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Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com

Enhanced cytotoxicity of gold porphyrin complexes after inclusion in cyclodextrin scaffolds adsorbed on polyethyleneimine-coated gold nanoparticles

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Gold nanoparticles
PEI
Gold porphyrins
Colorectal cancer
Microwave

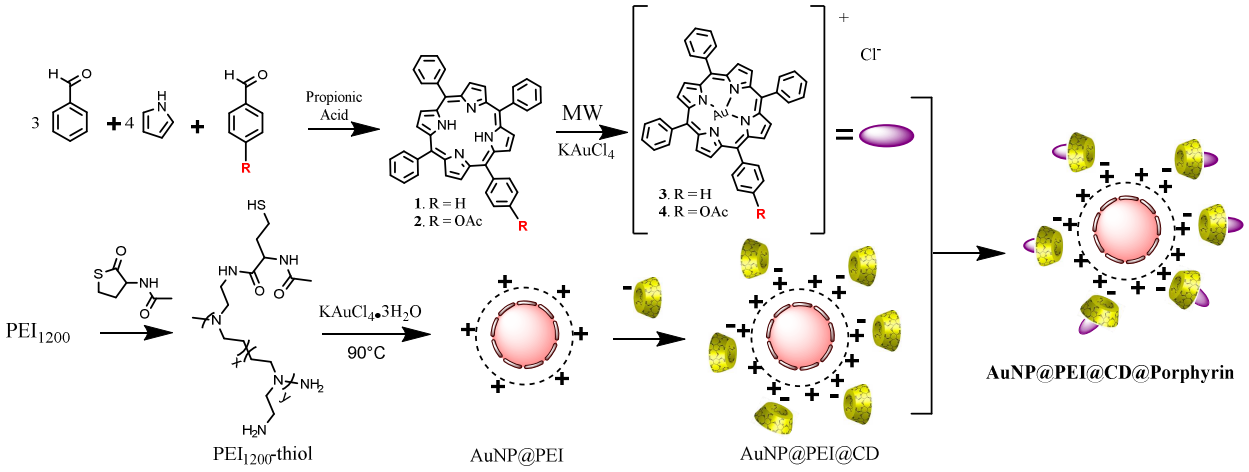
ABSTRACT

A new gold nanoparticle-based construct has been designed to hydrophobic drugs delivery into cancer cells. Cyclodextrin scaffolds adsorbed on polyethyleneimine-coated gold nanoparticles (AuNP@PEI@CD) have been used to encapsulate hydrophobic tetrapyrrolic compounds consisting of gold complexes of 5,10,15,20-tetraphenyl porphyrin (AuTPPCl) and 5-(4-acetoxyphenyl)-10, 15, 20-triphenyl porphyrin (AuTPPOAcCl). These two nanoparticles have been tested for their cytotoxic activities against the two colorectal cancer cell lines HT-29 and HCT-116 and have shown significant increases in toxicity when compared to the corresponding non-vectorized tetrapyrrolic macrocycles.

Colorectal cancer is the third most common cancer worldwide and is responsible for almost 30% of cancer deaths.[1] Combination chemotherapy is usually given when surgery has failed. First line chemotherapy for colorectal cancer is generally based on 5-fluorouracil or irinotecan in combination with oxaliplatin.[2] However, oxaliplatin, as well as cisplatin, which are among the most active drugs against colon cancer, may generate severe side effects along with induction of drug resistance; these drawbacks hamper a long term clinical use of these chemicals.[3]

For decades-long, gold derivatives have been used against various diseases.[4-7] Au^{III} and Pt^{II} lead to tetracoordinated complexes with the same square planar geometry. One could expect for Au^{III} complexes an activity comparable to that of cisplatin. In fact, a number of Au^{III} coordinates, tested against a broad spectrum of tumor cells, actually displayed inhibitory effects, although less prominent than those reported with platinum complexes.[7] On the other hand, Au^{III} coordinates have been shown to act upon more cellular or molecular targets as compared to cisplatin, e.g. inhibition of thioredoxin reductase, proteasome inhibition and modulation of specific kinases.[8,9] Moreover, they have displayed anti-cancer activity against both cisplatin- and multidrug-resistant xenografts.[10] Among these compounds, gold porphyrins complexes are particularly stable and have been the subject of some studies.[11-15] For example, Chen et al showed that five gold(III)-tetraphenylporphyrin derivatives are more cytotoxic that cisplatin against seven selected human cancer cell lines. In particular, gold(III) tetraphenylporphyrin complexe was the more efficient.[12]

