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## **N6L-Functionalized Nanoparticles for Targeted and Inhibited Pancreatic Cancer Cells**

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## Abstract.

Pancreatic cancer is a growing cause of concern, ~~with leading to an increasing trend number~~ of deaths worldwide. Most of ~~the~~ antitumor drugs ~~for pancreatic cancer~~ are given by systemic administration. ~~However, but~~ even if this is at therapeutic doses, its tends to ~~cause lead to~~ severe side-effects because of ~~the~~ nonspecific uptake by healthy tissues. In this context, we aim ~~at developing to develop~~ an innovative drug delivery approach for the treatment of pancreatic cancer based on tailor-made ~~nanomaterials~~ ~~nanomaterials with~~ presenting controlled size, chemistry and stiffness. ~~In this study, nanomaterials obtained by combining both templating method and self-assembly process on 20 nm and 60 nm size gold nanoparticles as a core template or capsules that have been functionalized with a the pseudopeptide N6L, which targets cancer cells and induced their apoptosis.~~ In this study, we have designed tailor-made N6L-nanomaterials, via the combination of both a templating method and a self-assembly process onto colloidal gold nanoparticles (diameters 20 nm and 60 nm) using a core template. The removal of the core leads to hollow particles, also called capsules. We judiciously functionalised the outer layer of the nanomaterial with N6L, which corresponds to a synthetic agent that is non-toxic to normal mammalian cells, targets surface nucleolin and induces tumour cell death by apoptosis. Our data demonstrated, *via* quartz crystal microbalance measurement, the possibility of directly using N6L in a self-assembly process through electrostatic interactions. ~~We evidenced via quartz crystal microbalance measurement the possibility to use directly N6L in a self assembly process through electrostatic interactions.~~ In addition, as shown by measuring zeta potential values, we ~~demonstrated showed~~ that electrostatic interactions between the protonated amine groups of poly (allylamine) macromolecules (+40 mV), the ~~sulfate sulphate~~ groups of ~~Dextran dextran~~ derivatives (-20 mV) and the protonated amine groups of N6L (+20 mV) are the driving attraction force during ~~the~~ multilayer fabrication onto colloidal gold. Transmission electron microscopy was used to confirm that the gold nanoparticles were coated and the nanocapsules ~~formation were formed.~~ ~~Finally, we showed that these nanostructures target cancer cells and inhibit in dose dependent manner cell survival in pancreatic cancer model highlight the potential antitumor activity of these nanostructures.~~ Finally, we successfully showed that these N6L-based nanostructures target cancer cells and inhibited, in a dose dependent manner, cell survival in pancreatic cancer model. This result clearly highlights the potential antitumor activity of the developed nanostructures.

**Keywords:** pancreatic cancer, templating method, nanocapsules, layer-by-layer assembly, nucleolin, gold nanoparticle

## 1. Introduction

Although overall cancer death rates continue to decline in both Western Europe and the United States, pancreatic cancer is one of the rare cancers whose mortality rates are expected to continue to increase over the next 10 years. [1, 2] This type of cancer has the worst prognosis among all cancers with an overall survival rate of 5 to 7% over 5 years ~~and~~. ~~Moreover~~, it is expected to become the second leading cause of cancer death ~~in over~~ the next decade. Surgery, with neoadjuvant therapy, is currently the most effective treatment for ~~resectable tumours~~. ~~and~~ However, this is only possible in 15% of patients with early-stage disease and if the patient's general condition allows it. [3] Nevertheless, most of the operated patients (about 80%) recur and quickly develop a local recurrence in the months or years following the intervention. [1, 4] ~~All these treatments reduce symptoms or increase life expectancy~~. Consequently, it is, therefore, ~~urgent~~ important to find new therapeutic treatments for this type of cancer.

Some more recent therapeutic approaches ~~are~~ ~~have been~~ documented in clinical trial databases: , ~~including~~ trials based on gemcitabine administered according to the folfirinox protocol,[3] ~~the~~ use of liposomal irinotecan,[5] or ~~different~~ combinations of existing active ingredients.[6]

Various experimental approaches using nanoparticles as carriers have already been investigated.[7] Among ~~these~~ ~~them~~, only one, based on (nab)-paclitaxel attached to albumin [8] in combination with gemcitabine,[9] has been approved by the Food and Drug Administration (FDA) for the treatment of pancreatic adenocarcinomas. In ~~these~~ approaches proposed so far, the majority of nanoparticles do not carry ligands that specifically ~~targets~~ cancer cells. As compared to classical treatments, the improvement observed by the use of nanoparticles [10] is therefore, ~~brought~~ mediated by passive targeting, the enhanced permeability and retention effect, the protection/activation of active molecules and, above all, by ~~a~~ better spatio-temporal control of ~~the~~ ~~ir~~ release of therapeutic molecules throughout the body. However, other approaches using surface ~~functionalized~~ ~~functionalised~~ nanoparticles that specifically target ~~tumour~~ cells are currently in progress. [7, 10]

In this context, we ~~proposed~~ to use a designed multivalent pseudopeptide (N6L) to target tumour cells through ~~the~~ nucleolin, which is a molecule overexpressed at the cell surface of tumour cells as compared to normal cells.[11-14] Nucleolin is a highly promising targeting and marker molecule, especially the molecular form ~~which is localised~~ ~~localised~~ at the cell surface.[14] It was first described in ~~the~~ 1970's as involved in the ~~remodelling~~ of nucleolar

chromatin, maturation of pre-RNA, rDNA transcription and ribosome assembly.[10] More recently, nucleolin has been shown to shuttle between **the** cytoplasm and cell surface. Each cell ~~localization~~ **localisation** of nucleolin is associated with a specific role of this multifunctional protein.[15] In the cytoplasm, it provides a posttranscriptional regulation of strategic mRNA and at the cell surface, ~~its~~ serves as a low affinity receptor for several ligands involved in **tumour** growth.[13] Interestingly, the expression of cell surface nucleolin is higher in cancer cells than in normal cells, [10] in which nucleolin is exclusively located in the nucleus, suggesting that this nucleoprotein constitutes a promising and specific target for cancer therapy. [13]

