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HIV-1 Tat peptide-Gemcitabine gold (III)-PEGylated complex –Nano flowers: A sleek thermosensitive hybrid nanocarrier as prospective anticancer.

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ABSTRACT

This paper focuses on the synthesis of novel metal-drugs nanoparticles based onto gold-gemcitabine complexes relevant for biomedical applications. Gold (HAuCl_4) are mixed with antitumor compounds (gemcitabine (GEM)) and dicarboxylic acid-terminated polyethylene-glycol (PEG) that plays a double role as surfactants and reducing agent. The proposed methodology is fast and reproducible and leads the formation of hybrid-metal nanoflowers called GEM ION-PEG-AuNPs, in which drug solubility and efficiency were improved. For therapeutic purposes, we introduce the emerging roles of GEM ION-PEG-AuNPs in the hyperthermic treatment of cancer through preliminary study based on temperature effect on colloidal solutions under specific experimental conditions. For this purpose GEM ION-PEG-AuNPs were decorated with HIV-1 Tat peptide that is a potent trans-activator of transcription from the viral promoter and is essential for viral replication containing an activation domain and a nucleic acid-binding domain. All products were evaluated by various spectroscopic techniques (Raman and UV-Vis spectroscopies) and transmission electron microscopy (TEM). This study, meaning an original chemical approach, will occupy an important position in the field of Nanomedicine, with novel perspectives that will be proposed for the development of new drugs and active targeting in cancer therapy.

KEYWORDS: AuNPs, Gemcitabine, HIV-1 Tat peptide, Raman Spectroscopy, gold complex, nanovector, hyperthermia.

1. Introduction

In the recent years, nanotechnologies applied to Medicine (e.g. Nanomedicine) have led to propose engineered tunable devices with the size of the order of nanometers as an intriguing tool potentially able to solve unmet problems in healthcare ^[1]. Smart nanomaterials and advanced nanotechnologies required the rapid development of original protocols for biomedical applications ^[2]. Among them, Gold Nanovectors are fascinating remarkable interest as workable biomedical materials and the research effort about this subject is continuously growing, due to their unique physical and biochemical properties ^[3]. The utilization of biomolecules and/or biopolymer-grafted AuNPs are widely used as biomarkers in cancer early detection and as *in vivo* drugs delivery systems in medicine/pharmacy applications, and also in beauty products ^[4].

Nanocarrier-based drug delivery systems afford key advantages to the conventional treatment modes: controlled release and targeted delivery. The ability to keep the drug inactive, while in transit, and release it in its active form, under the appropriate physiochemical conditions ^[5], mitigates the collateral damage to healthy tissue, which in conventional approaches is an inherent consequence that goes largely unchecked. Additionally, through multifunctionalization, nanocarriers can contain not only anticancer payloads but also ligands that preferentially bind to surface receptor proteins of cancer cells and mediate endocytosis of the nanocarrier. Recently, J.Spadavecchia et al. have developed original nanovectors based on drugs-gold-complex (i.e. doxorubicin, docetaxel, paclitaxel), according to a chelation methodology in which the drug was complex with tetrachloauric acid (HAuCl_4) and polymer molecules (PEG diacide) before reduction to final nanovector^[2c, 6]. Chemical-physical characterizations and biological studies of drugs-gold-complex, have showed a remarkable interest in its therapeutic activity to *in vitro* model of Pancreatic Ductal Adeno Carcinoma (PDAC) ^[2c, 4]. Other authors have studied the mechanism of hybrid nanoparticles using biocompatibles polymers as capping agent ^[7]. Behind anticancer drugs of interest,

Gemcitabine is a cytosine analogue (**Scheme S1 Supporting Informations**) that represent an antineoplastic agent used in the therapy of several forms of advanced, pancreatic, lung, breast, ovarian and bladder cancer^[8]. A serious limitation of the drug is its in vivo half-life of ~20 min, resulting from deamination by deoxycytidilate deaminase^[9].

In order to checked the release of GEM, several nanocarriers were carried out under specific conditions of the synthesis and surface functionalization^[10]^[9]. Many authors have been used micelles and metal (Au, Ag, or Cu) to grafted GEM as nanocarrier^[8c]^[10]. Others authors, have been grafted GEM on the NPs through a self-assembled monolayer (SAM) of functional thiols and folic acid (FA) or transferrin (TF) can improve targeted controlled-release of the drug, reducing the side effects of the drug and improving efficacy^[11]. During the last decade several cell penetrating peptides, such as the HIV-1 tat peptide, have been shown to traverse the cell membrane, where integral protein transduction domains (PTDs) are responsible for their cellular uptake, and to reach the nucleus while retaining biological activity^[12]. It has since been discovered that PTDs can enable the cellular delivery of conjugated biomolecules and even nanoparticles, but nuclear delivery has remained problematic^[12]. This study provides , for the first time, the basis for the development of a stable and biocompatible gemcitabine gold nanovector like flower-shape, conjugated with the HIV-1 Tat PTD with the objectif, to generate nuclear anticancer targeting agents . The particles were successively assayed in vitro with pancreatic adenocarcinoma PDAC cell line (PANC-1), in order to understand the bio-chemical behavior of peptide-gold nanovector after cell internalization. The therapeutic design introduced in this paper represent a novel approach to enhance the efficacy of gemcitabine, with the utilization of an active peptide targeting delivery system, employed as thermosensitive hybrid gold nanocarriers and prospective anticancer in nanomedicine field for direct targeting of tumour cells using peptide-conjugated gold NPs with gemcitabine as anticancer drug.