Recently, our team showed that gold (III) 5,10,15 triphenyl-20-(4-aryl) porphyrin complexes with aryl group substituted by an acetyl function or adamatanyl group were able to inhibit in vitro, proliferation of Colorectal Cancer (CRC) cell lines HT-29 and HCT-116.[15] Among the many gold (III) complexes of tetraarylporphyrin, tetraphenylporphyrin is one of the more lipophilic macrocycle and one of the most active of these compounds.[13] However this large activity enhancement is counterbalanced by the poor solubility of these complexes in aqueous media. A way to circumvent this difficulty could consist in a noncovalent association of the porphyrin complex with a carrier with a hydrophilic surface. [16,17] Moreover, *in vivo*, this carrier could selectively vectorize the active substance to the tumor, thanks to the enhanced permeation and retention (E.P.R.) effect.[18] Nanoparticles with a size comprised between 20 and 100 nm are selectively retained by the leaky structure of the tumor neovascularization and, conversely, are not absorbed by healthy tissues. Moreover, positively charged nanoparticles are prone to be actively endocytosed by cancerous cells.[19,20] Consequently, we resolved to design gold nanoparticles coated with a layer of polyethyleneimine (PEI). A one-pot synthesis of gold nanoparticles protected by polyethyleneimine was envisaged, since it has been recently shown that PEI can be used as both a catalyst and a capping agent for the synthesis of gold nanoparticles.[21,22] Unfortunately these gold nanoparticles suffer from poor stability unless high molecular weight PEIs are being used, but high molecular weight PEIs are somewhat toxic to normal cells.[23] So we envisaged to graft a thiol function on a low molecular weight PEI in order to obtain a stable binding of PEI to the gold surface, thus enhancing the stability of the nanoparticles. The layer-by-layer technique allowed us to introduce a capping with anionic β-cyclodextrin which would be able to encapsulate hydrophobic tetraarylporphyrins. Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six (α-), seven (β-) or eight (γ-) D-glucopyranose units linked by α-(1,4) glycosidic bonds. They are well known to form stable inclusion complexes with a variety of guest molecules[24]. β-CD is the most commonly used, due to its relatively easy synthesis, its low price and also to the large number of polar molecules which can fit into its internal cavity. Metallation of porphyrins by gold salts is usually realized in acetic acid at reflux for several hours.[11-13] However, in a recent work on the metallation of porphyrins by lanthanides, we showed that this reaction was greatly facilitated by microwave (MW) activation.[25] In addition, we showed that the reaction yield was largely improved by lithium ions. So, we thought to apply this methodology, in the presence of lithium ions, for the metallation of porphyrins by gold salts. [25,26] The synthesis of the different gold porphyrins and their encapsulation in cyclodextrin-capped gold nanoparticle is presented in scheme 1.



Scheme 1. General pathway of AuNP@PEI@CD@Porphyrin synthesis

Tetraphenylporphyrin (H_2TPP, **1**) was synthesized according to the Adler and Longo method starting from benzaldehyde and pyrrole.[27] The unsymmetrical 5-(4-acetoxyphenyl)-10, 15, 20-triphenyl porphyrin (H_2TPPOAc, **2**) was obtained by the mixed aldehydes method of Little.[28] *Para*-acetoxybenzaldehyde was synthesized by reaction of *para*-hydroxybenzaldehyde and acetic anhydride with DMAP (4-DimethylaminoPyridine) as catalyst.[15] Porphyrins were characterized by UV-Visible, NMR and mass spectrometry. In previous works, we have reported a catalytic effect of lithium ions on the metallation of porphyrins with copper, [26] erbium and gadolinium. [25] Moreover it has been shown that a considerable enhancement of the reaction yield was

obtained by microwave irradiation. [25] The goal of this work was to study these parameters on the metallation of tetraphenyl porphyrins by gold salts. All the experiments were conducted in acetic acid at 100 °C with a reaction time of 3 min and with 2 equivalents of $KAuCl_4$. The lithium amount added varied from 5 to 100 equivalents. The amount of metallated porphyrin was determined by UV-Visible spectroscopy by measuring absorbance at 526 nm (metallated) versus non metallated (acidic form) at 660 nm. The percentage of metallation of H_2TPP as a function of Li^+ equivalents is shown in Fig. 1.