It is within this framework that our study is presented by proposing a new innovative therapeutic option to treat pancreatic cancer. Indeed, we aimed to develop an original therapeutic approach for the treatment and targeting of pancreatic cancer based on N6L-nanomaterials. ~~Herein, we~~ We, thus, implemented a new strategy consisting of the development of ~~propose to develop~~ nanomaterials decorated with N6L on the surface with controlled size and stiffness (nanoparticles vs nanocapsules). **It is important to note that N6L is a “bioactive molecule”, i.e. it can enable pancreatic cancer cures. It also allows the targeting of tumour cells through nucleolin. The antitumor effect of N6L has been showed in several *in vitro* as well as *in vivo* models including breast cancer, [12, 16, 17] melanoma, [18] glioblastoma, [19] lymphoma [12] and pancreatic cancer [14]. In all of this tested preclinical models including xenograft in heterotopic, orthotopic or transgenics models, N6L induced a significant decrease of the tumor growth. Study of the mechanism by which N6L displays an antitumoral effect indicated that N6L is a proapoptotic [11, 12, 14] molecule and induced the normalization of the tumour angiogenesis. (the molecule overexpressed at the cell surface of tumour cells as compared to normal cells).The originality of this study lies in the use of the anticancer pseudopeptide N6L that targets tumor cells to functionalize nanoparticles.** The preparation of the carrier under consideration ~~is~~ **was** based on a key step of electrostatic interactions between the pseudopeptide N6L being positively charged and other polyelectrolytes, *i.e* layer-by-layer (LbL) build-up. This enables **one** to obtain well-defined chemical compositions and physical properties. This approach **also** presents the following advantages: (i) no specific equipment is required, (ii) no requirement ~~of~~ **for** organic solvents and (iii) ~~nanometer-ordered~~ **nanometre-ordered** functional layers can be easily fabricated. **Interestingly,** the LbL route is an efficient approach for fabricating carriers, as capsules, by combining both templating of particles and self-assembly methods on colloidal particles.

Colloidal gold nanoparticles are one such sacrificial template, ~~combining which combines~~ very interesting properties for the targeted application.[14] They present the advantage of being biocompatible and commercially available in a large range of sizes, including diameters starting from 5 nm. For instance, Gittins and Caruso [20, 21] reported ~~fabrication of nanocapsules nanocapsules fabrication~~ based on poly(diallyldimethylammonium chloride) and sodium poly(styrene sulfonate) via gold nanoparticles as ~~template-owing~~ templates with different diameters ( $11 \pm 2$  nm to  $54 \pm 13$  nm). Schneider and Decher [22] have shown how gold particles can efficiently be coated with up to 20 layers of a polycation (poly(allylamine hydrochloride, PAH)) and poly(styrene sulfonate) as a polyanion, ~~and~~ this was finally followed by the gold core removal. ~~Moreover~~, Belbekhouche et al. reported ~~on~~ the fabrication of ultra-low sized (20 and 60 nm) biodegradable capsules based on chitosan/poly(acrylic acid), ~~firstly~~ assembled on a gold particle.[23]

Thus, we proposed to self-assemble charged (macro-)molecules onto the surface of gold particles as a sacrificial template.[24-27] ~~Its~~ The selective removal of the gold particles ~~lead leads~~ to “hollow particles”, also called ~~or~~ capsules. ~~From~~ To the best of our knowledge, ~~no work there have been no previously published~~ reports on capsules/particles based on an N6L ~~based-on~~ templating method. Based upon our ~~current~~ knowledge, we ~~are were~~ convinced that this study ~~has had~~ great potential to succeed and ~~to~~ establish the proof of concept ~~consisting in~~ for obtaining antitumoral nanoparticles ~~functionalized functionalised~~ with N6L, a molecular known to allow efficient treatment and targeting of ~~to cure~~ pancreatic cancer. Indeed, ~~based on our results~~, we propose an easy and efficient approach for controlling the size, surface chemistry and stiffness ~~by involving a green process~~ (N6L-nanomaterials with (supposedly more rigid) or without gold matrix (nanocapsules, supposedly more flexible)) via an environmentally-friendly process (only water is used as a solvent for the preparation of the nanomaterial).

## 2. Materials and Methods

### 2.1. Materials

Poly(allylamine hydrochloride) (PAH, 50 000 g/mol), dextran sulfate (DexSO<sub>3</sub>, 40 000 g/mol) and mercaptoundecanoic acid were purchased from Sigma-Aldrich. Gold nanoparticles (NP) with a diameter of 20 nm ( $7 \times 10^{11}$  particles/mL) and 60 nm ( $2 \times 10^{10}$  particles/mL) stabilized in citrate buffer were purchased from Sigma-Aldrich. The N6L was synthesized as stated in a previous study.[12]

## 2.2. Elaboration of N6L-based multilayer film on flat substrate

Quartz Crystal Microbalance (QCM) measurement (Q-Sense E1, Q-Sense, Sweden) was performed to follow *in situ* to evidence to self-assemble polyelectrolytes with N6L. The gold coated QCM surface was first cleaned *via* a flushing with two successive solvents (water and ethanol) and then was subjected to a UV/ozone chamber (BioForce UV/Ozone ProCleaner) during 20 min which was finally followed by a rinsing with ethanol. Before introducing into the QCM flow chamber, the surface was overnight pretreated with a solution of mercaptoundecanoic acid (10 mg in 2 mL of ethanol). The surface was conditioned in water for 10 min. The self-assembly of PAH / dextran sulfate / N6L was achieved onto the gold pretreated substrate through a flushing of stock polymers solutions (10 mg/mL for PAH and dextran sulfate and 25 mg/mL for N6L, 15 min, flow rate of 250  $\mu$ L/min). A rinsing step was done with water between each polymer deposition step during 10 min. Wetting contact angle measurements were performed with a Kruss goniometer (room temperature, sessile drop configuration) on both pre-treated and modified gold surface.

## 2.3. Preparation of N6L- based particles via colloidal gold particles as a template

The nanomaterials ~~are~~ were obtained through a supramolecular approach involving the alternate deposition of polyelectrolytes with opposite charges (namely poly(allylamine hydrochloride), ~~Dextran dextran sulphate sulfate~~ and N6L) onto a sacrificial template, while the selective core destruction leads to hollow nanostructures (also called nanocapsules) (Figure 1). Herein, we focused on gold colloidal particles as the template. This material ~~presents presented~~ the advantages of combining several attractive properties ~~One may cite~~ , including: (i) their biocompatibility and (ii) their commercial availability in a wide range of sizes. This strategy ~~allows the~~ allowed control of the resulting nanocarrier size, surface chemistry, number of layers and mechanical properties.