2.RESULTS AND DISCUSSION

Formation mechanism of GEM ION-PEG-AuNPs

Recently, J.Spadavecchia et al. have designed some new nano-therapeutics agent based on drugs-gold-complex (i.e.doxorubicin, docetaxel, paclitaxel), according to a methodology named “Method IN”, in which the drug was complex with tetrachlorauric acid (HAuCl_4) before nanoparticle formation in presence of biocompatible polymer molecules (PEG diacide) as surfactant, and NaBH_4 as reducing agent. Chemical-physical characterizations and biological “*in vitro*” studies of drugs-gold-complex, have successfully showed a large influence in its therapeutic activity ^[4a, 13]. Other authors have studied the mechanism of hybrid nanoparticles and competition effect with different capping agent on the growth process^[2a, 14]. In this case we develop a modification of “Method IN”, in which, the main difference with previously reported synthetic procedures is that GEM participate to the stabilization of AuNPs *via* complexation and electrostatic interaction between your chetone and amino groups with chloride auric ions and dicarboxylic pegylated chaines.

In particular, the formation of gold NPs from AuCl_4^- includes some mains steps (**scheme 1**):

- (1) Complexation and initial reduction of AuCl_4^- ions by dicarboxylic acid-terminated PEG that adsorbs onto Au surface and generation of peg-gold clusters
- (2) Electrostatic Adsorption of Gemcitabine onto PEG-Au-complex
- (3) Reduction of metal ions in that vicinity and growth of gold particles and colloidal stabilization by molecules of PEG polymers.

In the first step, dicarboxylic PEG was added in HAuCl_4 aqueous solution to complex with him as described previously^[4b]. The addition of PEG-COOH increases the reduction process by complexation of Au ions, thought carboxylic groups, controlling the initial growth of nanoparticles^[2a]. Finally, the reduction by NaBH_4 , complete the reduction process of final nanoparticles (third step). When GEM molecules were mixed with PEG-Au solution, the charged residues opposite to the skeletal of GEM (preferentially $-\text{NH}_3^+$) were thought to

interact with the polar surface of PEG chains in such a way as the conformation structure of drug was turned of the gold surface (see **scheme 1**). This steric arrangement of GEM in PEG-Au clusters before reduction of HAuCl_4 during synthetic process of gold nanoparticles, was confirmed by UV-Vis absorption spectroscopy, TEM and Raman Spectroscopy reported in the following.

Comparative physico-chemical characterization of GEM ION-PEG-AuNPs:

Gemcitabine (2'-deoxy-2',2'- difluorocytidine ; GEM) is antimetabolite and deoxycytidine analogue (**Scheme S1 in the Supporting Informations**) that has been used as a chemiotherapeutic agent with antineoplastic activity for last two decades^[15]. In particular, GEM shows a hydroxyl and chetone functional group responsible of anticancer activity^[16]. Physicochemical properties of GEM do not allow for electrostatic interaction with all nanoparticle surface. For this reason, in previous studies, GEM was grafted onto iron-oxide nanoparticles by cicloaddition through Diels Alder reaction^[10].

In our study, GEM was electrostatically grafted onto pegylated complex to the AuCl_4^- solution, in order to form metallo-nanoflowers. TEM images of GEM ION-PEG-AuNPs display a snow-like shape, embedded in a shell of PEG, with a similar diameter around 50 ± 2 nm (**Figure 1-B**), compared with PEG-AuNPs as control^[4b] with an average size of 7.5 ± 1 nm (**Figure 1-A**). J.Spadavecchia et al. have reported the synthesis of similar nanostructures using dicarboxylic PEG^[2c, 4a, 13b] and drugs, while characteristic snowflakes nanoparticles were obtained by incorporating protoporphyrin molecules in the growth solution of AuNPs^[17]. On the basis of previous studies^[2a, 13b, 14] we presume that, when GEM was added to complex PEG-AuCl_4^- , the PEG change your chemical conformation switching from mushroom to brush mode conformation. The absorption spectra of GEM ION-PEG-AuNPs and PEG AuNPs were characterized by a small peak at 300 nm, assigned to the PEG absorption, and a surface plasmon band at 535 nm (**Figure 1-C red line**) for PEG-AuNPs and

608 nm for GEM ION-PEG-AuNPs (**Figure 1-C blue line**) respectively. PEG-COOH, as stabilizing polymers, provides to the formation of coordination bands between the Au ions and the chetone or amino groups of GEM with a good dispersion of the Au ions, and consequently growth of AuNPs like flowers. This behaviour is associated to π - π^* electronic transitions due to interactions between the pyrimidine ring and PEG-AuCl₄⁻ ions.

