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Novel synthesis of platinum complexes and their intracellular delivery to tumor cells by means of magnetic nanoparticles†

Alessandra Quarta,^{*a} Manuel Amorín,^b María José Aldegunde,^b Laura Blasi,^c Andrea Ragusa,^{a,d} Simone Nitti,^e Giammarino Pugliese,^e Giuseppe Gigli,^{a,f} Juan R. Granja^{id} ^{*b} and Teresa Pellegrino^{id} ^{*e}

Platinum-based drugs are popular in clinics as chemotherapeutic agents to treat solid tumors. However, severe side effects such as nephro- and neurotoxicity impose strict dosage limitations that can lead to the development of drug resistance and tumor relapse. To overcome these issues Pt(IV) prodrugs and platinum delivery systems might represent the next generation of platinum-based drugs. In this study four novel Pt(II) complexes (namely, **PEG-Glu-Pt-EDA**, **PEG-Glu-Pt-DACH**, **PEG-Mal-Pt-EDA** and **PEG-Mal-Pt-DACH**) were synthesized and a general strategy to covalently bind them to iron oxide nanoparticles was developed. The intracellular uptake and cell distribution studies of Pt-tethered magnetic nanoparticles on breast and ovarian cancer cell line models indicate that binding of the Pt complexes to the nanoparticles facilitates, for all the complexes, cellular internalization. Moreover, the magnetic nanoparticles (MNPs), as shown in a magnetofection experiment, enhance the uptake of MNP-Pt conjugates if a magnet is placed beneath the culture dish of tumor cells. As shown by a Pt release experiment, intranuclear platinum quantification and TEM analysis on cell sections, the presence of a pH-sensitive dicarboxylic group coordinating the Pt complex, triggers platinum dissociation from the NP surface. In addition, the triazole moiety facilitates endosomal swelling and the leakage of platinum from the endosomes with intranuclear localization of platinum release by the NPs. Finally, as assessed by MTT, caspase, calcein/ethidium bromide live/dead assays, among the four NP-Pt conjugates, the **NP-Glu-Pt-EDA** complex having a glutamate ring and ethylenediamine as a chelating amine group of the platinum showed higher cytotoxicity than the other three MNP-platinum conjugates.

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Introduction

Pt-Based drugs cover almost 50% of chemotherapeutic agents, and they are used to treat malignant solid tumors (*i.e.* pancreatic, testicular, cervical, ovarian cancers, *etc.*).^{1,2} Despite

cisplatin, only other two platinum based molecules, namely carboplatin and oxaliplatin, have been approved for use on humans.^{3,4} These drugs are all square-planar platinum(II) complexes with a Pt atom that is coordinated to two leaving (the carboxylic groups) and two non-leaving amine groups.⁵ The nature of these two types of chemical groups affects the solubility, stability, efficacy and toxicity of the drugs.⁶

Pt compounds however are hampered by dose-limiting toxic side effects, including nephrotoxicity, neurotoxicity, drug resistance and tumor relapse.⁷ The latter two effects generally arise from poor accumulation levels of platinum in tumor tissues, low cellular uptake,⁸ drug inactivation by intracellular glutathione,⁹ and the DNA repairing mechanisms developed by tumor cells.^{10,11} Nanocarriers could mitigate these side effects at different levels: (i) they improve the solubility of the drug while prolonging its circulation time; (ii) they penetrate deep the tumor; (iii) they enable a triggered release of the Pt complexes, which consequently reduce the drug resistance and allow lower drug doses to be used.^{12,13}

^aCNR NANOTEC – Institute of Nanotechnology, c/o Campus Ecotekne, via Monteroni, 73100 Lecce, Italy. E-mail: alessandra.quarta@nanotec.cnr.it

^bCentro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS) and Departamento de Química Orgánica, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain. E-mail: juanr.granja@usc.es

^cCNR, Institute for Microelectronics and Microsystems, Via Monteroni, Lecce, 73100, Italy

^dDipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università del Salento, Campus Ecotekne, via Monteroni, 73100 Lecce, Italy

^eIstituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italy. E-mail: teresa.pellegrino@iit.it

^fDipartimento di Matematica e Fisica E. De Giorgi, Università del Salento, Campus Ecotekne, via Monteroni, 73100 Lecce, Italy

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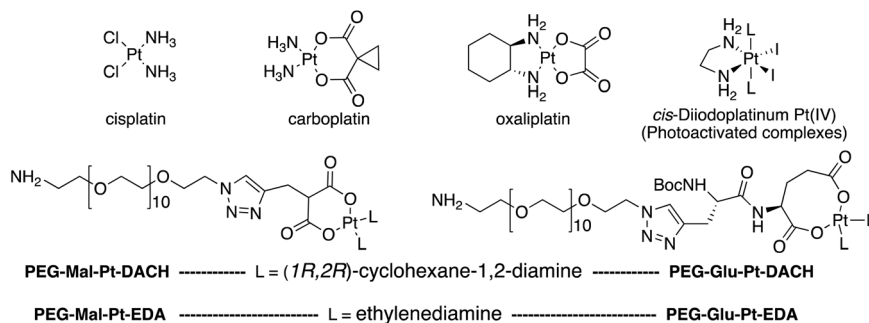
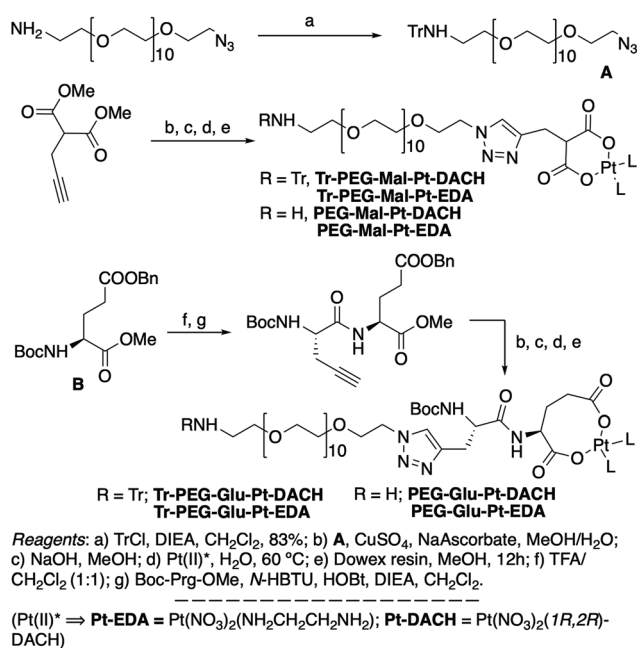


Fig. 1 Top: The structure of commercially available cisplatin derivatives and photoactivated diiodoplatinum(vi) complexes. Bottom: The platinum complexes prepared in this work.