More precisely, the surface modification of the negatively charged colloidal gold nanoparticles was ~~done~~ carried out by a method of ~~a~~ direct LbL saturation. This process is widely described in the literature [28-30] ~~This, and the~~ strategy is based on the possibility to directly incorporate the required amount of charged (macro-)molecules to efficiently coat the nanoparticle surface ~~and advantageously does not require~~ . It also has the advantage of not requiring a process of purification. The saturation concentration of each charged (macro-)molecule has to be empirically determined *via* zeta potential measurements. ~~In our study,~~

1000  $\mu\text{L}$  of the negatively charged gold nanoparticles templates were progressively covered with the polycation, namely, the PAH. Then, after 15 min, the polyanion ( $\text{DexSO}_3$ ) was similarly added and, finally, N6L was added as the outer layer. ~~The same approach was used with the N6L. The described process corresponded to the assembly of a single tri-layer, i.e. is then explained: PAH/DexSO<sub>3</sub>/N6L. During the process, where a solution of 10 g/L from of PAH and DexSO<sub>3</sub> and 25 g/L of N6L was used and one of 25 g/L from N6L.~~

All solutions were prepared in water. The volumes of the stock PAH solution required to form a stable first layer were 5  $\mu\text{L}$  and 9  $\mu\text{L}$  for the nanoparticles with a diameter of 20 nm and 60 nm, respectively. Deposition of  $\text{DexSO}_3$  was then performed to form the second layer; the volumes of the stock  $\text{DexSO}_3$  solution (1 g/L prepared in water) were 15  $\mu\text{L}$  and 20  $\mu\text{L}$  for the nanoparticles with a diameter of 20 nm and 60 nm, respectively. The required volume of N6L was 2  $\mu\text{L}$  (i.e. 50  $\mu\text{g}$ ) and 5  $\mu\text{L}$  (i.e. 125  $\mu\text{g}$ ) to saturate the nanoparticles with a diameter of 20 nm and 60 nm, respectively. The determination of these required volumes (or following of the adsorption process) was achieved via zeta potential and absorbance measurements. The method used to determine these conditions for preparation of the nanomaterials will be discussed in the text. ~~The detailed conditions of the preparation of the nanomaterials will be determined and then discussed in the text.~~

Dissolution of the gold template to obtain capsules was performed *via* treatment with 20  $\mu\text{L}$  of a potassium cyanide solution at 10 mg/mL. A dialysis method was applied to remove the gold cyanide complex from the capsules *via* a cellulose dialysis bag (3.5 kDa cut-off) against distilled water. Images of the capsules were taken with a Transmission Electron Microscope (TEM).

#### 2.4. Assay for antitumor activity of N6L-based particles

Human pancreatic carcinoma cell line (PANC-1; ATCC® CRL-1469™) was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Dulbecco's Modified Eagle Medium) 10% FCS (fetal calf serum) supplemented by 1mM pyruvate (complete culture medium, Gibco, France). For viability experiments,  $5 \times 10^3$  PANC-1 cells per  $\text{cm}^2$  were plated at day 0 in 48-wells cell culture plates (Falcon). The day after, cells were treated by the investigated molecules diluted in DMEM 5% FCS. Quantification of cells was performed using Alamar blue assay (Sigma-Aldrich, St Quentin Fallavien, France) after 72 hours of culture.

## 2.5. Quantification of gold internalization in tumor cells

PANC-1 cells ( $5 \times 10^5$  cells/well) were seeded in 6-well culture plates in complete culture medium. The day after, culture media were replaced by 5% FCS and cells were treated with gold nanoparticles with an amount equivalent of 1.8  $\mu\text{M}$  and 7  $\mu\text{M}$  N6L for 60 nm and 20 nm nanoparticles respectively during the indicated time. Cells were then washed two times in PBS (Phosphate-Buffered Saline), incubated with trypsin and harvested by centrifugation. Cell pellets were then treated with 12 N HCl (hydrochloric acid), solution was sonicated (30 min, 40°C), diluted 1/10 in H<sub>2</sub>O (water) and stored at -20°C until the quantification of gold. Note that the Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analyses (Au wt.%) were carried out with an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) spectrometer (Simultaneous Varian Vista Axial).

## 2.6 Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5.0 software. Statistical significance was determined using the unpaired *t*-test. Values of  $P < 0.05$  were considered significant. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$ .

## 2.7. Methods

*2.7.1. Zeta-Potential Measurements.* Zeta potential measurement was performed on a Zetasizer Nano-ZS (Malvern Instrument) to follow the adsorption process of the polyelectrolytes pair onto the gold core (Zetasizer 4700 Malvern Instruments, Brookhaven Instruments Corporation, USA).

*2.7.2. Wetting contact angle measurement.* Wetting contact angle measurements were performed with a Kruss goniometer (room temperature, sessile drop configuration) on gold modified surface.

*2.7.3. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D).* A Q-Sense E1 (Q-Sense, Sweden) system employing ATcut gold-coated quartz crystal sensors (Lot-Oriel, France) with a nominal frequency of about 5 MHz was used to monitor *in situ* formation of the self-assembly.

*2.7.4. Absorbance measurement.* Absorbance spectra were recorded with a UV-Visible spectrophotometer (a Varian Cary 100).

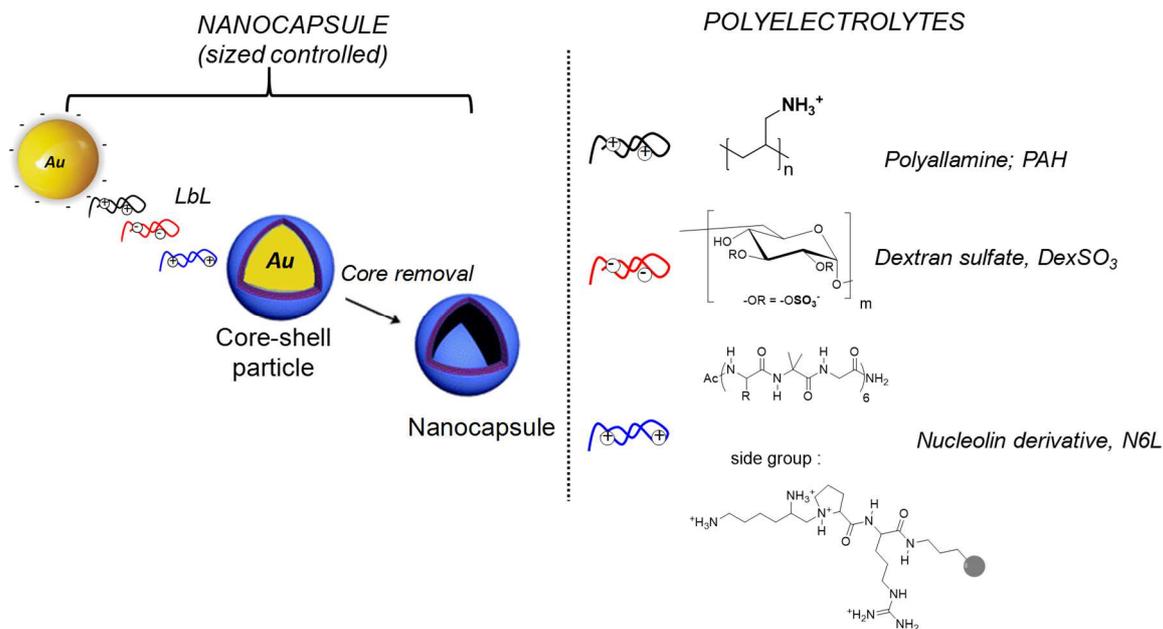
*2.7.5. Transmission Electron Microscopy (TEM).* Images of the capsules were taken with a Transmission Electron Microscopy. Observations were conducted on a Tecnai F20 ST microscope (field-emission gun operated at 3.8 kV extraction voltage) operating at an acceleration voltage of 200 kV and equipped with an X-ray energy-dispersive spectrometer (XEDS from Oxford Instruments) for chemical analysis. The samples were prepared as follows: a droplet of NP suspension and one of a solution of uranyl acetate (0.7 wt %) were deposited on a plasma pre-treated copper grid and left to dry at room temperature prior to analyze the NPs remaining on the copper grid.