The loading of GEM on the gold nanoparticles was confirmed by the presence of the peak at 270 nm, and the strong decrease of the band at 608 nm with consequently disappearance of the band at 300 nm in the UV-Visible spectra (**Figure 1-C blue line**) and the tendency of snow-like shape to agglomerate differently. This chemical behavior, is due to a different adsorption onto gold facets [110] of GEM, based on their different steric conformation of chemical groups (**Scheme S1**) during nucleation and growth process of AuNPs. In addition the color bright violet-blue of the obtained nanoparticles and the UV-Vis spectra remain unaltered after storage for more than six months at room temperature suggesting the formation of stable particle suspension. The chemical ad steric arrangement of GEM onto pegylated gold nanoparticles was confirmed by Raman spectroscopic analysis (**Figure 1-D**). T.Y Santiago et al. have synthetized and characterized GEM-gold nanoparticles by Raman spectroscopy^[11]. These authors, have immobilized GEM on the gold nanoparticle (AuNP) through covalently bonded thiol linkers and spectroscopic analysis showed that the specific functional groups of the GEM-AuNPs were chemically modified before the grafting of AuNPs. Contrary to this study, we have involved other ingredients (drug, polymer) chelated to gold ions, to form gold nanoparticles, in which the spectroscopical fingerprints of our final nano-drug are peculiar compared to the *free* drugs.

In detail, Raman spectra of free GEM and GEM ION-PEG-AuNPs in water exhibit many bands in the region 500-2000 cm⁻¹ (The wide band observed around 1600 cm⁻¹ on the GEM ION-PEG-AuNPs Raman spectra is assigned to the water) (**Figure 1-D**). In particular, the

peak at 823 cm^{-1} characteristic of the aromatic proton and C-F groups, the vibrations at 1250 cm^{-1} and 1300 cm^{-1} due to ν C-O-C stretching and the vibration at 1655 cm^{-1} due to protonated NH_2 . We observed an exaltation of the peak at 430 cm^{-1} due to the C-N vibrations of the pyperazynil group. In addition, we note the disappearance of a band present in the spectrum of "free" Gemcitabine located between 469 and 514 cm^{-1} when the GEM is grafted on gold nanospheres due to the C-C-O vibration. The prominent peak at 430 cm^{-1} is due to the vibrations $\delta(\text{OH}\dots\text{O})$, $\nu(\text{OH}\dots\text{O})$ of the PEG. Based on the spectrochemical and previously theoretical results, we assumed that Au^{3+} ions promoted the deprotonation of the GEM ciclopenthanol group at position C-5'. We have also observed that more peaks were exalted in the region between 1000 cm^{-1} and 1200 cm^{-1} due to a chemical and steric arrangement of GEM onto pegylalated gold nanoparticles previous complexation as described previously^[13a].

Drug loading and release of GEMION-PEG-AuNPs:

Recently, A.Oluwasanni et al. have studied the process of loading of GEM onto hybrid iron oxide-nanoparticles (HNPs) by Diels Alder reaction^[10]. This formulation was capable of high drug loading (86%) which appeared stable even upon reduction of pH and provided to a rapid liberation of the prodrug at elevated temperatures, through retro Diels Alder breakdown^[10, 18]. Previously Lele Li et al. have obtained a drug loading of about 55% after self-assembled of GEM onto Diphosphate-PEG based gadolinium nanoparticles (GdNPs)^[18]. In similar case, other authors have immobilized GEM on the nanoparticle (NP) through a pH-sensitive amide bond. This bond prevents the drug from being metabolized or acting on tissue at physiological pH 7.4, but breaks, releasing the drug at acidic pH, characteristic of cancer cells^[11]. In our case, after complexation of AuCl_4^- with PEG diacid chains, GEM was binding electrostatically onto complex before reduction of NaBH_4 to obtain GEM ION-PEG-AuNPs. In this novel formulation GEM assume a different chemical and sterical conformation compared to recent synthesis approach in which the drug molecules reside inside the AuNP