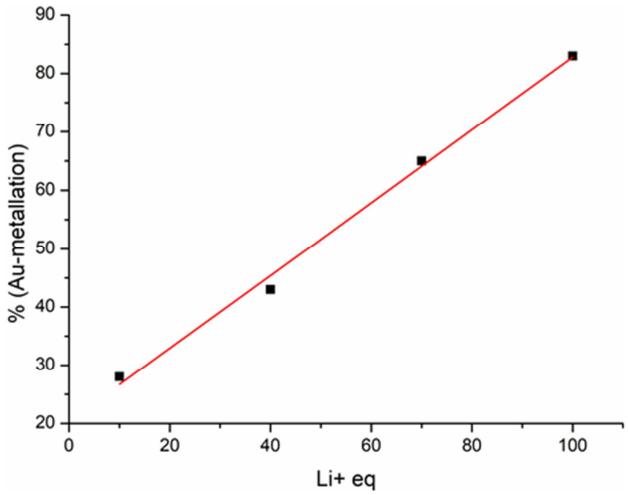


Figure 1: Percent metallation as function of Li^+ eq at 100 °C

The percentage of metallation increases linearly with the amount of added lithium. This result is in accordance with the effect previously reported for the metallation of tetratolylporphyrin with erbium or gadolinium.[25]

Moreover, microwave activation considerably reduces reaction time: only 3 minutes are required for a complete reaction instead of two hours at reflux as reported in the literature. [11-13] Gold metallation resulted in the formation of AuTPPCl (**3**) and AuTPPOAcCl (**4**).

One-pot synthesis of gold nanoparticles with high molecular weight polyethyleneimine (25 kDa PEI) has been recently reported; these particles benefit from a satisfying stability.[21,22] Unfortunately, this kind of PEI can hardly be considered for *in vivo* treatment, due to their significant toxicity. [23] Our previous works showed that the use of low molecular weight PEI (from 0.6 to 2 kDa) which displays low toxicity, led in the formation of unstable nanoparticles. [16] So, we decided to provide PEI with a thiol function, able to anchor the PEI chains onto the gold particle surface. In order to minimize the toxicity of polyamines against normal cells we chose to work with a very low molecular weight PEI (1.2 kDa). PEI_{1200} was reacted with 3-acetamidotetrahydro-2-thiophenone in dichloromethane at RT (Room Temperature) for 24 hours. After evaporation of dichloromethane and dissolution in water, a dialysis with a membrane with a 500 Da cutoff was realized. A degree of substitution of 1.3 was determined by elemental analysis (see Supplementary Data). Nanoparticles were synthesized by adding various amounts of PEI-thiol to a solution of $KAuCl_4$ at 90 °C for 30 minutes. The average diameters of the resulting AuNP@PEI, determined by UV-Visible spectroscopy [28] and by transmission electron microscopy (Fig. 2) are reported in Table 1. Particles diameter slightly drops while PEI–thiol concentration increases.

Table 1: Diameter of gold nanoparticles (AuNP@PEI) determined by UV-vis spectroscopy and TEM

Ratio (m_{PEI} / m_{Au})	0.36	0.4	0.46
UV-Visible (d.nm ; λ.nm)	46 ; 530	40 ; 527	22 ; 523
TEM (d.nm)	35	40	15

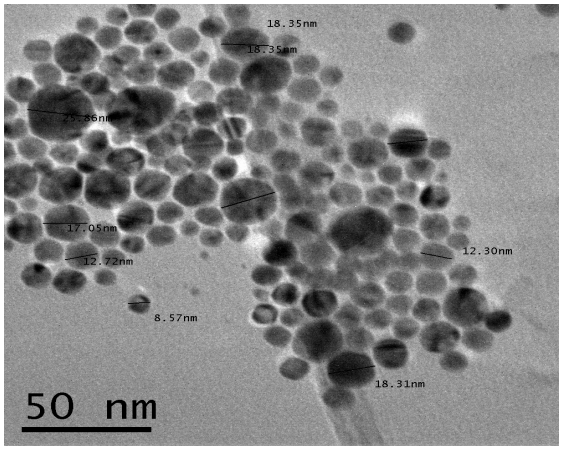


Figure 2: TEM images of gold nanoparticles (AuNP@PEI) with ratio (m_{PEI}/m_{Au} = 0.46)

Finally, commercial sulfated β-cyclodextrin was added to a solution of AuNP@PEI in water and the mixture was left 1 hour at RT, letting the negatively charged cyclodextrin to bind the positively charged AuNP@PEI. The resulting nanoparticles (AuNP@PEI@CD) were purified by centrifugation and resuspended in DI (Dionized) water. AuNP@PEI@CD were characterized by UV-Visible (Fig.3), dynamic light scattering (DLS) and zeta potential measurements. The metallated porphyrin was then encapsulated into cyclodextrin. The amount of entrapped porphyrin was calculated, after centrifugation, by subtracting the amount of porphyrin contained in the supernatant (determined by UV-Visible) from the total amount of porphyrin introduced.