Scheme 1 Synthetic strategy used for the preparation of PEG-Glu-Pt-DACH, PEG-Glu-Pt-EDA, PEG-Mal-Pt-DACH, and PEG-Glu-Pt-EDA platinum complexes.

second one, ethylenediamine (EDA), was chosen due to its structural simplicity and because it has already been shown to give promising results with regard to its use in photoactive Pt (iv) complexes.^{51,52}

The synthesis of the platinum complexes is illustrated in Scheme 1. The first step was the protection of the amino group on a commercially available PEG derivative that was treated with trityl chloride in the presence of diisopropylethylamine to provide azide **A** with a yield of 83% (as determined by NMR, see the ESI† for the NMR spectra of the compounds involved in the synthesis). In parallel, *N*-Boc-Glu(Bn)-OMe was deprotected with trifluoroacetic acid (TFA) and then coupled to Boc-protected propargylglycine to obtain dipeptide **B** with a yield of 93%. The click reaction between the azido derivative **A** and either dimethyl propargylmalonate or dipeptide **B** resulted in the corresponding triazole derivatives with a yield of 71% and

72%, respectively. The platinum complexes were prepared by reacting dinitroplatinum compounds, either $\text{Pt}(\text{NO}_3)_2\text{-DAE}$ or $\text{Pt}(\text{NO}_3)_2\text{-DACH}$, with the dicarboxylate salts of the triazole derivatives previously obtained by the basic hydrolysis of methyl esters. Finally, the trityl protecting groups were removed after treatment with the acidic Dowex resin thus yielding the four Pt-derivatives (PEG-Glu-Pt-DACH, PEG-Glu-Pt-EDA, PEG-Mal-Pt-DACH, and PEG-Glu-Pt-EDA) ready for conjugation to the magnetic nanoparticles (MNPs) through simple amide bonds (Fig. 2).

Preparation and characterization of the nanoparticles conjugated to the Pt-complexes

Magnetic iron oxide nanoparticles (with an average size of 10 nm) were prepared according to a thermal decomposition method,⁵³ and then transferred into water by means of a polymer encapsulation procedure with polymaleic anhydride octadecene.⁵⁴ After solubilization, the amphiphilic polymer exposes to the outer environment carboxylic groups, which were used to link the platinum derivatives. Before attaching the drug, it was necessary to pre-functionalize the surface of the nanoparticles with some PEG molecules to ensure their stability in DMSO, the solvent used during the coupling reaction to dissolve the Pt complexes. The final nanoparticle-platinum complexes were transferred into PBS buffer and kept at 4°C prior to use. It is worth mentioning that the choice of this polymer coated and PEG functionalized MNPs was mainly dictated by their safety and biocompatibility even at an injection dose as high as 0.8–1.0 mg of iron, as previously demonstrated by us in an *in vivo* biodistribution study on a murine model.⁵⁵

The four MNP-Pt conjugates were characterized by means of dynamic light scattering (DLS), gel electrophoresis, elemental analysis (Fig. 2), transmission electron microscopy (TEM) (Fig. 1SI†), and Fourier Transform Infrared (FTIR) spectroscopy (Fig. 3).

For their conjugation to the MNPs, the number of Pt molecules to be added per nanoparticle needed to be optimized in order to achieve the right balance between linking a sufficiently toxic amount of Pt complexes to each nanoparticle and to be able to preserve the stability and solubility of the nanoparticles under physiological conditions.



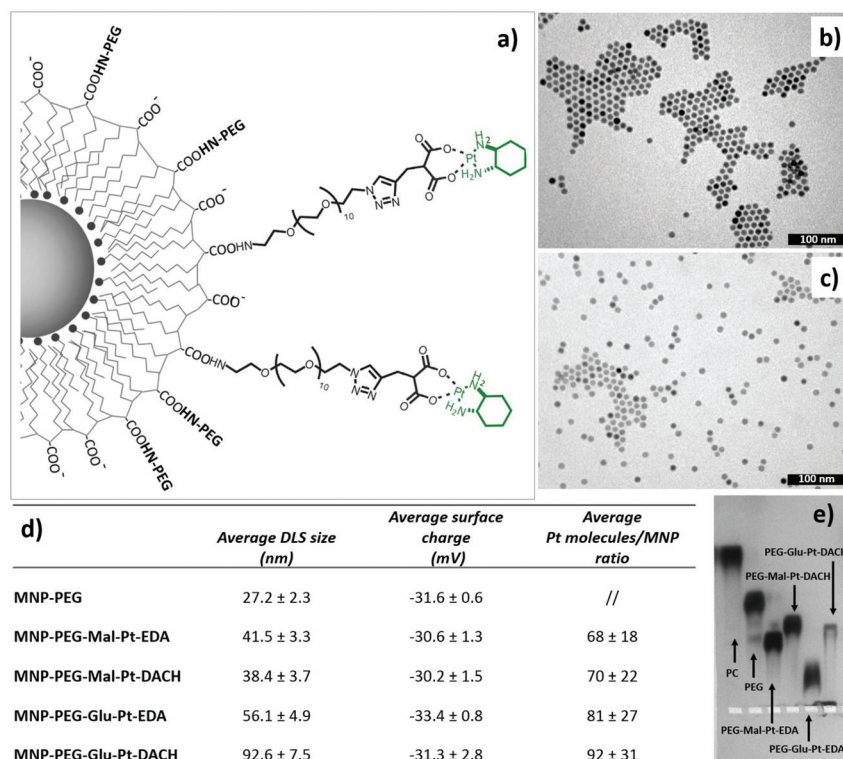


Fig. 2 (a) Representative drawing of the MNP-PEG-Glu-Pt-DACH conjugate. TEM images of the nanoparticles after water solubilization (b) and (c) surface functionalization with PEG-Glu-Pt-EDA. (d) The table reports the average hydrodynamic size and surface charge values for the nanoparticles conjugated to the different platinum complexes, as determined by DLS, as well as the average number of platinum complexes linked per type of nanoparticle, as assessed by elemental analysis using the Pt/Fe ratio. (e) Agarose gel electrophoresis characterization showing the mobility of the nanoparticles after each step of surface functionalization.

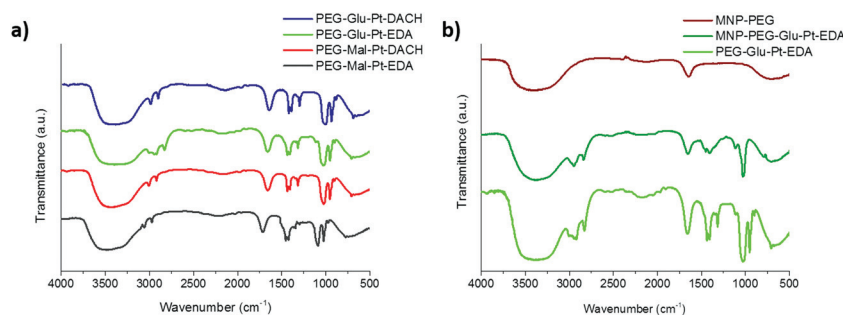


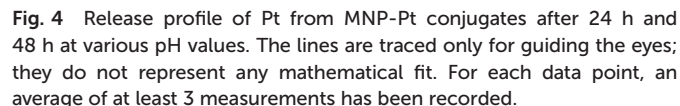
Fig. 3 FTIR spectra of (a) the synthesized Pt complexes and (b) the MNP before and after conjugation to PEG-Glu-Pt-EDA. In panel (b) the spectrum of PEG-Glu-Pt-EDA is also reported for comparison.