core^[6a, 13b]. The successful loading ratio of GEM onto PEG-AuNPs, were evidenced by the characteristic absorption peaks at 268 nm from GEM (**Figure S2 Supporting information**). The standard absorption of GEM was plotted in the inset of figure (**Figure S2 supporting information**) according to UV-Vis absorbance spectra of GEM at various concentrations. The loading efficiencies were estimated to be 86% with 8.5 μg present in 2.5×10^{-8} mol of NPs (data not shown). GEM releases were pH- and time-dependent (**Figure 2 A-B**). A sustained drug release was observed in the first 5 h for both drugs at pH 4 and 7, which is highly favorable for drug delivery as the cancer cells thrive in acidic conditions. We presumed that the release of GEM was checked by a ionic equilibrium between Au (III)-PEG complexes trapped into AuNPs by the hydrophobic interactions between PEG chains and ionic drug. As previously described, the mechanism by which acidic pH triggers drug release is probably associated to the presence of carboxylate groups in the chemical structure of PEG molecules. Such groups become protonated at acidic pH. This reduces the electrostatic interactions occurring between PEG and drugs that stabilize the GEM ION-PEG-AuNPs structure.

We suppose that, the ionic strength and protonation of GEM onto PEG chains improve the water solubility of the drug molecules, taking advantage of the drug release from AuNPs. Indeed, a remarkable moiety of GEM electrostatically binding onto dissociated complex is released, through PEG chain and diffusion phenomena^[2c, 19].

Stability under pH and electrolytic conditions.

In order to understand the behavior of GEM ION PEG-AuNPs and HIV-1 Tat GEM ION PEG-AuNPs as future diagnostic or drug delivery tools, comparative studies of nanoparticles exposed to different pH solution under ionic strength were carried out. Localized Surface Plasmon (LSP) band at 565 nm (was performed under different pH and ionic strength conditions over a reasonable period of time (3 months)). The synthesized GEM ION PEG-

AuNPs did show an almost negligible change in the LSP band position over a period of three months at the pH 5 and pH 7 (**Figure S1 in Supporting Information**). As described previously^[20], the stability of AuNPs at lower concentrations is crucial, in biomedical applications because chemical and photophysical properties were preserved under *in vitro* and *in vivo* environments^[21]. A change of around 1nm in λ_{max} was reputed, relevant to assess AuNPs stable over that period of time in a particular medium. As described previously, after cell internalization, Na^+ and Cl^- ions were applied to change the concentration of other compounds, by affecting in the direction in which water diffuses to form macromolecules in the human body^[22]. Other authors, have showed that pegylated nanoparticles functionalized with amount 16.8 and 25.2 $\mu\text{g/ml}$ of PEG, remain stable in 1M NaCl which is a value well in excess of the normal physiological salt concentration (0.15 M)^[23]. In our case, GEM ION PEG-AuNPs and HIV-1 Tat GEM ION PEG-AuNPs preserve your morphology and size under ionic strength conditions (2 M NaCl) as showed in **Figure 3 A**. A change in the LSP band position was negligible for few amount of NaCl and decrease dramatically at high dose rates. TEM Images (**Figure 3 B**) revealed a good homogeneity and stability due probably to a good equilibrium between ionic species ($\text{Na}^+ \text{Cl}^-$), polymer chains (PEG-COOH), Gemcitabine (GEM) and Peptide (HIV-1 TAT).

Peptide Active Targeting

Drug-gold nanoparticles (GEM IN PEG-AuNPs) was used as a support for the conjugation of HIV-1 Tat targeting moiety. HIV-1 Tat peptide is a strong *trans*-activator of transcription and play a key role during viral replication^[24]. The structure of the Tat peptide be alike a peculiar transcriptional activator including an activation domain and a nucleic acid-binding domain. HIV-1 Tat molecules were immobilized on the surface of activated nanoparticles through the formation of amide links between the COOH groups decorating the surface of the drug-gold nanoparticles and the NH_2 groups of the peptide (**Scheme 2**).

The binding between HIV-1 Tat protein and GEM-gold nanoparticles were monitored by detecting changes in the LSP band (**Figure 4A**). GEM ION PEG-AuNPs showed an LSP band centered at ~608 nm, typical of AuNPs ^[25]. After immobilization of anti HIV-1 Tat protein onto GEM ION PEG-AuNPs, the resulting solution was colorless and was characterized by a broad LSP peak at 630 nm, which could be assigned to a change of localized refractive index of PEG-AuNPs and to the formation of NP agglomeration. Based on the UV-Vis spectra, we suggest that the significant red shift and broadening of the LSP band in the UV-Vis spectra could be associated to the successful bioconjugation of the AuNPs surface with anti HIV-1 Tat protein and the subsequent AuNPs agglomeration associated to the formation of classical van der Waals inter-peptide interaction^[26]. Hypothesizing that the size of the HIV-1 Tat protein is about 20 nm ^[27], we can estimate that the peptide stabilised the inter-particle distance *via* a double combination of electrostatic and steric repulsions among the various proteins linked to the PEG-chain grafted onto the nanoparticles' surface. This phenomenon was confirmed by TEM images analysis (**Figure 4 B**) by means of which the size of gold-nanoflowers of about 60 nm, as well as the presence of agglomerates, was evaluated. In particular, it was observed that HIV-1 Tat GEM ION PEG-AuNPs were uniformly dispersed, confirming that the amphiphilic HIV-1 Tat GEM ION PEG layer grafted to the gold core of the nanoflowers stabilized the colloidal suspension. High-Resolution TEM (HR-TEM) image (**Figure 4 B**), of HIV-1 Tat GEM ION PEG-AuNPs clearly display the presence of an organic PEG layer of few nanometers among two gold nanoflowers through inter-peptide interaction. The achievement of peptide grafting and the covalent conjugation *via* EDC/NHS groups, were also confirmed by Raman spectroscopy (**Figure 4 C**). SERS effect can be observed. An enhancement of certain peaks corresponding to the C-S stretch (674 cm^{-1} ; 692 cm^{-1} ; 745 cm^{-1}) provides some informations about peptide's backbone^[28]. This is confirmed by the broadening of the peak, confirming a higher number of peptide conformations inside the PEG layer (higher porosity or lower charges) after GEM ION PEG-AuNPs binding^[26, 29].

