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Research paper

pH/redox dual-sensitive dextran nanogels for enhanced intracellular drug delivery

Manuela Curcio [a,*], Luis Diaz-Gomez [b], Giuseppe Cirillo [a], Angel Concheiro [b], Francesca Iemma [a,1], Carmen Alvarez-Lorenzo [b,1]

[a] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende, CS, Italy
[b] Departamento de Farmacología, Farmacia y Tecnología Farmacéutica, R+DPharma Group (GI-1645), Facultad de Farmacia, and Health Research Institute of Santiago de Compostela (IDIS), Universidade de Santiago de Compostela, 15782-Santiago de Compostela, Spain

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ABSTRACT

pH/redox dual-responsive nanogels (DEX-SS) were prepared by precipitation polymerization of methacrylated dextran (DEXMA), 2-aminoethylmethacrylate (AEMA) and N,N′-bis(acryloyl)cystamine (BAC), and then loaded with methotrexate (MTX). Nanogels were spherical and exhibited homogeneous size distribution (460 nm, PDI < 0.30) as observed using dynamic light scattering (DLS) and scanning electron microscopy (SEM). DEX-SS were sensitive to the variations of pH and redox environment. Nanogels incubated in buffer pH 5.0 containing 10 mM glutathione (GSH) synergistically increased the mean diameter and the PDI to 750 nm and 0.42, respectively. In vitro release experiments were performed at pH 7.4 and 5.0 with and without GSH. The cumulative release of MTX in pH 5.0 medium with 10 mM GSH was 5-fold higher than that recorded at pH 7.4 without GSH. Fibroblasts and tumor cells were used to tests the effects of blank DEX-SS and MTX@DEX-SS nanogels on cell viability. Remarkable influence of pH on nanogels internalization into HeLa cells was evidenced by means of confocal microscopy and flow cytometry.

1. Introduction

One of the most fascinating trends in drug delivery is the design of stimuli-responsive nanocarriers with tailored structural characteristics, able to release their payload only after "recognition" of pathological tissue modifications (acting as stimuli) for maximizing the efficacy and safety of the treatment [1]. When applied in cancer therapy, this approach acquires a number of potential advantages related to the possibility of minimizing the premature release of chemotherapeutics to normal tissues, improving drug accumulation in tumor cells and overcoming the multidrug resistance, that represents the main impediment to the success of chemotherapy [2]. The "intelligent" recognition of the pathological signals in a tumor tissue also underlies the passive targeting concept that exploits the enhanced permeability and retention (EPR) effect, typical of many rapidly growing solid tumors [3], and the variations of some cellular parameters [4]. For example, it is well known that the extracellular environment is more acidic (pH 6.5) in tumors than in blood and in normal tissues (pH 7.4), and that pH values of endosomes/lysosomes are even lower (5.0–5.5) [5]. Moreover, the remarkable differences in glutathione (GSH) concentration in cancer cells (approximately 2–10 mM), compared to normal extracellular matrix (approximately 2–20 μM), generate a high redox potential [6] that, together with the pH change, could serve as an ideal trigger for the selective release of anticancer drugs in tumor cells. Ultimately, on the basis of these considerations, an ideal stimuli-responsive vehicle for chemotherapy should present a nanosized structure, as a way to achieve high tumor accumulation, and should be able to change its structure in response to different environments in order to enhance cellular internalization and drug release [7]. Many examples of pH and redox responsive nanovehicles, including micelles [8–10], microcapsules [11] and nanoscaled hydrogels [12–15], have been reported. Generally, these nanocarriers contain pH-responsive moieties, such as reversibly ionizable carboxylic or amino groups or hydrazone groups [16], and redox-responsive functionalities consisting of disulfide linkages [17]. The disulfide bonds are stable in the presence of the low GSH levels of extracellular fluids, but are reversibly cleaved inside cells [18].

Among the wide range of nanocarriers, polysaccharide-based nanogels are receiving increasing attention as they can merge the features of hydrogels and nanosized entities (e.g. large surface

* Corresponding author.
E-mail address: manuela.curcio@unical.it (M. Curcio).
[1] Joint senior authors.