*2.7.6. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).* ICP analyses (Au wt.%) were carried out with a ICP-OES spectrometer (Simultaneous Varian Vista Axial).

### **3. Results and discussion**

The preparation of the systems under consideration ~~is was~~ based on a key step of electrostatic interactions between the pseudopeptide N6L (positively charged at physiological pH) and polyelectrolytes. A method to create degradable hollow nanoparticles (namely nanocapsules) based on the surface modification of colloidal template particles and their subsequent removal ~~will-be was~~ used (Figure 1). This strategy ~~enables-a~~ enabled direct control of the size/dispersity of the resulting nanoparticles and the structure of the bioactive macromolecules. Herein, we focused on gold colloidal particles as template. This material presents the advantages of combining several attractive properties. ~~One may cite, including~~ (i) their biocompatibility and (ii) their commercial availability in a wide range of sizes (20 to 100 nm).

A multilayer film was assembled by electrostatic interactions between polyelectrolytes of opposite charges. Our tests involved the use of poly(allylamine hydrochloride) as a polycation, dextran ~~sulfate~~ sulphate as a polyanion, followed by a layer of N6L. Finally, the core of the nanoparticles was selectively removed by etching without affecting the colloidal stability of the final assembly (Figure 1). ~~Gold dissolution can be obtained through cyanide-assisted etching.~~ A water-soluble salt of potassium gold cyanide complex ~~is was~~ then formed and ~~can-be was~~ efficiently eliminated by a diffusion process across the polyelectrolyte membrane.



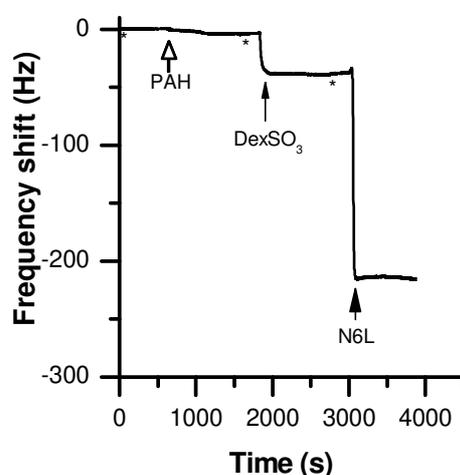
**Figure 1.** Schematic representation of the nanocapsules based on N6L, i.e. composed of poly(allylamine hydrochloride) / dextran sulfate / N6L.

This implemented approach was first shown *via* a pretreated flat gold surface by wettability measurement and adsorption test. Static contact angle assays ~~was done~~ were carried out on the flat surface before and after dipping in the electrolyte solutions, *i.e.* (PAH/ DexSO<sub>3</sub>/N6L), to qualitatively ~~proof the possibility to use the~~ prove the possibility of using N6L as a component of a multilayer film ~~onto~~ the surface. The contact angle value decreased from 75° to 20° upon coating, which could be linked to the hydrophilic nature of the macromolecules, ~~adsorbed on the surface~~. Then, stepwise adsorption of PAH, DexSO<sub>3</sub> and N6L was first investigated in real time by in situ QCM measurements on the flat gold surface, mainly to investigate the adsorption kinetics of each electrolyte, the possibility to absorb the N6L and then to show robust deposition of the implied electrolytes multilayers. ~~Quartz substrate pretreated~~ A quartz substrate, pre-treated with mercaptoundecanoic acid, was flushed in water and after an equilibrium time of 10 minutes, the polycation solution (PAH) was flowed through the negatively charged QCM sensor. A decrease of in the frequency signal was then observed (-5 Hz) indicating that mass was being added onto the surface (Figure 2). The surface was then flushed with water to remove all the unabsorbed polycation and then the polyanion (DexSO<sub>3</sub>) was flushed. Again, the frequency signal decreased (-38 Hz). The same process was applied with the N6L (-213 Hz). From the frequency shifts, we then use the Sauerbrey [31] equation to estimate the adsorbed masses per unit area *via* the following Equation (Equation 1):

$$\Delta m = -C \frac{\Delta f_n}{n} \quad \text{Equation 1}$$

with the mass sensitivity constant,  $C = 18.06 \pm 0.15 \text{ ng}\cdot\text{cm}^{-2}\cdot\text{Hz}^{-1}$  for sensors with a resonance frequency of  $4.95 \pm 0.02 \text{ MHz}$ , and the overtone number  $n$ . The normalized frequency shifts,  $\Delta f = \Delta f_n/n$ , for the third overtone were used here to estimate the deposited mass on the QCM sensor.

Then, after the deposition of PAH, DexSO<sub>3</sub> and N6L, the mass deposited on the QCM sensor is  $0.09 \text{ }\mu\text{g}/\text{cm}^2$ ,  $0.68 \text{ }\mu\text{g}/\text{cm}^2$  and  $3.85 \text{ }\mu\text{g}/\text{cm}^2$  respectively. The total mass deposited of N6L is then  $3.17 \text{ }\mu\text{g}/\text{cm}^2$ .