The NP sizes were confirmed by DLS measurements (**Table 1**). Zeta potential measurements show that GEM ION-PEG-AuNPs and HIV TAT-1 GEM ION-PEG-AuNPs were colloidally stable at physiological pH (z-potential = -25 ± 2 Mv and -30 ± 1 mV with a PdI equal to 0.3).

Table 1: z-potential and hydrodynamic diameter of GEM ION PEG-AuNPs and HIV-1 Tat GEM ION PEG-AuNPs

Synthetic product		Zeta potential (mV)	Hydrodynamic diameter (nm)	PdI
GEM ION PEG-AuNPs		-25 ± 2	50 ± 2	0.3
HIV-1 Tat GEM ION PEG-AuNPs		-30 ± 1	63 ± 2	0.3

Efficacy:

During the interaction between GEM and DNA, a single deoxynucleotide is incorporated and breaking off the chain elongation [19]. The non-terminal position of gemcitabine promote DNA polymerases, and inhibits removal of gemcitabine by exonucleases^[30]. Biological studies were performed to assess the targeting efficacy of HIV Tat-1 peptide and the functional activity of gemcitabine in the nanoconjugated form (GEM ION PEG AuNPS) against various PDAC (PANC-1) cell lines in vitro.

Tat peptide have been reported to translocate through the cell membrane and to carry exogeneous molecules into cytoplasm and nucleus^[31]. HIV Tat-1 is an 86 amino-acid protein and aa 47-57 of Tat possess a high net negative charge at physiological pH, with nine of its 11 aminoacids being either arginine or lysine. Fusion of several proteins and this 11 aa region of Tat protein enables the delivery of proteins into cells. Thus, this 11aa region is considered a protein transduction domain (PTD)^[31-32]. Previously, H. Hashida et al, have studied molecular conjugates of poly-lysine with natural or artificial ligands using the DNA-binding and condensing properties of poly-lysine to mediate interaction with DNA^[31]. Upon formation of DNA-polylysine ligand complex (polyplex)^[33], gene transfer is facilitated via receptor-mediated endocytosis. In our study, we investigated, for the first time, the potential

role of GEM onto pegylated gold complex fused Tat-1 PTD as a gene delivery agent. We tested, PANC-1 to understand the activity of gemcitabine had been retained in the nanoconjugated form. The percentage (%) of live PDAC cells (PANC-1 cell line) were evaluated by flow cytometry after exposure of 48 h with PEG-AuNPs^[34] as control, GEM ION PEG-AuNPs and HIV Tat-1 GEM ION PEG-AuNPs. As described previously, PEG-COOH do not impact on the toxicity of the final nanocarrier^[4]. It is evident from the **Figures S3 in Supplementary Information** that HIV Tat-1 GEM ION-PEG-AuNPs substantially inhibited proliferation of pancreatic cancer cells at lowest concentrations (from 1.7 μ M to 0.085 μ M). Otherwise, the inhibition of proliferation of PANC-1 cells by GEM ION-PEG-AuNPs was more evident at highest concentration (from 3.4 μ M to 17 μ M). This original biological behavior, is probably due to a chemical and steric arrangement of peptide onto GEM PEGylated gold nanoparticles that induce a different biological activity at lower concentrations. These data clearly suggest an original behaviour of HIV Tat-1 and gemcitabine in the nano-conjugated form retains its specific targeting ability expressing cancer cells in vitro. All nanoparticles were *free* endotoxin and suitable for further nanomedicine applications.

Photothermal heating :

A photothermal effect occurs when a metallic nanoparticles converts the absorbed photon onto heat. This conversion is a consequence of a plasmonic relaxation of the nanoparticle localized plasmons after a photon excitation^[35]. This effect depending on nanoparticle nature, shape and size, has been widely exploited to destroy cancer cells^[36], drug delivery and photodynamic therapy because of their ability to selectively drive temperature-sensitive chemical reactions^[37]. For an ideal PTT nanovector, several conditions should be fulfilled, including suitable size and shape for a NIR region illumination, but also a low cytotoxicity to healthy tissues^[38].

