC-UV analysis. J. B.-G. performed the immunofluorescence experiments and their analysis. A. G. extracted the proteins and quantified by western blot the amount of EGFR proteins. S. H. performed the imaging experiments. S. J. designed the molecular constructs. X. P., G. N., A. D., S. H., S. L. and S. B. designed the study and analyzed the results. M. A. R., X. P., S. H., A. D., and S. B. wrote the manuscript.

Data availability

The main data supporting the results in this study are available within the paper and the ESI.† The raw data are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

A. D. acknowledges funding by, in part, the European Research Council (ERC) Starting Grant TheranoImmuno (grant agreement #95010), ITMO cancer of Aviesan within the framework of the 2021–2030 Cancer Control Strategy, on funds administered by Inserm, and the labelisation by the Ligue Contre le

Cancer. S. H. acknowledges the Strasbourg Drug Discovery and Development Institute (IMS) for funding this study, as part of the Interdisciplinary Thematic Institute (ITI) 2021–2028 program of the University of Strasbourg, CNRS and Inserm, IdEx Unistra (ANR-10-IDEX-0002), and by the SFRI-STRAT'US project (ANR-20-SFRI-0012) under the framework of the French Investments for the Future Program. A. D. and S. H. also acknowledge support from the Institut of Cancerologie de Strasbourg Europe. B. F. acknowledges support from The Region Alsace, France, and the University of Mons for the doctoral fellowship. S. B. acknowledges funding from ANR (EURONANOMED2020-121 – THERAGET) under the umbrella of the ERA-NET EuroNanoMed (grant agreement #723770) of the EU Horizon 2020 and from Alsace Contre le Cancer. Additionally, the authors acknowledge support from Pascal Kessler at the PIC-STRA imaging platform from the University of Strasbourg. SuperBranche is thanked for providing dendron molecules.

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