The hydrodynamic diameters verified that the size of the MNP-Pt conjugates shifted towards larger values than that of the sample coated with only PEG molecules, but the overall size was smaller than 100 nm (Fig. 2, panel d). The MNPs bearing the PEG-Glu-Pt-DACH complex showed the largest diameter, likely due to the tendency to form clusters and interparticle interactions, as also observed by gel electrophoresis (Fig. 2, panel e). The surface charge was quite negative for all the samples, with an average value close to -30 mV. The average amount of Pt complexes linked to each nanoparticle was estimated by means of elemental analysis, ranging from

about 70 to 100 metal complexes attached per nanoparticle depending on the preparation conditions and on the type of Pt complex (last column of the table of Fig. 2). The pattern of gel migration clearly showed the different delays of the nanoparticle bands at each step of functionalization especially when comparing the polymer coated NPs with the PEG coated up to those conjugated to the Pt-complexes. As an example, in the agarose gel electrophoresis shown in Fig. 2e, the MNP-Pt loaded for the electrophoretic run carried an average amount of 50 Pt molecules per nanoparticle. The gel electrophoresis pattern of the four MNP-Pt conjugates showed that, under the



To study the pH-dependent release of the conjugated platinum, the MNP-Pt nanoconjugates were kept at 37 °C for either 24 or 48 h in solutions at a well-defined pH in the range of 3 to 7. The amount of Pt that separated from the nanoparticles was recovered by filtration and quantified by elemental analysis, and the data obtained were plotted as a percentage of the initial platinum amount (Fig. 4). Having proven the presence of platinum on the NPs, we further studied the Pt release for all the Pt-conjugates by keeping the MNP-Pt conjugates in solution while gradually reducing the pH towards acidic values and evaluating the free platinum after separation from the NP fraction. At a pH of 3, almost 40% of Pt was released into the solution after 24 h, and this value increased up to 80% and 85% after 48 h in the case of **MNP-Glu-Pt-EDA** and **MNP-Mal-Pt-EDA**, respectively. At a pH of 4.0–4.5, which corresponds to the lysosomal pH, approximately 20% of Pt was detected after 24 h, whereas the average free platinum for all the nanoconjugates ranged between 50 and 70% after 48 h. On the other hand, at the physiological pH 7 the amount of Pt released was as low as about 10% and 20% after 24 h and 48 h, respectively, suggesting a potential good stability of the drugs before cellular internalization.



It is widely known that in an endocytic pathway, the pH drops from 6.5 in early endosomes to 4 in mature lysosomes and might facilitate the dissociation of platinum from the nanoparticle surface.^{56,57}

Based on these Pt loading and release findings, cellular studies were performed to assess the cytotoxic activity of the free Pt complexes and of the MNP-Pt conjugates in two tumour cell lines that display different sensitivities to platinum-based chemotherapy. In detail, ovarian cancer cells (IGROV-1) and breast cancer cells (MDA-MB-231) were chosen as *in vitro* models, the latter being generally recognized as cells quite resistant to platinum-based drug treatments.^{58–60}

Viability and apoptotic cellular assays (namely MTT and caspase) were performed to investigate the efficacy of both free and nanoparticle-bound platinum complexes on both cell lines.

First, a MTT proliferation assay was carried out to determine the inhibitory concentration (IC_{50}) values of Pt complexes (either free or conjugated to the MNP) after 24 h of incubation with IGROV-1 and MDA-MB-231 cells. Cisplatin was also tested as an additional reference for being routinely used in the clinical treatment of several cancers. Remarkably, all the free Pt complexes showed greater toxicity (IC_{50} between 6 and 8 μM) as compared to cisplatin (IC_{50} around 98 μM and 80 μM for the MDA-MB-231 and IGRO-1 cells, respectively). This means that the bare Pt complexes are on average 10 times more toxic than standard cisplatin. In addition, the IC_{50} values further decreased when the Pt drugs were bound to the nanoparticles (Table 1). As expected, the IGROV-1 cells were more sensitive to the toxic activity of the Pt derivatives than the MDA-MB-231 ones. Indeed, in the case of IGROV-1 that had been administered with **MNP-Glu-Pt-EDA** and **MNP-Glu-Pt-DACH**, the IC_{50} dropped down to 1.6 and 1.2 μM , respectively,

Table 1 IC₅₀ (μM) values estimated for either free or MNP-bound Pt conjugates after 24 h of incubation with MDA-MB231 and IGROV-1 cells. Cisplatin was also evaluated as an additional reference. The assays were normalized with respect to the total amount of Pt. The data represent the average and the standard deviation of three independent measurements. Statistical analysis was performed via the *t*-test: *p* < 0.01 when comparing the data of free Pt complexes with IC₅₀ values of MNP-Pt in both cell lines; *p* < 0.01 when comparing the IC₅₀ values of MNP-Pt of MDA-MB231 with those of IGROV-1 cells

Tested sample	MDA-MB-231 IC ₅₀ (μM)	IGROV-1 IC ₅₀ (μM)
PEG-Glu-Pt-EDA	7.1 ± 0.2	5.8 ± 0.3
PEG-Glu-Pt-DACH	8.3 ± 0.1	7.9 ± 0.1
PEG-Mal-Pt-EDA	6.2 ± 0.1	6.8 ± 0.1
PEG-Mal-Pt-DACH	7.4 ± 0.1	7.5 ± 0.1
MNP-PEG-Glu-Pt-EDA	5.3 ± 0.2	1.6 ± 0.4
MNP-PEG-Glu-Pt-DACH	5.2 ± 0.4	1.2 ± 0.4
MNP-PEG-Mal-Pt-EDA	6.2 ± 0.1	4.2 ± 0.1
MNP-PEG-Mal-Pt-DACH	5.8 ± 0.1	4.0 ± 0.4
Cisplatin	97.6 ± 5.1	79.8 ± 0.3

while MDA-MB-231 cells that had been incubated with the same nanoparticles showed an IC₅₀ equal to 5.3 and 5.2 μM, respectively. This corresponds to a further 3–4 fold decrease of IC₅₀ as compared to the corresponding free drug. The same trend was also detected when the two cell lines were incubated with **MNP-Mal-Pt-EDA** and **MNP-Mal-Pt-DACH**, but the difference in the IC₅₀ was less significant (the values for the IGROV-1 were 4.2 and 4 μM, and the values for the MDA-MB-231 cells were 6.2 and 5.8 μM, respectively).

These results suggest that the Pt complexes and MNPs Pt conjugates affect cell survival significantly as compared to cisplatin and there are correlations between the structures of the nanoconjugates, platinum complexes and cell line types. Indeed, as a first observation, the Pt-based drugs seem to induce a more cytotoxic effect when bound to the nano-

particles, with respect to that of the free Pt complexes at the same dose of administration. These results are in line with the literature, as it has already been reported that the use of drug carriers could facilitate drug uptake and accumulation by overcoming several limitations of traditional drugs.^{3,61}

The cell response also depends on the genetic and epigenetic characteristics of the cell lineage and on the acquisition of drug resistance upon exposure.⁶² Several drug resistance mechanisms have already been reported, from the overexpression of efflux pump Pgp to the activation of DNA repair mechanisms.⁶³ Thus, it might be assumed that under the same experimental conditions, the MDA-MB-231 cells exhibited some resistance to the Pt-based drugs (or at least a reduced sensitivity to drug action), since this behaviour has been partially identified over the last few years.⁶⁴

In an attempt to define the interactions between MDA-MB-231 or IGROV-1 cells and Pt compounds, a caspase-3 apoptotic assay was performed. The two cell lines were incubated with either the free Pt complexes or the MNP derivatives at a Pt concentration equal to 2 μM – a value that is close to the IC₅₀ of the most effective nanoconjugates.

