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# Synthesis of biocompatible polymeric nano-capsules based on calcium carbonate: A potential *cisplatin* delivery system

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ABSTRACT

A smart nanocarrier system for cancer therapy, based on a recently developed technique for preparing pure nanometric calcium carbonate ($CaCO_3$), was studied. Different approaches were used to obtain sustained release of *cisplatin*: at first, pure $CaCO_3$ nanoparticles were evaluated as carriers, then the nanoparticles were functionalized with polymer or silanes, and finally they were employed as a substrate to build layer by layer (LbL) self-assembled polyelectrolyte nanocapsules. Loading efficiency and release kinetics were measured. The best loadings were obtained with the LbL nanocapsules, allowing for high loading efficiency and the possibility of controlling the release rate of the drug. The behavior of all the carriers was evaluated on four neoplastic cell lines, representative of different types of neoplastic disease, namely MCF-7 (breast cancer), SKOV-3 (ovarian cancer), HeLa (cervical cancer) and CACO-2 (human epithelial colorectal adenocarcinoma). Negligible cytotoxicity of the nanoparticles, functionalized nanoparticles, and nanocapsules was observed in experiments with all cell lines. Nanocapsules were functionalized with fluorescein isothiocyanate (FITC) in order to track their kinetic of internalization and localization in the cell line by confocal laser scanning microscopy (CLSM). The cytotoxicity of the loaded capsules was evaluated, showing cell survival rates close to those expected for non-encapsulated *cisplatin* at the same nominal concentration.



## 1. Introduction

Nano-sized drug carriers like polymer conjugates, liposomes, micelles, dendrimers, inorganic or other solid particles, and many others brought important contributions to cancer therapy over the last decade [1,2]. Advantages of such nanocarriers lie in their ability to improve drug solubility, prolong systemic drug half-life, provide means for sustained and environmentally responsive drug release, enable tumor specific delivery, reduce immunogenicity and systemic side effects, suppress development of drug resistance and also deliver simultaneously two or more drugs for combined therapy [3].

Among the large variety of nano/microparticles described in literature and optimized to increase efficacy and improve the delivery into cancer cells, one can find calcium carbonate ($CaCO_3$). Calcium carbonate is an extremely important biomaterial whose behavior is driven by its defined proprieties, such as morphology, structure, size, specific surface area and chemical purity. In our previous work we demonstrated that calcium carbonate microparticles can be considered an ideal drug carrier due to their excellent biocompatibility and ability to readily penetrate cancer cells [4]. Furthermore they allowed us to engineer microcapsules of polyelectrolytes multilayers starting from calcium carbonate micro-core templates, capable to encapsulate various classes of drug molecules, by using polymers that are biodegradable or that can respond and release their payload in response to well-defined stimuli [5–7]. To respond to the requirements of the biomedical field to deliver anti-tumor drugs, we have developed a smart method for the synthesis of particles of calcium carbonate of nanometric dimensions. This spray drying method allows synthesizing of pure and thermodynamically stable nanocalcium carbonate by a fast and atom economic method [8]. The technique is easily scalable allowing at the same time to tune the size and morphology without surfactants or other chemical species commonly used to stabilize the mixture and the reaction product.

In this work the nanometric $CaCO_3$ is used as both template and as direct carrier to load the most widely used platinum-based anti-neoplastic agent, *cis*-dichloro diammineplatinum(II) (*cis*-$[PtCl_2(NH_3)_2]$, *cisplatin*) [9]. It is a potent drug that is usually administered intravenously for treatment of solid malignancies [10]. Apart from its well established clinical value, it is one of the few approved transition-metal-based drug, inspiring generations of inorganic chemists to pursue application of their research in medical sciences [11]. However, a major

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obstacle to more widespread use of *cisplatin* is the persistence of severe toxic side effects [12]. Other disadvantages associated with *cisplatin* clinical use include short circulation period in the blood due to glomerular excretion [13], intrinsic or acquired resistance of some tumors to the drug and limited aqueous solubility (1.0 mg/mL) [14]. *Cisplatin* undergoes ligand exchange reactions kinetics which are largely determined by the nature of the leaving groups. In biological fluids *cisplatin* can react irreversibly with a variety of nitrogen- and sulfur-containing biomolecules that reduce its therapeutic concentration [15,16]. However, *cisplatin* also reacts with weaker nucleophiles, *i.e.* carboxylate ions, and the resulting species are able to undergo the reverse exchange reaction with chloride ions to regenerate *cisplatin* at physiological salt concentrations. It is estimated that up to 90% of *cisplatin* binds to plasma proteins [17], and as low as 1% of intracellular *cisplatin* manages to actually interacts with DNA [18,19] to give the intrastrand cross-links between adjacent purines [20], the lesions considered to be responsible [21] for the antitumoral activity. Creating a drug delivery system able to reduce the drug loss on the way could and targeting the cell would therefore be a dual advantage. We have investigated several ways to load the *cisplatin* into systems based on $CaCO_3$. The results reported demonstrated how the loading efficiency can be changed exploiting the calcium carbonate features, functionalizing their surface by silane, covering it with polymers in order to create a capsule [6]. The latter strategy was the one that gave the best results. The polymeric nanocapsules were obtained according to the layer by layer technique (LbL), a method used widely to fabricate hollow multilayered capsules by depositing polyelectrolytes onto cores afterwards sacrificed after film formation [22–31]. The majority of polyelectrolyte capsules described in literature are composed of pairs of synthetic not biodegradable polymers (*e.g.* polystyrenesulfonic acid and polyallylamine hydrochloride, PSS and PAH), or composed of biocompatible and biodegradable (*e.g.* protamine and dextran sulfate, PRM and DXS) polymers, which are more suitable to medical therapy. Of the two pairs of polyelectrolytes we selected, PRM/DXS and protamine/alginate (PRM/ALG), the best loading efficiencies were obtained with the latter.