Recently, highly crystalline and monodisperse NPs have demonstrated excellent properties that can be applied to areas such as drug delivery, cell separation and hyperthermia^[39]. To evaluate the photothermal effect of our NPs in object (GEM ION PEG-AuNPs, HIV-1 Tat GEM ION PEG-AuNPs, PEG-AuNPs (i.e.control), we investigate the temperature changes under 808 nm and 1 W.cm² laser irradiation after the 3 steps of NPS synthesis (**Figure 5a**). The temperature elevation rose rapidly after 5 minutes in the three solutions. The temperature increase was stabilized after 15 minutes and reached the value of +8°C for PEG AuNPs (control), +35°C for GEM ION PEG-AuNPs and +27°C for HIV-TAT GEM ION PEG-AuNPs, while the temperature elevation of the buffer increased only by 1°C. With a LSPR peak close to the 808 nm laser irradiation in the case of GEM ION PEG-AuNPs (50 nm) and HIV-TAT GEM ION PEG-AuNPs (63 nm), the absorption of photons is rapidly converted to heat, which results in a fast increase of temperature compared to PEG AuNPs (7.5 nm) and which is not possible in the buffer solution. The temperature elevation was measured after 15 minutes irradiation by varying the laser power from 0.12 to 3 W after the 3 steps of NPs synthesis (**Figure 5b**). We noticed that the temperature elevation increased linearly until 1W.cm² irradiation power. After this irradiation level, the temperature seemed to be increasing slowly between 1W and 3W laser power. We attribute this phenomenon to a maximum conversion of the optical energy to heat by NPS until 1W laser power. Further, this conversion becomes less efficient and the temperature increase was measured to +52°C for GEM ION PEG-AuNPs and +47°C for HIV-1 TAT GEM ION PEG-AuNPs at 3W irradiation power. While for PEG-Au NPs, the temperature elevation was lower +32°C and negligible for the buffer solution +4°C. By increasing the laser power and exposition time, we observed that the temperature increase of HIV-1 TAT GEM ION PEG-AuNPs was slightly lower than GEM ION PEG-AuNPs with a maximum temperature difference of 5°C, while the rate of the temperature rise was approximately the same. These last results showed that the

energy transfer from the NPs to the surrounding media was weakly affected by the presence of the HIV-1 TAT molecules.

3. CONCLUSIONS

In the present article three objectives were carried out: first, we designed and synthesized GEM based gold nanovector (GEM ION PEG AuNPs) using a “simple” method implying hybrid NPs with biocompatible capping materials (diacide PEG). Second, we combined an original active targeting onto GEM ION PEG AuNPs through HIV-1 Tat peptide functionalization in order to , investigated the efficacy of HIV-1 Tat GEM ION PEG AuNPs , before and after internalization in PDAC cells . Third, we suggest a preliminary photothermal heating study of these nanostructure in order to develop a multitask nanovector with a highly effective cancer therapy. These findings and speculations main headlines the potential of the chemical approach to study GEM–gold interactions in relation to drug delivery and drug targeting activity. Further studies are still envisaged, focused on assessing the *in vivo* assessment toxicity, pharmacokinetics and dynamics on relevant pancreatic-cancer model.

4. EXPERIMENTAL SECTION

Materials and methods

All chemicals were reagent grade or higher and were used as received unless otherwise specified. Tetrachloroauric acid ($\text{HAuCl}_4 \cdot \text{H}_2\text{O}$), sodium borohydride (NaBH_4), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), dicarboxylic PolyEthylene Glycol (PEG)-600 (PEG), phosphate buffered saline (PBS, 0.1 M, pH from 4 to 13), Gemcitabine (98%), HIV-1 Tat peptide were purchased from Sigma Aldrich.

Synthesis of GEM ION-PEG-AuNPs

The main steps of synthesis of GEM ION-PEG-AuNPs colloids are described in **Scheme 1**. 10 ml HAuCl₄ aqueous solution (2.5×10^{-4} M) was added to 250 μ l of dicarboxylic PEG. mixed by magnetic stirring for 20 min at room temperature. After 20 min, 2 ml of GEM solution (1.72×10^{-4} M) was added under magnetic stirring for 1h at room temperature. Finally, 1.2 ml of aqueous 0.01 M NaBH₄ was added at once. The formation of the GEM IN-PEG-AuNPs was observed as an instantaneous colour change of the solution from pale yellow to blu-violet after addition of the reducing agent. Products of each synthetic step were stored at 27-29 °C and characterized by UV-Vis spectroscopy, Transmission Electron Microscopy, and Raman spectroscopy. The “as-prepared” GEM IN-PEG-AuNPs solution was centrifuged at 11,000 rpm for 15 min for three times; then, the supernatant was discarded. This was repeated twice to remove excess of not-conjugated dicarboxylic PEG.