As reported in Fig. 5, the activation of caspase 3, which is a common killing factor in the apoptotic process, was detected by using a fluorogenic substrate which is cleaved upon stimulation. It is evident that, while MNP-PEG induced an activation close to 20% in both cell lines, the free Pt compounds and their nanoconjugates triggered a significant activation of caspase 3. This process seemed to be more prominent in IGROV-1 cells than in MDA-MB-231 ones, and it was boosted in cells that had been administered with MNP bearing the Pt complexes with respect to those that had been incubated with free Pt molecules. These data were in accordance with those obtained by the MTT assay, and they confirmed that IGROV-1 cells are more sensitive than MDA-MB-231 cells to the Pt drugs.



Fig. 5 Caspase-3 assay performed with IGROV-1 and MDA-MB-231 cells incubated for 24 h with the free Pt complexes, the four MNP-Pt conjugates, and MNP-PEG. The results are reported as a percentage of the fluorescence intensity of each sample with respect to the untreated control cells. The values and the SD were obtained from 3 independent experiments. The *t*-test carried out on the two cellular data sets revealed a significant difference (*p* < 0.01) among the cells incubated with the free Pt complexes and those administered with the Pt-MNP conjugates.



To elucidate the contribution of the nanoparticle-mediated delivery in combination with the magnetic guidance, a quantitative estimation of Pt internalized by the cells was performed by elemental analysis (Fig. 7). Both IGROV-1 and MDA-MB-231 cells were treated for 24 h with either **PEG-Glu-Pt-EDA** or **MNP-PEG-Glu-Pt-EDA** with or without the application of a magnetic field. Then the cells were processed to detect Pt internalization for the Pt-MNP conjugates, the free Pt complexes and the free cisplatin. After 24 h, the highest intracellu-



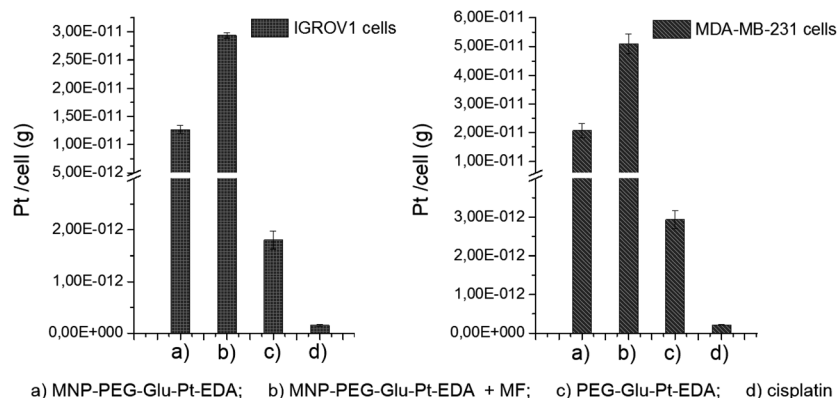


Fig. 7 Pt determination via elemental analysis on IGROV-1 cells (left panel) and MDA-MB-231 cells (right panel) incubated with either **MNP-PEG-Glu-Pt-EDA** (a) without and (b) with the application of a magnetic field (MF), or (c) the free **PEG-Glu-Pt-EDA** complex or (d) cisplatin for 24 h.

lar content of Pt was detected when the Pt complex was linked to the nanoparticle (reaching 1.2×10^{-11} g per cell in IGROV-1 and 2.0×10^{-11} g per cell in MDA-MB-231, respectively) and it was more than double when a magnetic field was applied under the well (2.9×10^{-11} g per cell in IGROV-1 and 5.0×10^{-11} g per cell in MDA-MB-231, respectively). However, the intracellular amount of Pt was around one order of magnitude smaller when the cells were administered with the free **PEG-Glu-Pt-EDA** complex (1.8×10^{-12} g per cell in IGROV-1 and 2.9×10^{-12} g per cell in MDA-MB-231, respectively). Remarkably, in the case of cisplatin the intracellular uptake was even less, since the Pt amount detected was 1.5×10^{-13} g per cell in IGROV-1 and 2.1×10^{-13} g per cell in MDA-MB-231, respectively.

These results may be explained on the basis of the poor solubility of the free cisplatin under physiological conditions, which would explain the low cytotoxicity measured by the MTT assay.

In contrast, the magnetic nanoparticles accelerated the Pt internalization, facilitating its uptake even without applying a magnetic field, probably through endocytosis, as already reported in the literature.¹² According to this process, the nanoparticles, once engulfed within vesicular structures, are usually confined to the perinuclear region where they remain localized unless a physicochemical process (mediated by the chemical composition or stimuli responsiveness of the nanoparticles, such the triazole moiety of the platinum complexes) is activated. The triazole moieties by reincorporating protons and counter ions from the cytoplasm into the endosomes may cause rupture or destabilization of the endolysosome membranes, and consequently the release of their content inside the cytosol.⁶⁶

With the aim of following the intracellular pathway of the MNP-Pt complex, structural analysis of the cells incubated with either **MNP-PEG-Glu-Pt-EDA** or MNP-PEG at sub-lethal doses (Pt concentration equal to 0.25 μ M) was performed by electron microscopy (Fig. 8). After 1 h of incubation, the nanoparticles were clearly present in the endosomal vesicles, and

the morphology of the endosomes containing either MNP-PEG (Fig. 8a) or **MNP-PEG-Glu-Pt-EDA** (Fig. 8b) was similar.

In another experiment, after the cells were incubated for 1 h with **MNP-PEG-Glu-Pt-EDA** or MNP-PEG, the cell medium was replaced with a fresh one that did not contain nanoparticles or the Pt complex, and the cultures were kept for an additional 24 h at 37 °C. The TEM images of the intracellular uptake pathway showed that the endosome structure was dramatically altered, they appeared swollen and exhibited the nanoparticles in a corona-like distribution (Fig. 8e and f). Indeed, it seems that the **MNP-PEG-Glu-Pt-EDA** conjugate promoted an osmotic imbalance, accompanied by water diffusion and a consequent swelling of the endosomes. This effect was not detected in the case of MNP-PEG that was incubated under the same conditions (Fig. 8c and d; additional TEM images in Fig. 4 and 5 of the ESI†). By analyzing and comparing the size distribution of the endosomal vesicles (Fig. 6SI†), it became apparent that they have a broad distribution in the cells incubated with the MNP-Pt conjugate, ranging from 100 to 800 nm, with two intense peaks at 250 and 500 nm, the latter population being the most abundant. In the case of MNP-PEG, the analysis showed a much more uniform distribution, between 200 and 400 nm, with a median endosomal size of 400 nm.

