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Multifunctionalization of magnetic nanoparticles for controlled drug release: A general approach

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ABSTRACT

In this study, a general approach for the multifunctionalization of magnetic nanoparticles (MNPs) with drugs (Doxorubicin and Gemcitabine) and targeting moieties (Nucant pseudopeptide) for controlled and selective release is described. The functionalization is achieved by the formation of disulfide bonds between MNPs and drugs derivatives synthesized in this work. Our strategy consists in the introduction of a pyridyldisulfide moiety to the drugs that react efficiently with sulfhydryl groups of pre-activated MNPs. This approach also allows the quantification of the covalently immobilized drug by measuring the amount of the 2-pyridinethione released during the process. The linkers developed here allow the release of drugs without any chemical modification. This process is triggered under highly reducing environment, such as that present inside the cells.

Complete release of drugs is achieved within 5–8 h under intracellular conditions whereas negligible percentage of release is observed in extracellular conditions.

We propose here a modular general approach for the functionalization of nanoparticles that can be used for different types of drugs and targeting agents.

1. Introduction

The use of nanoparticles as carrier systems for therapeutic molecules has been explored during the last 20 years [1] aiming to improve the therapeutic effect of the drugs and their administration, as well as, to reduce their side effects. Different types of nanostructures (metallic nanoparticles, polymeric core–shell nanoparticles or micelles) have been evaluated particularly in cancer therapy, whose vast side effects compromise the health of patients. In this regard, nanoparticles can be designed as multifunctional platforms that can be loaded with several drugs and also modified with targeting molecules to direct them to cancer cells [2]. Targeted therapies have been developed to diminish side effects of current approaches [3] where specific cell membrane receptors overexpressed by cancer cells are used as targets, improving the efficacy of classical treatments [4,5].

Nanoparticles for medical applications, and particularly for targeted cancer therapy, must be (1) non-toxic (2) with a good colloidal stability in physiological conditions; (3) easy to load with known amounts of therapeutic agents and targeting molecules and (4) able to release the cargo efficiently inside the cells. Therefore, the development of strategies for the functionalization of nanoparticles is a crucial point for the future clinical use to improve anticancer therapies.

In this study, immobilization strategies for the functionalization of magnetic nanoparticles (MNP) using tailored linkers have been developed. The systems described have the required properties to be successfully employed in biomedical applications.

In particular, dimercaptosuccinic acid coated magnetic nanoparticles [6,7] (DMSA-MNPs) have been functionalized with two chemotherapeutic drugs and a targeting molecule. The controlled release of the drugs has been evaluated. One of the drugs employed is Doxorubicin (DOX), which is widely used to treat a broad spectrum of cancers (breast, stomach, non-Hodgkin's lymphoma, and bladder cancer) [8]. One advantage of DOX is its strong visible absorption and fluorescence emission that makes it easy to monitor during the different steps of the functionalization strategy. The other chemotherapeutic drug used is Gemcitabine (GEM) that is employed in several cancers such as pancreatic cancer [9–11], non-small cell lung cancer, bladder cancer, soft-tissue sarcoma, metastatic breast cancer and ovarian cancer and acts as an antineoplastic

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agent. Both drugs are cell-cycle specific therapeutic agents and therefore need to reach the nucleus. DOX acts by intercalating DNA whereas GEM replaces cytidine during DNA replication. Despite positive results with these drugs in clinics, classical chemotherapy still presents several problems. For example, doxorubicin has shown great efficacy in both solid and liquid tumors, but the emergence of drug resistance and several side effects such as heart muscle damage are important limitations for successful cancer treatment [12]. Gemcitabine undergoes rapid deamination into the inactive uracil derivative, resulting in a short half-life. Also in the case of Gemcitabine, drug resistance has been observed in *in vitro* and *in vivo* preclinical models, which is critical for future clinical usage [13]. The use of nanoparticle-based delivery systems has been shown to overcome multidrug resistance in tumors and to increase the stability immobilized molecules [14,15]. Therefore, more efficient nanoparticle-based formulations that additionally incorporate targeting for local administration are needed. In this sense, to introduce targeting capabilities in the multifunctional formulations the pseudopeptide Nucant (N6L) has been used together with DOX and GEM. The Nucant pseudopeptide, which acts both as an anticancer drug and as a targeting agent is nowadays in clinical trials. N6L binds nucleolin, which is a protein overexpressed in the membrane of cancer cells, and nucleophosmin [16,17], and can enter the cell nucleus to induce apoptosis [18].

As a first approach to improve cancer therapy non-covalent functionalization of nanoparticles, mainly through electrostatic or hydrophobic interactions [19,20] has been explored intensively in the past due to the ease of application, but has some concerns and drawbacks. The most important limitation is the poor control on the release of the drug immobilized onto such nanostructures. The drug release occurs as a passive process based on the high concentration of salts and biomolecules *in vivo* or on pH changes. This strategy is suitable for *in vitro* assays or *in vivo* assays using intratumoral injection but it should not be applied intravenously. Another drawback is the fact that neutral drugs under physiological conditions, such as Gemcitabine cannot be immobilized electrostatically.

