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Versatile quarto stimuli nanostructure based on Trojan Horse approach for cancer therapy: Synthesis, characterization, in vitro and in vivo studies

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ABSTRACT

Nanostructured delivery and diagnostic systems that induces specific targeting properties by exploiting the local physicochemical tumour characteristics will be evaluated is the present work. It is well known that cancer cells have specific physicochemical characteristics, which can be taken into consideration for the design of a broad spectrum of drug delivery systems (DDS). Some of those characteristics including the different temperature environment their susceptibility when temperature ranges between 40 and 43 °C where cell apoptosis is induced, the intra- and extra-cellular pH which varies from 6.0 to 6.8, for cancer cells, and 6.5 to 7.4 for normal cells respectively, (lysosomes acidic pH ranges 4–5). Additional significant factors are the overexpressed receptors on the tumour surface. Loading and release studies were carried out by using the anthracycline drug Doxorubicin and their cytotoxicity was evaluated by using the MTT assay in healthy and diseased cell lines. The highlight of this work is the in vitro and in vivo studies which were performed in order to evaluate different nanostructures as for their biodistribution, pharmacokinetic and toxicity per se.

1. Introduction

Currently, there has been a range of synthetic approaches of novel and smart drug delivery systems (DDS) aiming at improving cancer therapies as well as the patient's quality of life. The development of these systems is substantial in order to achieve more effective and targeted treatments [1–10]. Conventional treatments, such as chemotherapy and radiotherapy, show a lack of selectivity, as they also target normal cells and healthy tissues, thus causing serious side effects. The potential contribution of DDS in cancer treatment focuses not only on reducing toxicity by targeting only the tumour tissue, but also on improving the drug efficacy by increasing drug bioavailability and circulation time. It is well known that cancer cells have specific physicochemical characteristics, which can be taken into consideration for the design of a broad spectrum of DDS. Some of the characteristics include: 1) the different temperature environment in the surrounding area of cancer cells due to their rapid proliferation, 2) their susceptibility when temperature ranges between 40 and 43 °C [10–15] where cell apoptosis is induced, in contrast to normal cells, 3) the different intra- and extra-cellular pH which varies from 6.0 to 6.8, for cancer cells, and 6.5 to 7.4 for normal cells respectively, (lysosomes acidic pH ranges 4–5) [16] due to lactic acid production, 4) the enzyme's abundance which creates specific reduction and oxidizing (redox) conditions inducing glutathione in 10-fold greater concentration than normal cells. By combining all these factors, a smart nanocarrier can be fabricated aiming at targeting the cancer cells through the physicochemical characteristics in which the carriers are sensitive releasing the desired drug in a controlled manner. The idea of the aforementioned nanocarrier has been studied for the past few years and a great number of ideas came to forefront [17–19]. Thermo-, pH-, and redox sensitivities were used as a single property. By combining two of the above properties, the targeted polymer nanocarriers were achieved [17–30]. These responsive nanocarriers can be in various shapes and types such as microspheres, micelles, nanorods, nanoparticles, nano-containers, etc. and are based on desired polymer's properties for cancer treatment. One of the most sophisticated characteristics for the nanocarriers' fabrication is

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thermo-sensitivity [17,31–33]. Monomers such as Hydroxy Propy Methacrylamide (HPMA) and Dimethyl Amino Ethyl Methacrylate (DMAEMA) consist of polymers desirable for their thermosensitive behaviour. Similar to thermo-sensitive monomers are, pH-sensitive monomers [37–39] such as Acrylic Acid (AA) and redox-sensitive monomers [40–42] such as *N,N′*-(disulfanediylbis(ethane-2,1-diyl))bis(2-methylacrylamide). The combination of the most appropriate monomers results into the fabrication of a copolymer that comprises thermo- and pH-sensitivity, or thermo- and redox-sensitivity, or any other combination of the above desired properties. A way to enhance the efficacy of the nanocarriers in cancer therapy is to introduce magnetic nanoparticles either on the nanocarriers' surface or in their interior. Magnetic nanoparticles, such as Fe_3O_4 (magnetite), have been used extensively in medicine for cancer therapy, [37–41] either in Magnetic Resonance Imaging (MRI) contrast agents or in local hyperthermia. By using an alternating magnetic field (AMF) the temperature of the nanocarriers doped with magnetic nanoparticles increases in a controlled manner, thus inducing an additional capability to the smart DDS. Great progress has been also achieved in the design of nano- and micro-carriers, which selectively carry radionuclides, aiming at the improvement of cancer diagnosis outcome, staging and treatment [23,42].

In this study, we present the biological evaluation of a versatile DDS, quarto stimuli nanocontainer, PMMA@ P(MMA-*co*-DMAEMA-*co*-DVB-*co*-PEG360-*co*-AA-*co*-DS)@Fe_3O_4 (Q-NCs) with pH-, thermo- and redox sensitivity that can be used in hyperthermia treatment. The fabricated system was assessed for, a) its loading and release behaviour using the drug Doxorubicin (DOX) as a model drug, b) its hyperthermia impact, c) its cytotoxicity profile against cancer and healthy cells and finally d) its in vivo biodistribution in mice.