As suggested by our data, this peculiar behaviour can be attributed to the structure of the Pt complexes. Indeed, the presence of a triazole group has been often used as a pH-sensitive group to trigger endosomal protonation and drug escape in others types of cargo systems (*i.e.* cation lipids and modified antibody structures).^{67,68}

The triazole group in the Pt complex may facilitate the entrance of protons, anions and water, favouring the high osmotic pressure inside the endosome. The consequent swelling of the endosomal vesicles and the acidic hydrolysis of the platinum(II) complex enable the metal release and therefore the endosomal escape, as shown in Fig. 8SI.†⁶⁹

Interestingly, the TEM images suggest that the swelling process did not lead to the rupture of the endosomal vesicles but only to the increase of their volume, which likely allowed



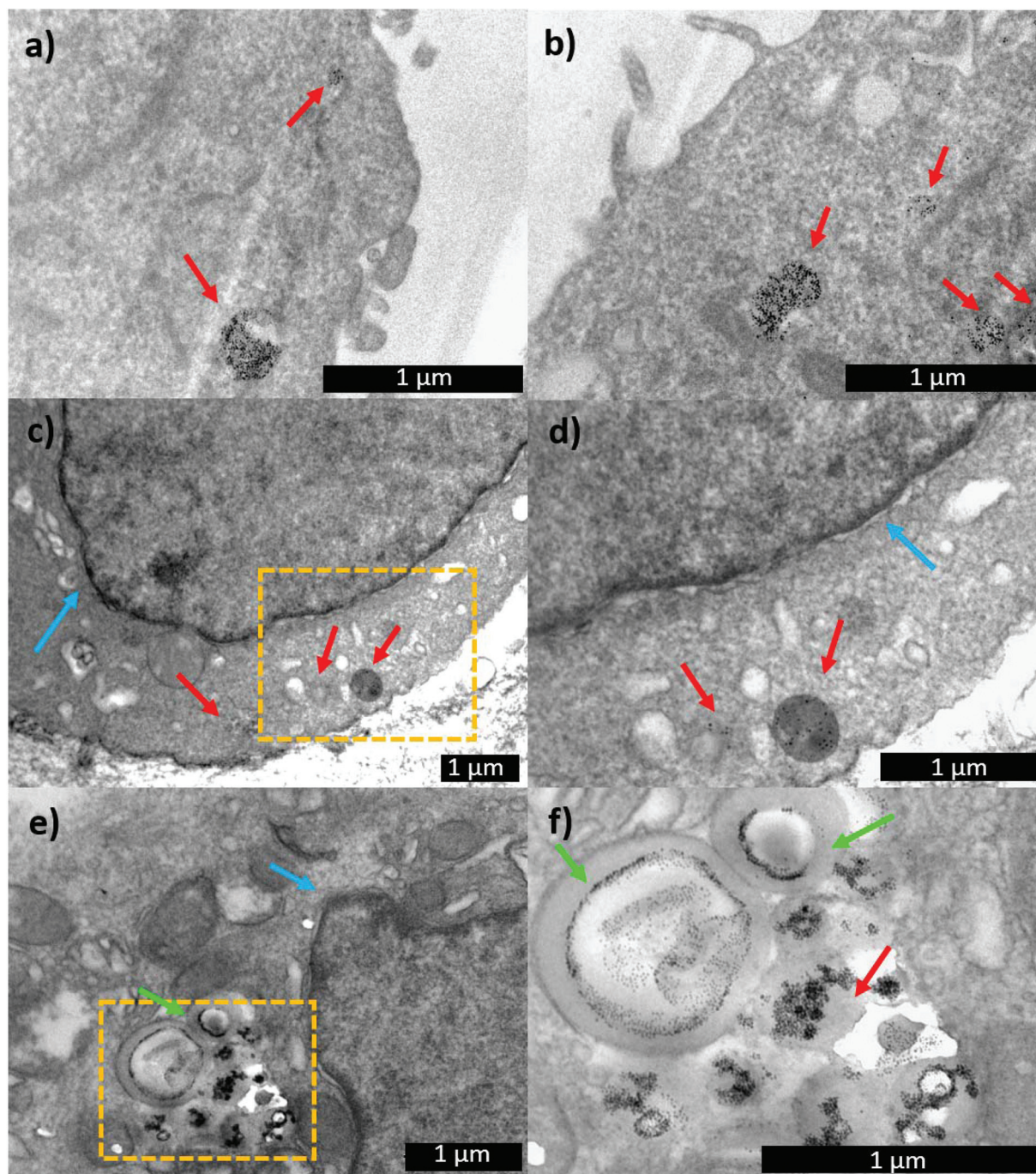


Fig. 8 TEM images of IGROV-1 cells incubated for 1 h with (a) MNP-PEG and (b) MNP-PEG-Glu-Pt-EDA. TEM images of IGROV-1 cells incubated for 24 h with (c and d) MNP-PEG and (e and f) MNP-PEG-Glu-Pt-EDA. The red arrows indicate the endolysosomes containing the nanoparticles. The green arrows indicate the swelling of the vesicular structures, observed only for MNP-PEG-Glu-Pt-EDA, in which the nanoparticles appear to be arranged in circular geometries. The blue arrows point to the nuclear membrane. (d) and (f) are the zoomed areas of (c) and (e), respectively.

for the Pt escape, while the bulkier MNPs remained confined to the endosomes (at least at sub-lethal doses), as already reported by some of us.⁷⁰

After 96 h, the enlarged vesicles were still visible in the cytosol, although they looked less swelled, likely as the consequence of the cellular attempt to restore the physiological endosomal sizes (Fig. 7SI†).

To verify if the platinum complex reaches the nucleus and binds itself to the deoxyguanosine residues, subsequently hindering the DNA replication process, as already reported,⁷¹ we have quantitatively determined the amount of intra-nuclear platinum. To this aim, the DNA of the cells incubated with either MNP-PEG-Glu-Pt-EDA or PEG-Glu-Pt-EDA (under the same conditions used for the estimation of the intracellular



Table 2 Nuclear Pt determination *via* elemental analysis on IGROV-1 cells and MDA-MB-231 cells incubated with either **MNP-PEG-Glu-Pt-EDA** or the free **PEG-Glu-Pt-EDA** complex for 24 h

	Sample name:	Pt (g per nucleus)
IGROV-1	MNP-PEG-Glu-Pt-EDA	$1.5 \times 10^{-12} \pm 1.2 \times 10^{-12}$
	PEG-Glu-Pt-EDA	$2.1 \times 10^{-13} \pm 2.6 \times 10^{-14}$
MDA-MB-231	MNP-PEG-Glu-Pt-EDA	$7.8 \times 10^{-13} \pm 1.7 \times 10^{-14}$
	PEG-Glu-Pt-EDA	$1.6 \times 10^{-13} \pm 7.6 \times 10^{-15}$

Pt) was extracted prior to the detection of Pt *via* elemental analysis. The results show that the absolute amount of intranuclear Pt is higher when the cells were incubated with the MNP bearing the Pt complex (1.5 and 0.78 pg in the case of IGROV-1 and MDA-MB-231, respectively) than with the free complex (0.21 and 0.16 pg in the case of IGROV-1 and MDA-MB-231, respectively) (Table 2).

Conclusions

In this study, four novel platinum complexes with amino terminated PEG chain linkers were synthesized and then attached to the surface of iron oxide nanoparticles. The platinum-based drugs were specifically designed to have a chelating dicarboxylic acid moiety, as in the case of oxaliplatin, either malonate or glutamate, and a diamino group, either EDA or DACH. The amino-PEG on the side chain of the platinum complex allowed its binding to iron oxide nanoparticles. The cytotoxicity assays showed that the MNP-Pt complexes were generally more cytotoxic than the corresponding free Pt-compounds and cisplatin used as a gold standard. This was directly related to the enhanced drug uptake when it is linked to the MNP rather than the free complex. In the case of the MNP-derivatives, internalization was further accelerated when an external magnetic field was applied.