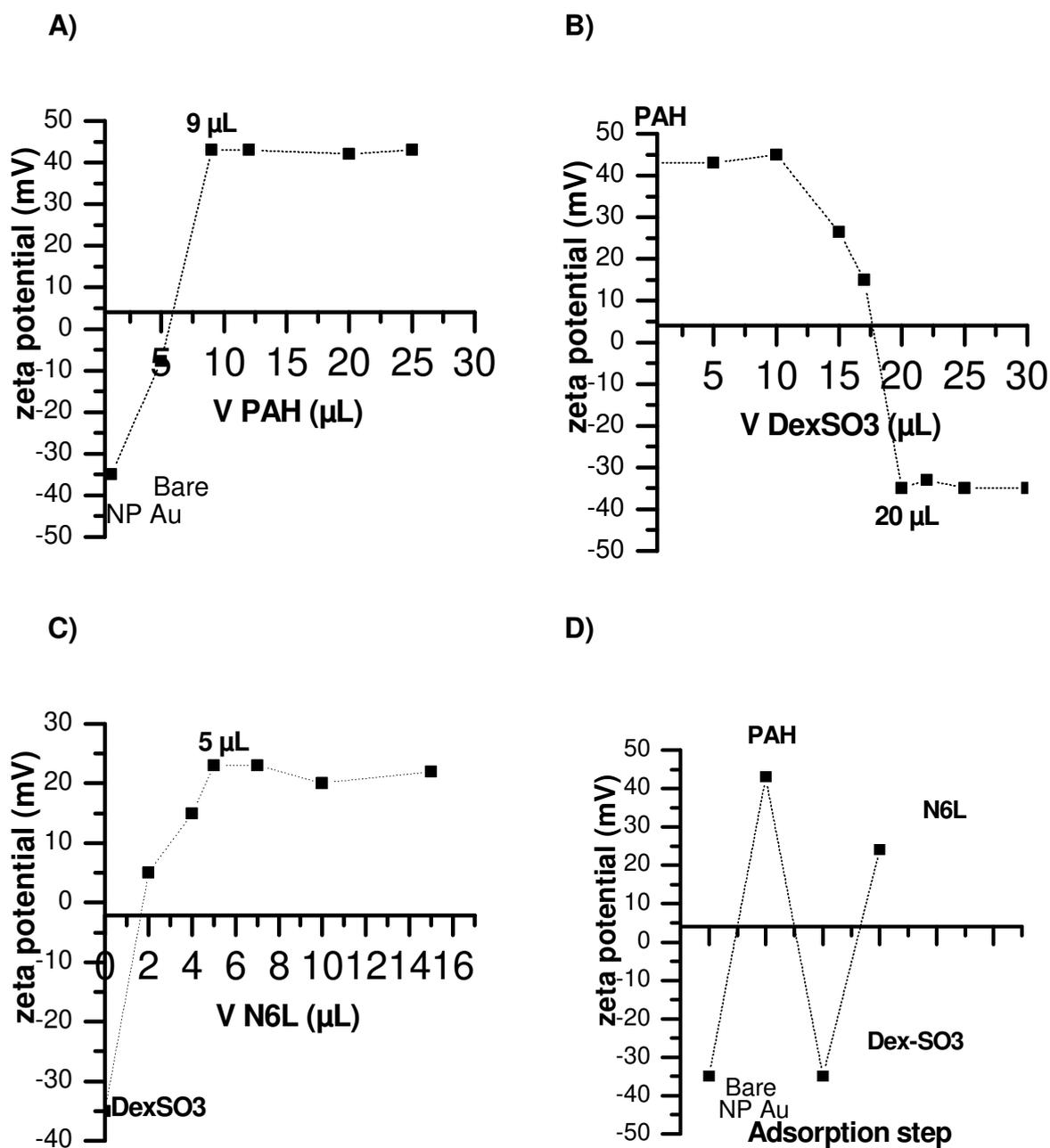


**Figure 2.** Frequency variation during layer-by-layer assembly on a flat surface (poly(allylamine hydrochloride) / dextran sulfate / N6L); (PAH: (poly(allylamine hydrochloride)), DexSO<sub>3</sub>: dextran sulfate and N6L) (\*:rinsing with solvent).

One of the most frequently used techniques to separate adsorbed polyelectrolyte from free polyelectrolyte is the centrifugation process. However, this way method has some drawbacks, for example, it is like time-consuming. The saturation method is a more attractive approach, as it presents the has a great benefit to in that it can present post-processing procedures. One In this regard, one may cite washing and centrifuge steps. The concentration of each electrolyte (called “saturation concentration”) needed to form each layer of the nanomaterial shell was estimated experimentally via zeta-potential measurements. [30, 32] The addition of either PAH or DexSO<sub>3</sub> or N6L was stopped as soon as the measured zeta potential value of the suspension of the coated nanoparticles reached a value near to those obtained with the polyelectrolyte alone in solution. Thus, it is was hypothesised that the quantity of free

polyelectrolyte in the suspension of particles was very low/insignificant. The required volumes of stock PAH solution (10 g/L prepared in water) to form a stable first layer were of 5  $\mu$ L and 9  $\mu$ L for the nanoparticles with a diameter of 20 nm and 60 nm, respectively. The polyanion deposition was then ~~done~~, carried out, and the volumes of the stock DexSO<sub>3</sub> solution (1 g/L prepared in water) to form the second layer were of 15  $\mu$ L and 20  $\mu$ L for the nanoparticles with a diameter of 20 nm and 60 nm, respectively. According to these data, in the experimental conditions described in Materials and Methods, the required volume of N6L ~~is was~~ 2  $\mu$ L (*i.e.* 50  $\mu$ g) and 5  $\mu$ L (*i.e.* 125  $\mu$ g) to saturate the nanoparticles with a diameter of 20 nm and 60 nm, respectively. As an example, Figures 3A-C show how the volume of each polyelectrolyte ~~is was~~ determined for the gold nanoparticles with a size of 60 nm.

Figure 3D shows the variation of the zeta potential value of coated nanoparticles after the layer adsorption of the PAH/ DexSO<sub>3</sub>/N6L shell with the required amount on the colloidal gold core. The negatively charged gold particles (-35 mV) were first coated with the positively charged layer of the PAH (+40 mV), ~~alternated~~–alternating with a layer of negatively charged DexSO<sub>3</sub> (-40 mV) and, finally, with the N6L (+20 mV). This charge reversal after each deposition clearly highlighted the coating of the colloidal gold nanoparticles *via* electrostatic interactions and, more importantly, the deposition of ~~the~~ N6L as the last layer.