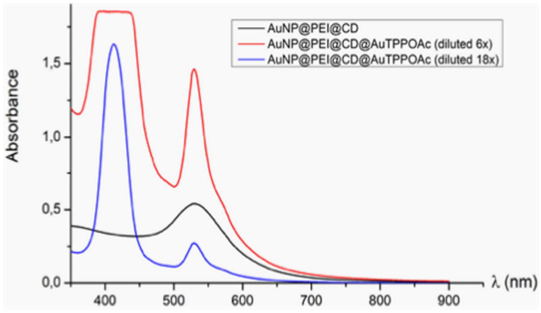


Figure 3: UV-Visible spectra of gold nanoparticles AuNP@PEI@CD alone and with encapsulated gold porphyrin AuTPPOAcCl.

Encapsulated AuTPPCl and AuTPPOAcCl represented 6 and 9.5% (w/w) of the total mass of the respective nanoparticles. The acetate moiety contributes to an enhanced encapsulation yield of the porphyrin into the cyclodextrin ring. The measured diameters of the different nanoparticles (AuNP@PEI, AuNP@PEI@CD, AuNP@PEI@CD@AuTPPCl, and AuNP@PEI@CD@AuTPPOAcCl) determined by Dynamic Light Scatting (Fig. S1, S2 and S3), are reported in table S1. AuNP@PEI size observed by DLS corresponds to a diameter of 30 nm (Table S1). Addition of β-cyclodextrin resulted in an increased size (up to 45 nm) and a second increase was observed (up to 70 nm) after inclusion of gold porphyrins. AuNPs bearing polyethyleneimine exhibited a large positive surface charge (ζ potential) of 43.2 mV. After addition of the negatively charged β-cyclodextrin this value dropped down to 16 mV but remained relatively unchanged after addition of gold porphyrins.

Gold porphyrins (compounds **3** and **4**) and nanoparticles bearing gold porphyrins were evaluated for their cytotoxicity against two colorectal cancer cell lines, HT 29 and HCT-116. Cell viability was assessed by the 3-(4,5-dimethyl-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) staining method after a 48 hour incubation. Cell viability *versus* gold porphyrin concentration (AuTPPCl, AuTPPOAcCl and AuNP@PEI@CD@AuTPPCl, AuNP@PEI@CD@AuTPPOAcCl) is reported in Fig. 4 for each cell line.