Finally, a possible explanation of the mechanism through which the Pt complex detaches from nanoparticles and escapes from the endosomal compartment has been provided. As a first step, the pH-hydrolysis of the platinum–ligand complex on the platinum–MNP conjugates, also verified in a test tube, enables the uncoupling of the Pt ions from the nanoparticle surface, while the presence of a triazole moiety in the platinum complex promotes the swelling of the cellular endosomes, the latter observed under TEM analysis, allowing the endosomal escape of the platinum.

Indeed, the endocytic pathway often limits the intracellular transport of drugs/nanoparticles since most of the carriers and cargos become trapped within the nano-meso scale, generally entering the cell *via* an endocytosis mechanism and remaining confined within the vesicles without reaching their biological target.⁷²

Finally, the platinum intranuclear localization, as measured by platinum associated with the DNA, enables the execution of the anti-proliferation action. The highest cytotoxic effect was observed for the Pt-nanoconjugates containing the glutamate

ligand as the chelating dicarboxylate and EDA as the diamino group, yielding a nanodrug that proved to be more than order of magnitude more efficient than standard cisplatin. This nanoconjugate also reached lower IC₅₀ concentrations when compared to previous literature studies on Pt-based drugs bound to iron oxide nanoparticles, such as porous hollow Fe₃O₄ nanoparticles⁴³ and dumbbell-like Au–Fe₃O₄ nanoparticles⁷³ for cisplatin delivery, or other magnetic nanoparticles designed to deliver different platinum(IV) prodrugs,^{46,50} thus being a promising candidate for future *in vivo* studies. As a next step, efforts should be made to develop a single magnetic Pt-nanoconjugate that can merge the physical magnetic accumulation and cytotoxicity effects of the platinum compound with biomolecular targeting that can recognize receptors overexpressing on the specific tumor under treatment (such as AFRA antibody fragments that can link folate receptors overexpressing on ovarian cancer cells⁵⁵). This would make the anti-tumoral treatment very specific sparing healthy cells that do not express the receptors, and at the same time, it would require fewer doses of anti-tumoral agents thus minimizing side effects.

Materials and methods

Chemicals

1-[Bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (*N*-HBTU), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), alpha-amino acids, amines, *etc.* were purchased from Novabiochem, Applied Biosystems, Global Sales Manager, GL Biochem (Shanghai) Ltd, China, Sigma-Aldrich or Across. The azido-PEG-amine [N₃–CH₂–CH₂–(O–CH₂–CH₂)₁₀–O–CH₂–CH₂–NH₂] was purchased from Polypure AS (Oslo, Norway), and the Pt compounds [PtCl₂–EDA and K₂PtCl₄] were purchased from Alfa Aesar. QuadraSil® AP was purchased from Aldrich. All reagents and solvents were used as received unless otherwise stated. Solvent mixtures for chromatography are reported as v/v ratios. Column chromatography was performed on EM Science silica gel 60 (230–400 mesh). CH₂Cl₂ and DIEA were distilled from CaH₂ over argon immediately prior to use.

General techniques

¹H NMR spectra were recorded on a Bruker AMX 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. Chemical shifts (δ) were reported in parts per million (ppm, δ) relative to tetramethylsilane (δ = 0.00 ppm) or by the deuterium solvent. ¹H NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t), or quartet (q). Splitting patterns that could not be easily interpreted are designated as multiplet (m) or broad (br). ¹³C NMR spectra were recorded on a Bruker AMX 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. Carbon resonances were assigned by using DEPT spectra obtained with phase angles of 135°. Electrospray ionization (ESI) mass spectra were recorded on a Bruker BIONTOF II mass spectrometer.



Synthesis of Pt complexes

Azido-PEG-amine-Trt (A). A solution of **Azido-PEG-amine** [$\text{N}_3\text{-CH}_2\text{-CH}_2\text{-(O-CH}_2\text{-CH}_2\text{)}_{11}\text{-NH}_2$] (55 mg, 0.10 mmol) in CH_2Cl_2 (1 mL) was treated with trityl chloride (56 mg, 0.19 mmol) and DIEA (33.4 μL , 0.19 mmol). The mixture was stirred at room temperature for 12 h, and then an aqueous solution of NH_4Cl (sat. sol., 1 mL) was added. The solution was poured into a separation funnel and extracted with CH_2Cl_2 (3×1 mL). The combined organic layers were dried with anhydrous Na_2SO_4 and then they were filtered and concentrated under reduced pressure. The resulting residue was purified by flash chromatography (1–2% MeOH in CH_2Cl_2) to give 65 mg of **A** as a light-yellow oil [83%, $R_f = 0.40$ (5% MeOH/ CH_2Cl_2)]. $^1\text{H NMR}$ (CDCl_3 , 300 MHz, δ): 7.55–7.43 (m, 6H), 7.30–7.16 (m, 9H), 3.76–3.50 (m, 42H), 3.39 (t, $J = 5.1$ Hz, 2H), 2.36 (t, $J = 5.3$ Hz, 2H), 2.08 (br, 2H). $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz, δ): 146.1 (C), 128.7 (CH), 127.7 (CH), 126.2 (CH), 77.6 (C), 71.3 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 70.1 (CH_2), 70.0 (CH_2), 50.7 (CH_2), 43.1 (CH_2) ppm. **MS** (ESI-TOF $^+$) [m/z (%): 851.4 ([MK] $^+$, 12), 835.4 ([MNa] $^+$, 87), 813.5 ([MH] $^+$, 4), 593.3 ([MNa – Trt] $^+$, 48), 571.3 ([MH – Trt] $^+$, 100). **HRMS** (ESI) calculated for $\text{C}_{43}\text{H}_{64}\text{N}_4\text{NaO}_{11}$: 835.4464, found: 835.4474.

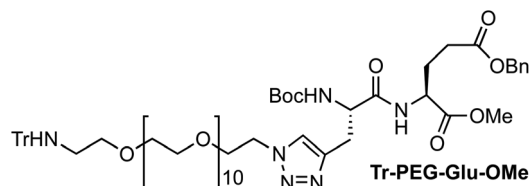
Boc-L-Pra-L-Glu(OBn)-OMe (B).