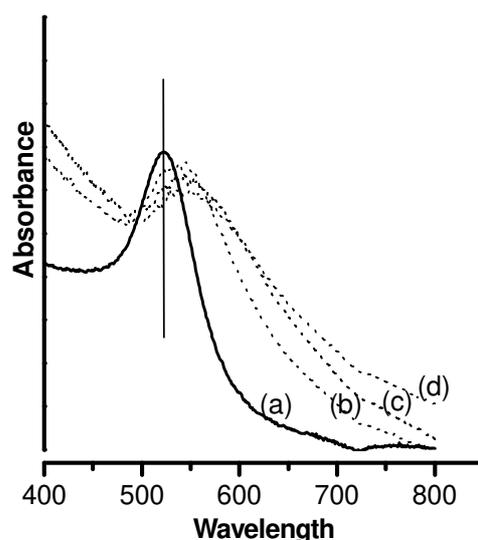


**Figure 3.** Determination of the saturation concentration of over zeta potential measurements (A) PAH, (B) DexSO<sub>3</sub>, (C) N6L. (D) Variation of the zeta potential value after each deposition step layer by layer of the assembly PAH/DexSO<sub>3</sub>/N6L, (PAH: poly(allylamine hydrochloride), DexSO<sub>3</sub>: dextran sulfate and N6L).

**A to C)** Determination of the saturation volume, via zeta potential measurements, of A) PAH, B) DexSO<sub>3</sub> and C) N6L.

**D)** Zeta potential variation during the elaboration of a PAH/DexSO<sub>3</sub>/N6L multilayer on gold nanoparticles.

Moreover, the successive deposition of the studied (macro-)molecules onto colloidal gold nanoparticles [33, 34] can also be followed by exploiting the sensitivity of the gold nanoparticle's surface Plasmon band,[35] i.e. UV-vis spectroscopy measurement enables to monitor each stage of surfaces' coating onto such nanoparticles. [35, 36] UV-vis spectra of gold nanoparticles (20 nm) coated with PAH/DexSO<sub>3</sub>/N6L coatings are shown in Figure 4. The absorption peak above 525 nm is assigned to the transverse surface Plasmon band of the bare nanoparticles. When coated with PAH/DexSO<sub>3</sub>/N6L layers, a red-shift of around 14 nm in the Plasmon band maxima is observed: 525 nm for uncoated gold nanoparticles (Figure 4, curve a), 532 nm for a coating with PAH (Figure 4, curve b), 536 nm PAH/DexSO<sub>3</sub> (Figure 4, curve c) and 539 nm for PAH/DexSO<sub>3</sub>/N6L (Figure 4, curve d). Such red-shifts in Plasmon band maxima are in agreement with the literature Caruso and co-workers, [37-39] Gole and Murphy [40] and Belbekhouche et al [23]. Furthermore, no additional Plasmon band in the UV-spectra strongly suggests the absence of nanoparticles aggregation upon the polyelectrolytes adsorption. [40]



**Figure 4.** UV-vis spectra of polyelectrolyte-coated gold nanoparticles (20 nm) as a function of the number of adsorbed polymer layers. Curves a, b, c and d depict for uncoated gold nanoparticles, (NP Au/PAH), (NP Au/PAH/DexSO<sub>3</sub>) and (NP Au/PAH/DexSO<sub>3</sub>/N6L); (PAH: (poly(allylamine hydrochloride), DexSO<sub>3</sub>: Dextran sulfate and N6L).

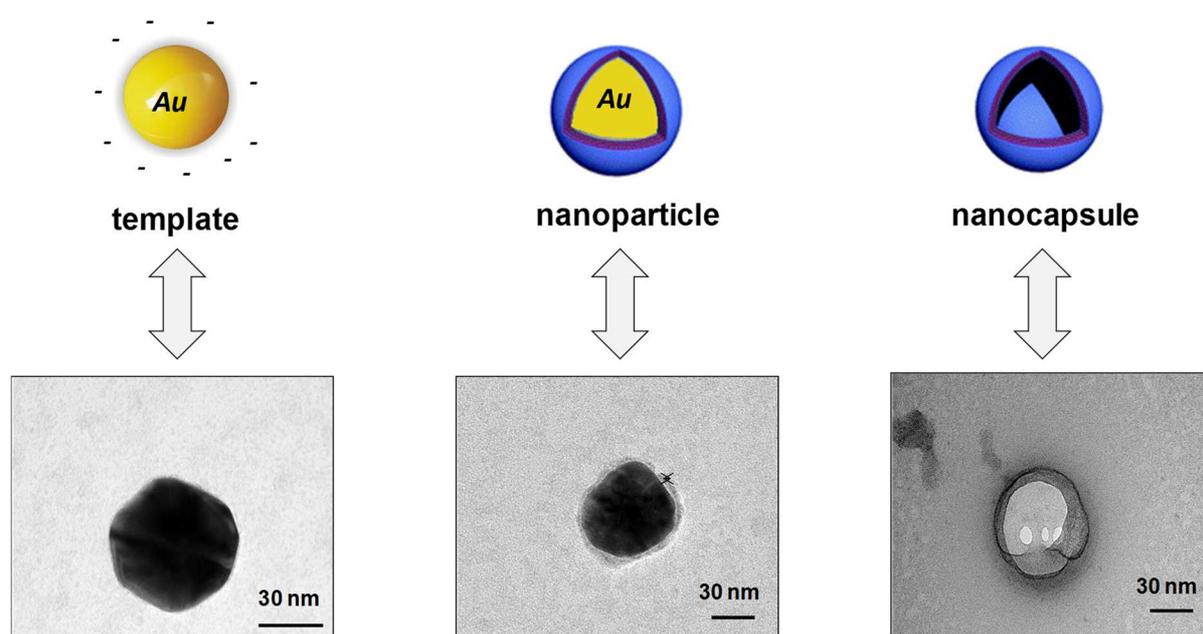
Nanocapsules can further easily be fabricated from modified gold nanoparticles and those simply by dissolving the Au<sup>0</sup> core. Indeed, cyanide-assisted etching in the presence of KCN enables the gold hydrolysis (Equation 2). A water-soluble salt complex is then obtained and

can easily and efficiently be removed via diffusion process across the polyelectrolyte shell. [21, 41]



At the end of this procedure, no presence of atomic gold was detected by Inductively Coupled Plasma Optical Emission Spectrometry analysis in the last dialysat solution.

Transmission electron micrograph (TEM) images of (A) uncoated gold nanoparticles, (B) (PAH/DexSO<sub>3</sub>/N6L) multilayer-coated gold nanoparticles and (C) (PAH/DexSO<sub>3</sub>/N6L) nanocapsules are depicted on Figure 5. These TEM observations strongly suggest the success of the studied strategy shown in Figure 1.