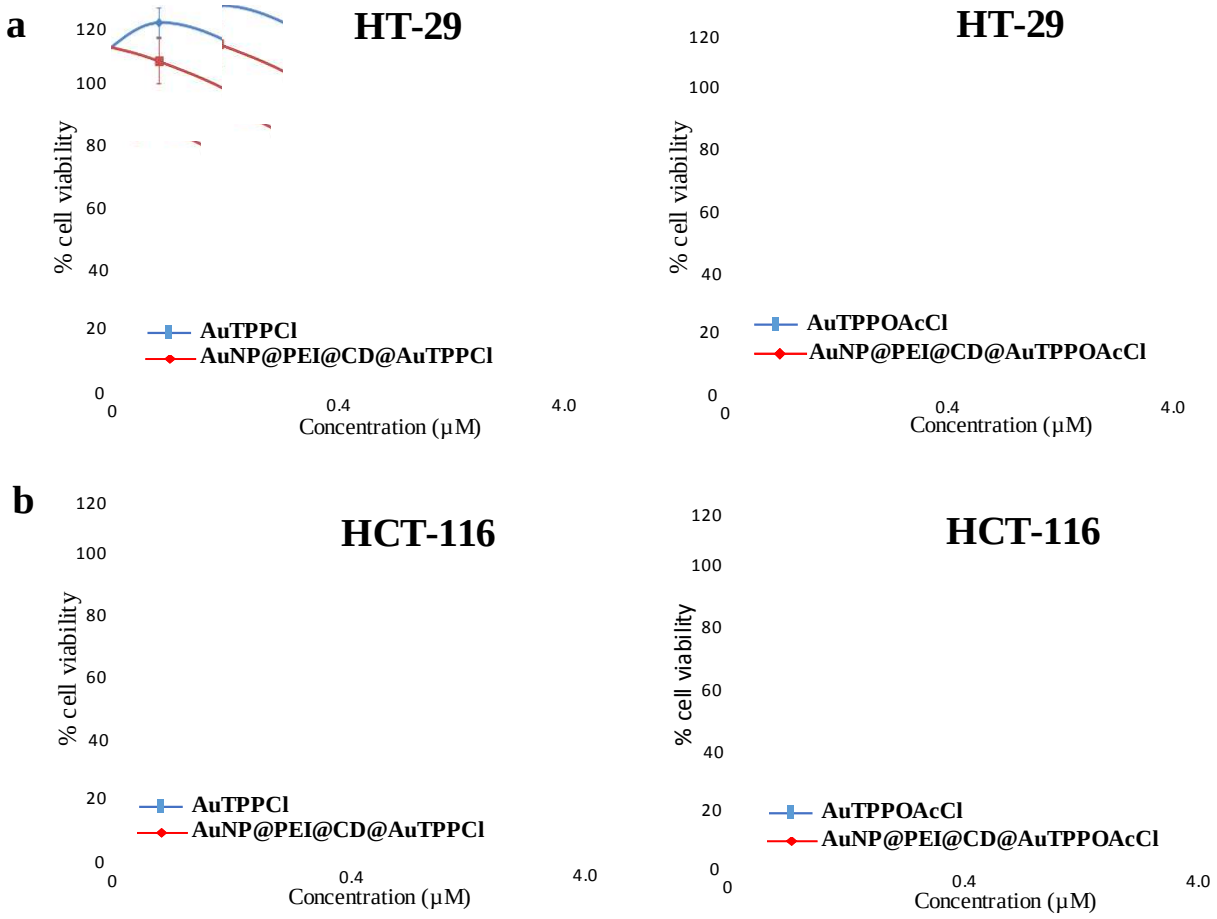


Figure 4: Cell viability of HT-29 (a) and HCT-116 (b) for 48h in the presence of increasing amounts of gold porphyrins (3 and 4) and the same porphyrins entrapped in gold nanoparticles. Each value represents the mean±SD of three separate experiments. The concentration of AuNP@PEI@CD@AuTPPCl and AuNP@PEI@CD@AuTPPOAcCl were expressed as equivalent of gold porphyrin (3 or 4) concentration.

Controls conducted in presence of gold nanoparticles coated with PEI and cyclodextrin (AuNP@PEI@CD) showed that these particles did not exhibit any cytotoxicity (up to a concentration corresponding to a value of 5 µM in porphyrin) against either of the two cell lines. IC_{50} deduced from these curves are shown in Table 2.

Table 2: IC_{50} of free AuTPPCl (3), AuTPPOAcCl (4) and AuNP@PEI@CD@AuTPPCl and AuNP@PEI@CD@AuTPPOAcCl complex after 48h Incubation in HT-29 and HCT-116 cells. [a] Concentration corresponding to a value of 5µM of PS.

	IC_{50} (µM of PS)				
Cell lines	AuTPPCl	AuNP@PEI@CD@AuTPPCl	AuTPPOAcCl	AuNP@PEI@CD@AuTPPOAcCl	AuNP@PEI@CD[a]
HT-29	2.23 ± 0.1	0.69 ± 0.02	3.65 ± 0.1	0.74 ± 0.05	> 5.0
HCT-116	0.42 ± 0.02	0.33 ± 0.01	0.48 ± 0.03	0.22 ± 0.01	> 5.0

IC_{50} values ranged from 0.42 to 2.3 µM. These values are similar to those found elsewhere for this compound tested against a panel of colon cancer cells (0.20 – 3.43 µM).[30] The introduction of the acetoxy group does not modify significantly the cytotoxicity of this molecule. Encapsulation of either of the two gold porphyrin resulted in strong increases in cytotoxicity (3 and 5 fold) against the HT-29 cell line. Smaller effects (1.5 and 2 fold) were observed against HCT-116; however this cell line is more sensitive to the free compounds (0.69 – 0.74 µM). These results are comparable to previous findings where encapsulation of curcumin by cyclodextrin-cellulose-nanocrystals complexes resulted in a 3 fold increase in cytotoxicity against HT-29 cell line.[16]

We describe in the present work and for the first time, a new, easy and efficient method for the synthesis of gold porphyrins which uses microwave activation and the catalytic effect of lithium ions. These gold porphyrin complexes where successfully encapsulated in β-cyclodextrin-capped gold-PEI nanoparticles. This encapsulation resulted in an enhancement of gold porphyrin cytotoxicities against two colon cancer lines by increasing the drug penetration into the cells. This new platform (AuNP@PEI@CD) could be a very efficient new anti-cancer hydrophobic drug delivery system, for *in vivo* application.

Acknowledgements

We thank the 'Conseil Regional de Nouvelle Aquitaine' for financial support. The authors are indebted to Dr. Michel Guilloton for help in editing the manuscript, Pierre Carles (CARMALIM-IRCER— Limoges) for MET analysis and Yves Champavier from BISCEm core facility for NMR analysis.

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Graphical Abstract

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