Taking into account all these parameters, the covalent functionalization of anticancer drugs onto targeted nanoparticles seems to be a suitable way to tackle these problems. Ideally, only cancer cells should be targeted by drug-loaded magnetic nanoparticles, where anti-tumoral agents are inactivated until they are released inside the cell. In this regard, different linkers sensitive to certain intracellular triggering stimulus such as pH [21,22] and the presence of some enzymes [23–25], or external stimuli such as temperature [26,27] have been employed to connect and release drugs from magnetic nanoparticles in a controlled manner. On the other hand, disulfide bond based linkers have excellent properties for this application [28] because allow the formation of a covalent bond between the nanoparticle and the required molecule. Then, the disulfide bond can be broken by specific reducing agent such as endogenous glutathione (GSH). It is well known that the intracellular level of GSH in cells is in the millimolar range (0.5–10 mM), whereas just micromolar concentrations are typically found in blood plasma and the extracellular medium [29]. Moreover, it has been shown by clinical studies that tumor tissue is often higher in glutathione content than normal tissue [30,31]. By designing the functionalization with a disulfide linker, the attached molecule will be released only under highly reducing environment such as the tumor cells' intracellular environment. Another advantage of the strategy described here relies on the design of a modification attached to the molecules in order to quantify the immobilization. By anchoring a linker ending with a thiol activated by 2-mercaptopyridyl group, it is possible to quantify the amount of molecules linked to the nanoparticles by following the UV–Visible absorption of the byproduct released during the functionalization reaction. Finally, the linkers have been designed in order to release the molecule without any chemical modification, after an internal rearrangement [32,33]. Therefore, the initial molecule will be released within the cell and its activity will not be affected.

The work presented here is a successful example of efficient drug's covalent functionalization of nanoparticles for an intracellular controlled release. The method herein presented could be applied to other drugs (charged or neutral, fluorescent or not) and targeting agents (peptide, antibodies, etc.) for multiple biomedical applications.

2. Experimental

2.1. Materials

All reagents were purchased from Aldrich and used without further purification. José Courty's group from CRRET-CNRS laboratory provided cysteine modified Nucant pseudopeptide (N6L-Cys). Ultrapure reagent grade water (18.2 MΩ, Wasserlab) was used in all experiments. DMSA-MNPs have been provided by Dr. Gorka Salas' group at IMDEA Nanociencia.

2.2. Measurements

Thin layer chromatography (TLC) was carried out using Silica Gel 60 F254 plates. Column chromatography was performed using Silica Gel (60 Å, 230 × 400 mesh). All NMR spectra were recorded on a Bruker instrument (MHz indicated in brackets) as solutions in $CDCl_3$, and the chemical shifts are reported in parts per million (ppm). Coupling constants are reported in hertz (Hz). MALDI-TOF mass spectrometer analysis was performed using a Voyager DE Pro (AB Applied Biosystems). *UV–Vis* and fluorescence spectra were recorded on a Synergy H4 microplate reader (BioTek) using 96-well plates. Hydrodynamic diameter and zeta potential measurements were determined using a Zetasizer Nano-ZS device (Malvern Instruments). Hydrodynamic diameter and zeta potential were measured from dilute sample suspensions in water at pH 7.4 using a zeta potential cell. HPLC: Agilent Technologies, 1260 Infinity. Column ZORBAX 300SB-C18, 5 μm, 9.4 × 250 mm.

2.3. Synthesis and characterization

2.3.1. Synthesis of intermediates and drugs derivatives

2.3.1.1. Synthesis of 2-(pyridin-2-yldisulfanyl)ethanol (1) [32]. To a solution of aldrithiol (300 mg, 1.36 mmol) in MeOH (1.5 mL) under Ar, 2-mercaptoethanol (53 μL, 0.75 mmol) was added slowly and stirred for 16 h. Then, the solvent was evaporated in vacuum and the residue purified by flash chromatography (CH_2Cl_2/AcOEt 5:1) to obtain compound **1** (Fig. 1) as a colorless oil in 86% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, J = 4.3 Hz, 1H), 7.58 (td, J = 8.0, 1.7 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.17–7.13 (m, 1H), 5.72 (bs, 1H), 3.80 (dd, J = 10.4, 6.5 Hz, 2H), 2.97–2.94 (m, 2H).

2.3.1.2. Synthesis of 4-nitrophenyl 2-(pyridin-2-yldisulfanyl)ethyl carbonate (2) [32]. To a solution of compound **1** (100 mg, 0.53 mmol), and bis(4-nitrophenyl) carbonate (241 mg, 0.79 mmol) in CH_2Cl_2 (2 mL) under Ar, DIPEA (158 μL, 0.79 mmol) was added and stirred for 5 h. The mixture was washed with water, and the organic phase dried with $MgSO_4$. After solvent evaporation, the residue was purified by flash chromatography (Hexane/AcOEt 4:1 and then 2:1) to obtain compound **2** (Fig. 1) as a colorless oil 67% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (d, J = 4.8 Hz, 1H), 8.28 (d,