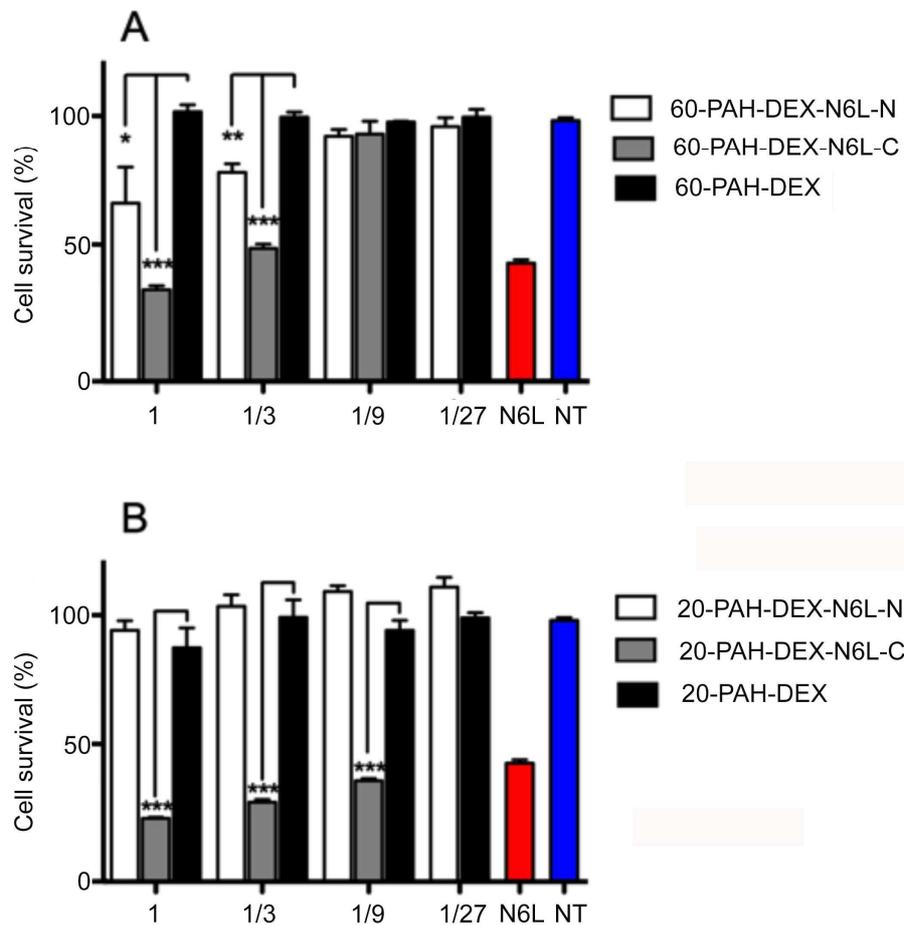
**Figure 5.** TEM Electron microscopy images with transmission of a gold nanoparticle unmodified (60 nm), modified by the layer-by-layer assembly PAH/DexSO<sub>3</sub>/N6L. (PAH: poly(allylamine hydrochloride), DexSO<sub>3</sub>: dextran sulfate and N6L) and capsules.

We next investigated the effect of these nanoparticles on the survival of human tumour cells derived from pancreatic cancer. In this context, **were investigated** the survival of PANC-1 cells treated with various concentrations of nanoparticles or nanocapsules of Au/PAH/DexSO<sub>3</sub>/N6L built with a nucleus of either 20 or 60 nm diameter **have been carried out**. As shown in Figure 6, only the nanoparticles assembled with the Au nucleus of 60 nm diameter induced in a dose dependent manner, a significant decrease **of in** the cell viability as compared to the control performed without N6L (Figure 6A). **As In** contrast, whatever the

considered dilution, no effect on cell viability ~~is was~~ observed for nanoparticles assembled with the Au nucleus of 20 nm (Figure 6B). Interestingly, whatever the size of the nanoparticles used in this study, nanocapsules significantly decreased, with a higher efficacy than nanoparticles, the viability of the tumour cells in dose dependent manner as compared to the control performed with non-~~functionalized~~ ~~functionalised~~ nanoparticles (Figures 1A and 1B). ~~Correlation~~ The correlation between the size, ~~the~~ shape and ~~the~~ surface chemistry of the nanoparticles and their biological response has been already studied.[14] To illustrate this point, it has been reported that, in *in vitro* experiments, nanoparticles ~~functionalized~~ ~~functionalised~~ with herceptin binds to the ErbB2 receptor with an affinity of  $5.5 \times 10^{-12}$  M on a 10-nm nanoparticle, and  $1.5 \times 10^{-13}$  on a 70-nm nanoparticles. Interactions between the ligands linked to the nanoparticles and ~~its~~ cell surface receptors are dependent ~~of on~~ several factors ~~like~~, including the geometry of the nanoparticles and the density of the ligands on the nanoparticles. [14] As a consequence, there ~~are-is~~ no clear correlation between the size and/or the shape of the nanoparticles and their biological activity. In our study, we ~~have-not-been able were unable~~ to quantify the concentration of ~~the~~-N6L linked to the nanoparticles. However, this amount ~~can could~~ be estimated from the QCM data, ~~which~~ indicated that we ~~can-link linked~~  $3.17 \mu\text{g}/\text{cm}^2$  of nanomaterial. Calculating the surface of the nanoparticles and knowing the concentration of those, we can evaluate that for the higher concentration used in the bioassay, cells ~~have-been were~~ treated with  $7.2 \mu\text{g}/\text{ml}$  ( $1.8 \mu\text{M}$ ) and  $28 \mu\text{g}/\text{ml}$  ( $7 \mu\text{M}$ ) of N6L for 60 nm and 20 nm nanoparticles/nanocapsules, respectively. This result is in accordance with the fact that the 20 nm nanocapsules ~~are were~~ more efficient ~~on in~~ impeding cell survival than the 60 nm ones nanocapsule. The cytotoxicity of nanoparticles as well as nanocapsules is given by the presence of N6L which is a ligand of cell-surface nucleolin that induces the apoptosis of tumour cells. [42] As previously described, binding of this ligand results in clustering of cell-surface nucleolin in lipid raft membrane microdomains before endocytosis of the ligand-nucleolin complex and induction of the apoptosis of tumor cells.[42] This is a dynamic mechanism and since the nanocapsules are more flexible than nanoparticles, [43, 44] we can presume that will be more efficacy in inducing apoptosis of tumour cells as presented in the figure 6.