A solution of Boc-L-Glu(OBn)-OMe (108 mg, 0.31 mmol) in a mixture of TFA/ CH_2Cl_2 (1 : 1, 4 mL) was stirred at room temperature. After 15 min, the solvent was removed under reduced pressure and the resulting solid was dried under high vacuum for 3 h. The resulting TFA salt was dissolved in CH_2Cl_2 (3 mL), and then Boc-L-Pra-OH (66 mg, 0.31 mmol), *N*-HBTU (129 mg, 0.34 mmol) and DIEA (0.22 μL , 1.20 mmol) were successively added. The mixture was stirred at room temperature for 2 h and the solution was poured into a separation funnel and washed with an aqueous solution of HCl (10%, 3×3 mL) and NaHCO_3 (sat. sol., 3×3 mL). The organic layer was dried with anhydrous Na_2SO_4 , and then it was filtered and concentrated under reduced pressure. The resulting yellow oil was purified by flash chromatography (50% EtOAc in hexanes) to give 129 mg of the dipeptide **B** as a white foam [93%, $R_f = 0.55$ (50% EtOAc in hexanes)]. $^1\text{H NMR}$ (CDCl_3 , 500 MHz, δ): 7.29 (m, 5H), 7.12 (d, $J = 7.8$ Hz, 1H), 5.42 (d, $J = 8.0$ Hz, 1H), 5.06 (s, 2H), 4.61 (td, $J = 7.9$ and 5.1 Hz, 1H), 4.29 (br, 1H), 3.67 (s, 3H), 2.72 (ddd, $J = 17.0$, 5.8 and 2.7 Hz, 1H), 2.57 (ddd, $J = 17.0$, 5.8 and 2.3 Hz, 1H), 2.51–1.91 (m, 4H), 2.01 (t, $J = 2.7$ Hz, 1H), 1.43 (s, 9H) ppm. $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz, δ): 172.4 (CO), 171.8 (CO), 170.2 (CO), 155.3 (CO), 135.7 (C), 128.5 (CH), 128.4 (CH), 128.2 (CH), 80.4 (C), 79.3 (C), 71.7 (CH), 66.4 (CH_2), 52.9 (CH), 52.5 (CH), 51.6 (CH_3), 30.1 (CH_2), 28.2 (CH_3), 27.3 (CH_2), 22.3 (CH_2) ppm. **MS** (ESI-TOF $^+$) [m/z (%): 469.2 ([MNa] $^+$, 100), 413.1 ([MNa – *t*Bu] $^+$, 7), 369.1 ([MNa – Boc] $^+$, 8).

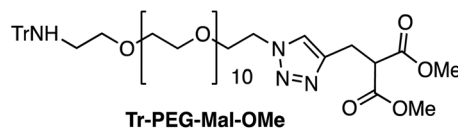
HRMS (ESI) calculated for $\text{C}_{23}\text{H}_{30}\text{N}_2\text{NaO}_7$: 469.1945, found: 469.1945.

Boc-L-Ala(triazole-PEG-amine-Trt)-L-Glu(OBn)-OMe (Tr-PEG-Glu-OMe).



A solution of Boc-L-Pra-L-Glu(OBn)-OMe (116 mg, 0.26 mmol) in a mixture of H_2O /MeOH (1 : 1, 40 mL) was treated with CuSO_4 (104 mg, 0.65 mmol) and sodium L-ascorbate (620 mg, 3.1 mmol). The mixture was stirred at room temperature for 15 min, and then a solution of azido-PEG-amine-Trt (**A**) (211 mg, 0.26 mmol) in a mixture of H_2O /MeOH (1 : 1, 5 mL) was added. The mixture was stirred at room temperature for 24 h, and then the solution was treated with QuadraSil® AP resin to remove any excess Cu. After stirring for 20 min, the resin was filtered and washed with MeOH, and then the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography (1–3% MeOH in CH_2Cl_2) to give 236 mg of **Tr-PEG-Glu-OMe** as a colorless oil [72%, $R_f = 0.60$ (10% MeOH/ CH_2Cl_2)]. $^1\text{H NMR}$ (CDCl_3 , 300 MHz, δ): 7.54 (s, 1H), 7.49–7.46 (m, 6H), 7.35 (m, 4H), 7.29–7.14 (m, 12H), 6.09 (br, 1H), 5.10 (s, 2H), 4.59–4.48 (br, 2H), 4.38 (q, $J = 5.1$ Hz, 2H), 3.77 (t, $J = 5.2$ Hz, 2H), 3.69 (s, 3H), 3.64–3.51 (m, 42H), 3.30 (dd, $J = 14.9$ and 5.2 Hz, 1H), 3.07 (dd, $J = 15.0$ and 5.4 Hz, 1H), 2.35 (t, $J = 5.4$ Hz, 2H), 2.24–2.08 (m, 4H), 1.45 (s, 9H) ppm. $^{13}\text{C NMR}$ (CDCl_3 , 75 MHz, δ): 172.3 (CO), 171.7 (CO), 171.2 (CO), 146.2 (CO), 143.3 (C), 135.8 (C), 128.7 (CH), 128.6 (CH), 128.4 (CH), 128.3 (CH), 127.8 (CH), 126.2 (CH), 123.6 (CH), 80.3 (C), 77.6 (C), 71.3 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 70.1 (CH_2), 69.4 (CH_2), 66.5 (CH_2), 54.0 (CH), 52.5 (CH), 51.5 (CH_3), 50.1 (CH_2), 42.1 (CH_2), 29.9 (CH_2), 28.3 (CH_3), 27.6 (CH_2), 27.4 (CH_2) ppm. **MS** (ESI-TOF $^+$) [m/z (%): 1281.7 ([MNa] $^+$, 12), 1259.7 ([MH] $^+$, 6), 1039.5 ([MNa – Trt] $^+$, 20), 1017.6 ([MH – Trt] $^+$, 100). **HRMS** (ESI) calculated for $\text{C}_{66}\text{H}_{94}\text{N}_6\text{O}_{18}$: 1259.6697, found: 1259.6718.

Tr-PEG-Mal-OMe.

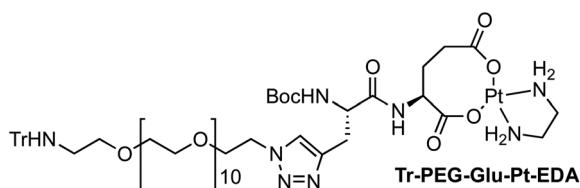


A solution of dimethyl 2-(prop-2-yn-1-yl)malonate (48 mg, 0.28 mmol) in a mixture of H_2O /MeOH (1 : 1; 46 mL) was treated with CuSO_4 (204 mg, 1.27 mmol) and sodium L-ascorbate (1.26 g, 6.37 mmol). The mixture was stirred at room temperature for 15 min, and then a solution of azido-PEG-amine-Trt (**A**) (207 mg, 0.25 mmol) in a mixture of H_2O /MeOH (1 : 1, 4 mL) was added. The mixture was stirred at room temperature for 24 h, and then the solution was treated with QuadraSil® AP resin to remove any excess Cu. After stirring for 20 min, the resin was filtered and washed with MeOH,



and then the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography (1–10% MeOH in CH₂Cl₂) to give 178 mg of **Tr-PEG-Mal-OMe** as a light-yellow oil [71%, *R_f* = 0.54 (10% MeOH/CH₂Cl₂)]. ¹H NMR (CDCl₃, 300 MHz, δ): 7.62 (s, 1H), 7.51–7.47 (m, 6H), 7.30–7.16 (m, 10H), 4.51 (t, *J* = 5.0 Hz, 2H), 3.93 (br, 1H), 3.86 (t, *J* = 5.0 Hz, 2H), 3.74 (s, 6H), 3.66–3.51 (m, 42H), 3.35 (br, 2H), 2.36 (t, *J* = 5.4 Hz, 2H) ppm. ¹³C NMR (CDCl₃, 75 MHz, δ): 168.9 (CO), 145.9 (C), 128.5 (CH), 127.6 (CH), 126.0 (CH), 71.0 (CH₂), 70.4 (CH₂), 70.2 (CH₂), 69.9 (CH₂), 69.2 (CH₂), 52.5 (CH₃), 51.1 (CH), 50.2 (CH₂), 42.8 (CH₂), 24.8 (CH₂) ppm. MS (ESI-TOF⁺) [*m/z* (%): 1005.5 ([MNa]⁺, 9), 983.5 ([MH]⁺, 8), 763.4 ([MNa – Trt]⁺, 11), 741.4 ([MH – Trt]⁺, 100). HRMS (ESI) calculated for C₅₁H₇₅N₄O₁₅: 983.5223, found: 983.5257.