Synthesis of PEG-AuNPs

Synthetic procedures were carried out as previously described ^[4b].

Stability of GEM ION-PEG-AuNPs as function of pH

The stability of nanoparticles was detected by UV VIS. GEM ION-PEG-AuNPs nanoparticles were dissolved in PBS solution 0.1M at different pH (pH 5, 7, 8, 9, 12, 13) during 18h (**Figure S1 Supporting Informations**).

HIV-1 Tat Peptide immobilization onto GEM ION-PEG-AuNPs

The binding of HIV-1 Tat peptide onto GEM ION-PEG-AuNPs surface was performed at pH 9.0 in PBS. Briefly, 20 μ l of EDC/NHS (80/20 w/w ratio) aqueous solution was added into 5 ml of GEM ION-PEG-AuNPs dispersion. After 2 h, 50 μ L (0.5 ng/ml) of HIV-1 Tat in PBS was added in 2 ml of the reaction mixture and stirred for 3 h at room temperature. The

obtained HIV-1 Tat -GEM ION-PEG-AuNPs were centrifuged two times at 9,000 rpm for 10 min to remove excess of not-conjugated HIV-1 Tat and dried under nitrogen.

Physico-chemical characterization

All the measurements were performed in triplicate in order to validate the reproducibility of the synthetic and analytical procedures. All the measurements were carried out as previously described ^[4b, 13b].

UV/Vis measurements

Absorption spectra were recorded using a Perkin Elmer Lambda UV/Vis 950 spectrophotometer in plastic cuvettes with an optical path of 10 mm. The wavelength range was 200–900 nm

Transmission Electron Microscopy (TEM)

All microscopy analysis were realized as previously described ^[4b, 13b].

Raman Spectroscopy The Raman experiments have been performed on an Xplora spectrometer (Horiba Scientifics-France). The Raman spectra have been recorded using an excitation wavelength of 785 nm (diode laser) at room temperature. For measurements in solution, a macro-objective with a focal length of 40 mm (NA = 0.18) was used in backscattering configuration. The achieved spectral resolution is close to 2 cm⁻¹.

Dynamic light scattering (DLS): The size measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a He-Ne laser (633 nm, fixed scattering angle of 173°) at room temperature.

Zeta potential measurements: All measurements were carried out as previously described [4b].

Drugs loading efficiency: The amount of the drug (GEM) incorporated onto GEM ION-PEG-AuNPs was measured by UV-Vis absorption spectroscopy. Absorption at 268 nm was used to extrapolate GEM concentrations based on a calibration curve (**Figure S2** in the **Supporting Information**). The Drug loading efficiency was calculated as follows (**Equation 1**):

$$\text{Drug loading efficiency}(\%) = \frac{C_1 - C_2}{C_1} \times 100 \quad \text{Equation 1}$$

Where C_1 is the initial drug content and C_2 is the amount of free GEM in the filtrate after separation of the nanoparticles by ultrafiltration with an Ultrafree MC centrifugal filter units, (30,000 NMWL, Millipore, USA).

Drug release from AuNPs: GEM releases was evaluated at physiological temperature (37°C). Drug-loaded AuNPs was dispersed at concentration of 1.8×10^{12} particles/ml in 1.3 ml PBS and performed by dialysis as previously described [13b]. The concentration of drug released from AuNPs was expressed as a percentage of the total drug concentration present in the sample (**Equation 2**) and plotted as a function of time.

$$\% \text{ Drug released} = \frac{\text{Concentration of drug released}}{\text{Initial drug concentration}} \times 100 \quad \text{Equation 2}$$

Endotoxin content in Drug-gold nanoparticles:

As routine quality control of the materials prior to biological testing, the Pierce LAL Chromogenic Endotoxin Quantitation kit (Thermoscientific, France) was used to determine the LPS contamination in Drug-gold nanoparticles (GEM ION-PEG-AuNPs) before and after conjugation with HIV-1 Tat peptide as described previously^[4a]. Results, are reported in Table

1 as average ($n_{test} = 2$) $\pm$ standard deviation. Endotoxin contamination results were kept into account when defining the type of cytotoxic endpoint to be examined.

Sample	Endotoxin Units (EU/ml)
PEG-AuNPs (control)	ND
HIV-1 Tat GEM ION PEG-AuNPs	>1
GEM ION-PEG-AuNPs	ND
HIV-1 Tat peptide	>1

Table 1. Endotoxin units detected by means of the LAL Chromogenic Endotoxin Quantitation kit (ND = not detectable; >1= above detection limit of the assay).