Another interesting result is that the nanocapsules ~~have had~~ a higher specific activity than N6L alone. While in this experimental model, the value of IC50 of N6L alone (*i.e.* not associated with nanomaterials) ~~is was~~  $30 \mu\text{M}$ , in the nanocapsule form the IC50 value ~~is was~~ an equivalent to  $1.8 \mu\text{M}$  and  $7 \mu\text{M}$  of N6L, for 60 nm and 20 nm nanocapsules, respectively

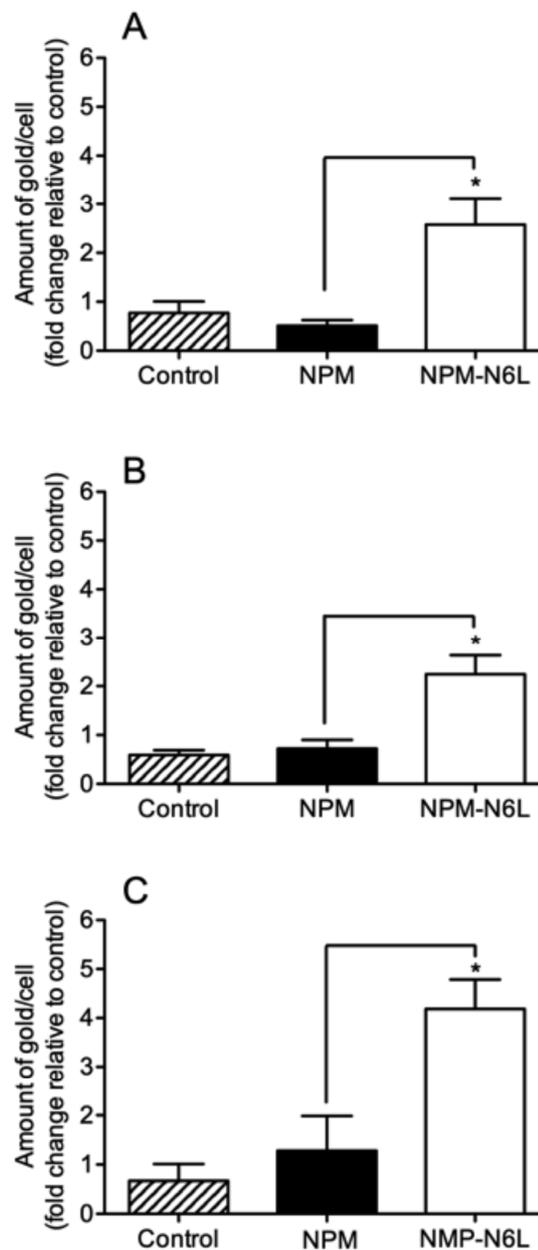
(Figure 6). Similar observations have been already reported showing that multivalent interactions between the nanoparticles and their targets **can** increase the affinity of target binding.[45]



**Figure 6.** Effect of (A) 60 nm or (B) 20 nm nanoparticles or nanocapsules functionalized or not with N6L on the survival of PANC-1 cells (DEX = DexSO<sub>3</sub>). The undiluted sample corresponds to an N6L concentration of 1.8 μM and 7 μM for 60 nm and 20 nm nanoparticles. Controls have been performed with 30 μM of N6L (red) and the viability of untreated cells was shown (blue). **Note that 'N' and 'C' refer to N6L-nanomaterials with (labeled “N”) or without gold matrix (labeled “C”, i.e. capsule)**

We next investigated the capacity of the N6L ~~functionalized-functionalised~~ nanoparticles to target tumour cells by quantification of Au by ICP. PANC-1 cells were incubated with 60 nm - Au/PAH/ DexSO<sub>3</sub>/N6L or 60 nm - Au/PAH/ DexSO<sub>3</sub> during three different times, cells were then lysed, and the amount of Au ~~amount~~ quantified by ICP. ~~Data are~~ The data (Figure 6) showed ~~presented in the Figure 6 and show~~ that whatever the incubation time, a significantly higher amount of Au ~~has been~~ was found in N6L ~~functionalized functionalised~~ nanoparticles

treated cells as compared to those treated with non functionalized non-functionalised these nanoparticles, validating the data obtained from the cell survival assay. These results are in agreement with those obtained with functionalized-functionalised iron oxide nanoparticles, showing that after 1 hour of incubation with tumour cells, the quantity of iron in the cells was significantly greater compared to the non-functionalized-functionalised nanoparticles. Taken together, these data show that the N6L functionalized-functionalised nanoparticles are able to target tumour cells through N6L. [17]



**Figure 7.** Time course of internalization of 60 nm nanoparticles functionalized or not with N6L. PANC-1 cells were treated for (A) 1 hour, (B) 3 hours or (C) 24 hours with 60 nm nanoparticles functionalized (~~NMP-N6L~~-NPM-N6L) or not (~~NMP~~ NPM) with N6L. Quantification of gold has been performed by ICP as described in Materials and Methods.

#### 4. Conclusion

In summary, an easily controllable continuous templating and self-assembly-based method was developed to produce N6L-~~functionalized~~ functionalised particles. In this regard, colloidal gold nanoparticles ~~were~~ templates were used and modified *via* a self-assembly approach. Compared with conventional methods, which are generally time-consuming and require organic solvents, the presented approach has several attractive features, **as including** simple fabrication steps, ~~green process, an environmentally-friendly process~~ and fast and easy particle production, ~~allow the control of~~, and, additionally, allows control over the size and the composition of the carrier. More precisely, multilayer films have been fabricated by an immersion-assisted approach on nanoparticles templates, *i.e. via* electrostatic interactions between oppositely charged electrolytes: PAH (polycation), DexSO<sub>3</sub> (polyanion) and N6L (polycation and bio-active agent). The sacrificial template ~~will finally be~~ was finally removed without affecting the colloidal stability of the carrier, which ~~allows us to functionalize~~ will allow the functionalisation of these nanocapsules with other therapeutic molecules ~~and their use supports their application~~ in *in vivo* studies. Studies are currently in progress to evaluate this possibility. Each step ~~implied~~ applied has been carefully studied *via* physicochemical ~~characterization~~ characterisation, leading to noteworthy data on the size, ~~the~~ surface charge and ~~the~~ amount of the N6L embedded. Compared to ~~commercialized~~ commercialised particles, these novel systems ~~present the advantages to obtain a well-defined carrier and also to specifically target tumor cells (nanoparticle concentration, size, N6L loaded...)~~ have the advantage of enabling the production of a well-defined carrier and inducing apoptosis of tumour cells

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