## 2. Experimental section

### 2.1. Chemicals

The following reagents were used (abbreviations, if used, and source in parentheses): dextran sulfate sodium salt from Leuconostoc s (DXS, Sigma, USA), protamine sulfate salt, grade III (PRM, Sigma, USA), polystyrenesulfonate sodium salt (PSS, Sigma, USA), alginic acid sodium salt (ALG, Sigma, USA), calcium chloride dehydrate 99.99% ($CaCl_2$•$2H_2O$, Aldrich, USA), sodium hydrogen carbonate ($NaHCO_3$, pro analysis, Merck, Germany), ethylenediaminetetraacetic acid disodium salt 99 + % (EDTA, Sigma, USA), fetal bovine serum (FBS, Sigma, USA), penicillin–streptomycin solution (Sigma, USA), sodium pyruvate (Sigma, USA), DMEM medium (Sigma, USA), [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide ≥97.5% TLC (MTT, Sigma, USA), phosphate buffer solution, Dulbecco A (PBS, Oxoid), sephadex G25 (Sigma, USA), fluorescein isothiocyanate isomer I (FITC, Aldrich, USA).

### 2.2. Synthesis of nano-$CaCO_3$

Pure $CaCO_3$ nanoparticles were synthesized by an atomization process [8], which involves the mixing of two aqueous solutions: $NaHCO_3$ and $CaCl_2$ at molar ratio 2:1 (Scheme 1). The reaction is carried out in atmosphere and temperature-controlled.

The two solutions are mixed using two pumps which allow a fine control of flow rate of each reagent. Then they are atomized in a hot air flow at 140 °C. The high temperature causes a rapid evaporation of the reaction mixture water, and allows the direct production of the powder of calcium carbonate and of sodium chloride, which are accumulated in a collection vessel; the reaction by-product (NaCl) can be easily removed by means of later water washings.

$$2NaHCO_3 + CaCl_2 \rightarrow 2NaCl + Ca(HCO_3)_2$$

$$Ca(HCO_3)_2 \rightarrow CaCO_3 + H_2O + CO_2$$

**Scheme 1.** Chemical reaction.

### 2.3. Silane modification of nano-$CaCO_3$

A minimal amount of anhydrous ethanol was added to the nano-calcium carbonate power. Then, the mixture was ultrasonic dispersed for 30 min. Under vigorous magnetic-stirring at 30 °C, 0.75 mL silane (Aminopropyltriethoxysilane, APTES, methyltrimethoxysilane MTMS or methyltriethoxysilane MTES) solution was added dropwise. After stirring at 30 °C for another 5 h, the mixture was centrifuged at 4000 rpm/min for 15 min to separate the APTES, MTMS, or MTES modified nano-calcium carbonate from the reaction medium. Finally, the silane modified nano-calcium carbonate was washed with ethanol for 5 times, dried at vacuum and kept for application.

### 2.4. Capsules fabrication

Polyelectrolyte nanocapsules were prepared by alternating incubation of $CaCO_3$ nanoparticles in DXS and PRM aqueous solutions (2.0 mg $mL^{-1}$), or in ALG and PRM solutions (2.0 mg $mL^{-1}$). The pH of the polymer solutions was adjusted to 6.5 by addition of HCl/NaOH. Capsules were fabricated in a two-step procedure. In a first step, the $CaCO_3$ nanoparticles were coated by using a LbL technique. 1.0 mg of powder of $CaCO_3$ was dispersed in a solution containing the polyanion (DXS or ALG). The dispersion was continuously shaken for 10 min. The excess polyanion was removed by three centrifugation/washing steps with deionized water. Thereafter, 1.0 mL of solution containing the polycation (PRM) was added and the dispersion was continuously shaken for 10 min, followed again by three centrifugation/washing steps. This procedure was repeated several times for each polyelectrolyte resulting in the deposition of four or six polyelectrolyte layers on the $CaCO_3$ particles. Coated colloids were cross-linked by 0.5% glutaraldehyde (GA) at room temperature (20–25 °C) for 30 min followed by three centrifugation/washing steps. In a second step, the $CaCO_3$ core was removed by complexation with EDTA. Cross-linked coated particles were shaken for 2 min with 1.0 mL of a 0.2 M EDTA solution pH 5.5 was adjusted by HCl, followed by centrifugation and redispersion in 1.0 mL of a fresh EDTA solution pH 7.5. The hollow nanocapsules obtained in this way were washed four times with deionized water and stored at 4 °C in water. To fabricate capsules with fluorescent-labeled polyelectrolytes, the same procedure was used with DXS-FITC. In order to study the localization and internalization of these nanoparticles, polymeric colloids of nano-$CaCO_3$ nanoparticles were prepared using a couple of synthetic polyelectrolytes, PSS and PAH-FITC.

### 2.5. Transmission Electron Microscopy (TEM)

TEM images were recorded with a Jeol Jem 1011 microscope operated at an accelerating voltage of 100 kV. We prepared samples for analysis by dropping a diluted nanoparticle dispersion in water onto carbon-coated copper grids and then allowing water to evaporate.

### 2.6. Cell culture

MCF-7 (human breast cancer), HeLa (human cervical cancer), SKOV-3 (human ovarian cancer), and CACO-2 (human epithelial colorectal adenocarcinoma) cell lines were used. Cancer cell lines were maintained in DMEM medium supplemented with FBS (10%), penicillin

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