Measurement of photothermal heating:

We irradiated 1mL of 1 nM nanoparticles in aqueous solution inside a quartz cuvette with a 808 nm continuous laser (FocusLight, China) at different power densities (0.12 to 3 W.cm⁻²) for 15 minutes at room temperature (23 °C). The temperature of colloids was recorded at 0.5 minute intervals using a thermocouple probe (Hanna Instruments, USA) with an accuracy of 0.1 °C. The sample volume, the nanoparticles concentration and the spot size (1 cm²) were remained constant and the temperature increase was measured as a function of the laser power and time.

In vitro biological activity of GEM IN-PEG-AuNPs and HIV-1 Tat-GEM IN-PEG-AuNPs

Cell culture

PANC-1 cells, a human pancreatic carcinoma cell line, were obtained from the American Tissue Culture Collection (ATCC). PANC-1 cells were cultured in DMEM (Gibco, BioSciences Ltd, Ireland) under previously reported^[4a].

Cell treatment

PANC-1 cells were exposed to a series of GEM ION-PEG-AuNPs, HIV-1 Tat -GEM IN-PEG-AuNPs and PEG-AuNPs (control) dilutions in complete media (DMEM+10% FBS) (concentrations ranging from 0 to 17 μ M of GEM ION -PEG-AuNPs and HIV-1 Tat GEM IN -PEG-AuNPs) for 48 h in 12 well plates as the procedure previously described^[4a]. Cells were seeded at a density of 5×10^3 cells/well, 100 μ L/well in a 96-well plate. After 48 hours, cells were washed with PBS and incubated with CCK8 dilutions in complete media (100 μ L/well) for 4h at 37°C and 5% CO₂ and OD450 were analyzed using a Microplate Reader.

ASSOCIATED CONTENT

Supporting Information. Supplementary figures, schemes and tables are provided. This material is available free of charge *via* the Internet at <http://.....>

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Figures and scheme

Scheme 1. Schematic representation of the synthesis of GEM ION-PEG-AuNPs *via* polymer reduction (Please note that drawings are not in scale and are not intended to be representative of the full samples composition).

Figure 1. TEM images of PEG-AuNPs (A) and GEM ION PEG-AuNPs (B); (C) normalized UV-Vis absorption and (D) Raman spectra GEM ION PEG-AuNPs products compared to free GEM as control. (A-B) Scale bars: 20 nm. (D) Raman spectra. Experimental conditions: $\lambda_{\text{exc}} = 785$ nm; laser power 20 mW; accumulation time 180s.

Figure 2: A) Drug release percentage (%) of GEM ION PEG-AuNPs time in PBS (37 °C) at pH = 4.0 or at pH = 7.0. B) Schematic diagram of GEM release under pH conditions.

Figure 3: TEM images of PEG-AuNPs (A) and GEM ION PEG-AuNPs (B); (A) normalized UV-Vis of GEM ION PEG-AuNPs before (red line) and after (black line) treatment under ionic strength conditions. (B) TEM images of GEM ION PEG-AuNPs (low and high magnification) in NaCl (0.9%; 50 µl; 48h)

Scheme 2. Schematic representation of HIV-1 Tat protein bio-conjugating onto GEM IN-PEG-AuNPs surface (Please note that drawings are not in scale and are not intended as representative of the full samples composition).

Figure 4: (A) Extinction spectra of GEM ION PEG-AuNPs before (blue line) and after conjugation to HIV-1 tat peptide (black line)

Figure 5: (a) Time-dependant temperature elevation of PEG-Au NPs, GEM ION PEG-AuNPs, HIV-1 Tat GEM ION PEG-AuNPs and buffer under 808 nm laser (1 W.cm^{-2}). (b) Laser power effect on photothermal heating on NPS compared to the control solution (buffer alone) after 15 minutes irradiation time. The temperature elevation of NPS is higher than the minimum temperature required for cell killing in PTT ($+4^\circ\text{C}$).

Supporting Information

HIV-1 Tat peptide-Gemcitabine gold (III)-PEGylated complex –Nano flowers: A sleek thermosensitive hybrid nanocarrier as prospective anticancer.

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ADDITIONAL SCHEMES

Scheme S1. Chemical structure of Gemcitabine (GEM). Rings and chemical groups are identified with number; this nomenclature is used for Raman band assignments. A blue ring is associated to site of complexation with gold ions; red and green rings are associated to ionic interactions.

ADDITIONAL FIGURES

Figure S1. UV vis spectra of GEM ION-PEG-AuNPs in buffer solutions at different pH

Figure S2 : UV-Vis absorption spectra of GEM at increasing known concentrations. (b) calibration curve extrapolated from such measurements. The amount of GEM molecules grafted onto GEM ION-PEG-AuNPs, was estimated based on this curve.

Figure S3: Proliferation PANC-1 cells of GEM ION-PEG-AuNPs, HIV-TAT GEM ION-PEG-AuNPs, and PEG-AuNPs (i.e. control) during 48h h at different concentrations.