2. Experimental

2.1. Materials and methods

Doxorubicin·HCl was provided by Pharmacia & Upjohn and used as received. Phosphate Buffer Saline (PBS) was used as a buffer solution for loading ant release study. Citrate buffer was used for release study.

2.2. Equipment

Scanning electron microscopy (SEM) and Transmission Electron Microscopy (TEM) images were obtained on an FEI Inspect microscope operating at 25 kV and a FEI CM20 microscope operating at 200 kV, equipped with a Gatan GIF200 Energy Filter utilized for EF-TEM elemental mapping respectively. For MTT assay the absorbance was measured at 540 nm (reference filter 620 nm) using a microplate reader (Sirio S, SEAC Radim group).

2.3. Synthesis of multi stimuli Q-NCs

The detailed procedure about the synthesis of quarto stimuli Q-NCs was published elsewhere (Tapeinos, C., Colloids and Surfaces B: Biointerfaces, http://dx.doi.org/10.1016/j.colsurfb.2016.08.019, (2016), 148, 2016, 95–103). Briefly, Q-NCs were fabricated through different steps of emulsion polymerization method. In the first step the polymeric core was fabricated and in the second step the cross-linked shell produced aiming at introducing pH, Thermo and redox sensitivity with simultaneous removal of the core. Iron NPs were doped on the Q-NCs surface for hyperthermia application [34–36].

2.4. Synthesis of core

In a 500 mL spherical vial introduced 240 mL water and 10 mL of MAA and the mixture was left for stirring under nitrogen for 1 h. When temperature kept 80 °C, 200 mg of KPS was added and the polymerization initiated. After 12 h the mixture warm down and purified by centrifugation (10,000 rpm × 3 times).

2.5. Loading and release

A typical procedure about the drug loading in the nanostructured materials is described below. Equal amounts of the Q-NCs and drug were treated under neutral conditions (pH = 7.4, PBS buffer solution) and the mixture was gently stirred for three days in the dark at room temperature. After that, the sample centrifuged three times (10,000 × 5 min) and the isolated product washed with PBS three times. The unloaded drug was estimated in the supernatant according to a DOX free standard curve in PBS [46]. The loading capacity (LC %) and encapsulation efficiency (EE %) of the Q-NCs were calculated according to the standard curve. The exact mass of the drug in Q-NCs was found to be 486 ± 2 μg/mg of the polymer, the LC % = 94.7 and EE% = 94.7. In the release study a standard amount of drug loaded Q-NCs was suspended in two different pH solutions, pH = 4.5 and pH = 7.4, and/or in combination of glutathione. After that, 0.5 mL of the suspension was centrifuged and the released drug was calculated in the supernatant. DOX concentration was calculated according to a standard curve, as previously described [50,51].

2.6. In vitro cytotoxicity studies

2.6.1. Cell cultures

Human Caucasian breast adenocarcinoma cell line (MCF-7) was maintained in DMEM medium, containing 10% FBS (PAA), 2 mM L-glutamine (PAA), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA), at 37 °C, growing in a 5% CO_2 atmosphere. Human embryonic kidney cell line (HEK-293, Cells contain adenovirus) were treated in DMEM medium, in similar conditions with MCF-7 cells. Both cell lines grew as monolayers.

The growth inhibition of MCF-7 and HEK-293 cells for all compounds was tested by using the MTT assay as described elsewhere [48,52]. Briefly, cancer cells were seeded in 96-well plates (1.5 × 104 cells in 100 μL of medium/well) in triplicate, allowed to attach overnight, and then treated with tested compounds for 24 h. After incubation the medium was replaced with 100 μL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution and the plate was incubated for another 4 h. The dark-blue formazan crystals which precipitate by viable cells were dissolved in 100 μL of DMSO. Results are expressed as the percentage (%) of treated cells versus untreated cells, using data from three independent experiments that had each been repeated two times. About the HEK-293 cell viability measurements, the MTT assay was measured as outlined elsewhere [53]. Briefly 1.5 × 10^4 cells in 100 μL of medium/well was seeded in 96-well culture plate for 24 h. After 24 h the medium was removed and cells were treated for additional 24 h with 100 μL media containing the treated compound in different concentrations in triplicate. 20 μL of the MTT (2 mg/mL) solution was added and the cells were incubated for additional 4 h at 37 °C. Cells were then treated with 150 μL DMSO and the absorbance was measured at the microplate reader. Cells were incubated in various concentrations of free DOX, DOX-loaded Q-NCs and empty Q-NCs (ranging from 0.1 to 100 μM for DOX and 0.1 to 39 μg/mL for Q-NCs corresponding to the polymeric amount that hosted DOX, the experiment was replaced three times and the results expressed as the median of them) (Fig. 3).

2.7. Radiolabelling

Radiolabelling of the Q-NCs was performed, with a gamma emitting radionuclide (99mTc), in order to attend their in vivo course by using scintigraphic imaging. Their biodistribution profile at 1 h and 24 h post injection (p.i.) in normal mice was also investigated [54]. Radiolabelling of the Q-NCs was performed using stannous chloride