Tr-PEG-Glu-Pt-EDA

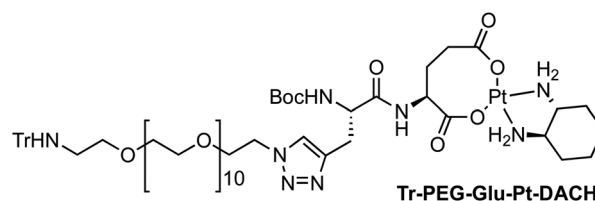


A solution of dichloro(ethylenediamine)platinum(II) (31 mg, 0.10 mmol) in H₂O (2 mL) was treated with AgNO₃ (32 mg, 0.19 mmol). The mixture was protected from light using aluminium foil, and it was stirred under Ar at 60 °C for 20 h. After this time, the mixture was cooled down to room temperature, filtered through a Celite pad and washed with H₂O (3 × 2 mL). The solvent was concentrated under reduced pressure until approximately 1 mL.⁶⁰

Compound **Tr-PEG-Glu-OMe** (40 mg, 0.032 mmol) was dissolved in MeOH (1 mL) before being treated with an aqueous solution of NaOH (0.5 M, 320 μL, 0.16 mmol), and then the mixture was stirred at room temperature for 12 h. After HPLC-MS analysis had confirmed the disappearance of the starting material, the solvents were concentrated under reduced pressure and the solid was dried under high vacuum.

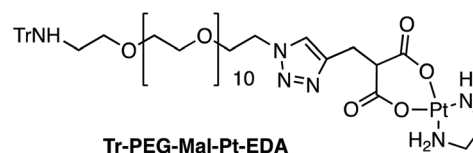
The resulting residue was treated with the [Pt(NO₃)₂EDA] aqueous solution, which had previously been prepared, and the solution was stirred at 60 °C for 24 h. Then the reaction mixture was cooled down to room temperature and concentrated under reduced pressure. The resulting residue was dissolved in H₂O, and the solution was cooled in an ice/water bath. This formed a precipitate, which was then removed by centrifugation and decantation. The solution was lyophilized, giving 45 mg of **Tr-PEG-Glu-Pt-EDA** as a yellow solid (quantitative). ¹H NMR (D₂O, 300 MHz, δ): 7.96 (s, 1H), 7.44–7.18 (m, 6H), 7.15–6.79 (m, 9H), 4.62 (br, 2H), 4.23 (m, 1H), 3.95 (br, 2H), 3.80–3.22 (m, 43H), 3.00–2.55 (m, 6H), 2.52–1.87 (m, 6H), 1.38 (s, 9H) ppm. MS (ESI-TOF⁺) [*m/z* (%): 1190.5 ([MNa – Trt]⁺, 3), 1168.5 ([MH – Trt]⁺, 100), 935.5 ([MNa – Trt – Pt-EDA]⁺, 6), 913.5 ([MH – Trt – Pt-EDA]⁺, 584.2 ([MH – Trt]²⁺, 1). HRMS (ESI) calculated for C₄₁H₈₀N₈O₁₈Pt: 1167.5236, found: 1167.5256.

Tr-PEG-Glu-Pt-DACH



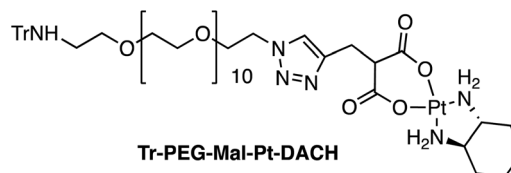
This compound was prepared following the same procedure described above for **Tr-PEG-Glu-Pt-EDA**, but [Pt(Cl₂)(1*R*,2*R*)-DACH] (36 mg, 0.10 mmol) and compound **Tr-PEG-Glu-OMe** (30 mg, 0.024 mmol) were used to give 35 mg of **Tr-PEG-Glu-Pt-DACH** as a yellow solid (quantitative). ¹H NMR (D₂O, 300 MHz, δ): 7.97 (s, 1H), 7.40–7.18 (m, 6H), 7.11–6.81 (m, 9H), 4.65 (m, 2H), 4.24 (m, 1H), 3.98 (br, 2H), 3.79–3.12 (m, 43H), 2.60–1.85 (m, 10H) 1.83–1.06 (m, 17H) ppm. MS (ESI-TOF⁺) [*m/z* (%): 1484.6 ([MNa]⁺, 1), 1462.7 ([MH]⁺, 1), 1242.5 ([MNa – Trt]⁺, 7), 1220.6 ([MH – Trt]⁺, 100), 1177.6 (2), 935.5 ([MNa – Trt – Pt-DACH]⁺, 8), 913.5 ([MH – Trt – Pt-DACH]⁺, 13). HRMS (ESI) calculated for C₆₄H₉₉N₈O₁₈Pt: 1462.6724, found: 1462.6692.

Tr-PEG-Mal-Pt-EDA

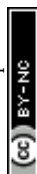


This compound was prepared following the same procedure described above for **Tr-PEG-Glu-Pt-EDA**, but dichloro(ethylenediamine)platinum(II) (29 mg, 0.09 mmol) and compound **Tr-PEG-Mal-Me** (30 mg, 0.03 mmol) were used to give 36 mg of **Tr-PEG-Mal-Pt-EDA** as a dark-yellow solid (quantitative). ¹H NMR (D₂O, 300 MHz, δ): 8.06 (s, 1H), 7.45–7.15 (m, 6H), 7.08–6.76 (m, 9H), 4.66 (br, 2H), 3.99 (d, 2H, *J* = 16.3 Hz), 3.80–3.13 (m, 45H), 3.01–2.44 (m, 6H), 2.22 (m, 2H) ppm. MS (ESI-TOF⁺) [*m/z* (%): 967.4 ([MH – Trt]⁺, 5), 922.4 ([MH – Trt – CO₂]⁺, 100), 713.4 ([MH – Trt – Pt-EDA]⁺, 18), 691.4 ([MNa – Trt – Pt-EDA – CO₂]⁺, 23), 669.4 ([MH – Trt – Pt-EDA – CO₂]⁺, 62). HRMS (ESI) calculated for C₃₂H₆₄N₆O₁₅Pt: 967.4077, found: 967.4063.

Tr-PEG-Mal-Pt-DACH



This compound was prepared using the same procedure described above for **Tr-PEG-Glu-Pt-EDA**, but [Pt(Cl₂)(1*R*,2*R*)-DACH] (34 mg, 0.09 mmol) and compound **Tr-PEG-Mal-Me** (30 mg, 0.030 mmol) were used to give 35 mg of the mentioned compound as a dark yellow solid (quantitative). ¹H NMR (D₂O, 300 MHz, δ): 8.03 (s, 1H), 7.61–7.16 (m, 6H), 7.12–6.72 (m, 9H), 4.67 (br, 2H), 4.01 (br, 2H), 3.83–3.15 (m, 43H), 2.84–1.92



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