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area, good mechanical stability, high drug loading capacity and water affinity) [19] with the benefits of the natural renewable resources [20,21]. Polysaccharides are nontoxic, intrinsically biodegradable, and cost-effective natural polymers with a well-known chemical structure that allows its modification for obtaining advanced functional materials [16,22]. As a remarkable example, dextran (DEX) is a bacterial-deriving glucose homopolysaccharide consisting of consecutive α(1–6) linkages in the major chain. It is widely employed in drug delivery due to its favorable properties such as high water solubility, biocompatibility, biodegradability and resistance to protein adsorption. In addition, the presence of reactive hydroxyl groups, susceptible to chemical modification, allows the obtaining of materials with tailored features. In literature, several pH- or redox-responsive drug delivery vehicles based on modified DEX have been described [23–27], but only a few examples of dual pH/redox responsive materials can be found. Zwitterionic pH/redox responsive DEX nanogels have recently been obtained by an auto-assembling process of DEX previously functionalized in a two-steps procedure with succinic acid and cystamine [28]. The zwitterionic nature of the particles facilitated their cell internalization *in vivo* and therefore the intracellular uptake of the loaded anticancer drug. Moreover, the free carboxylic acid and amino groups at the surface conferred excellent anti-protein adsorption ability.

The aim of the present study was to prepare dual pH/redox responsive nanogels (DEX-SS) by precipitation polymerization of methacrylated dextran (DEXMA) with 2-aminoethylmethacrylate (AEMA) as pH-responsive moiety, and N,N′-bis(acryloyl)cystamine (BAC) as redox-responsive crosslinker. The main novelty of our approach relies on that the synthesis protocol involves only a single functionalization of DEX with methacrylic anhydride for the subsequent radical polymerization. The obtained nanogels were characterized by DLS and SEM, and then the pH/redox-triggered destabilization and in vitro release of methotrexate (MTX) from MTX-loaded DEX-SS (MTX@DEX-SS) were investigated. Cytotoxicity of DEX-SS and MTX@DEX-SS was evaluated on fibroblasts and tumor cells. Finally, cellular uptake of fluorescent-labeled DEX-SS in HeLa cells was investigated in culture medium covering a wide range of pH values in order to elucidate whether typical changes in pH that occur in cancer cells alter the effective internalization of the nanogels.

2. Experimental section

2.1. Materials

Dextran (DEX) from *Leuconostoc* spp. (1 g, MW 6000 Da), trimethylamine (99.5%), methacrylic anhydride, cystamine dihydrochloride, acryloyl chloride, 2-aminoethylmethacrylate hydrochloride (AEMA), 2,2-azoisobutyronitrile (AIBN), fluorescein isothiocyanate–dextran (FITC-DEX, MW ~4000 Da, FITC:glucose 1:250), DMSO-d_6, and NaOH were obtained from Sigma-Aldrich, St. Louis, MO, USA. RPMI medium was from Gibco (Life Technologies, Bleiswijk, The Netherlands). Fetal bovine serum and penicillin-streptomicin were purchased from Sigma-Aldrich (St Louis, MO, USA). Other reagents were analytical grade.

2.2. Synthesis of DEXMA

DEXMA was obtained following a protocol reported in literature [29] with modifications. Dextran (200 mg) was dissolved in 5 mL of distilled water at room temperature. After complete dissolution, 0.07 mL of the trimethylamine catalyst was added to the DEX solution and stirred for 15 min. Then, 0.82 mL of methacrylic anhydride was slowly injected into the reaction flask. The reaction was conducted at 60 °C for 24 h. Afterward, the solution was introduced into dialysis tubes (Spectra/Por, MW cut-off 3.5 kDa, Spectrum, Canada) and dipped into a glass vessel containing distilled water at 20 °C for 48 h; water was exchanged every 6 h. The resulting solution was freeze-dried (Modulyo freeze driers, Edwards, UK). The obtained DEXMA was analyzed using FT-IR spectroscopy (Jasco FT-IR 4200, Easton, MD, USA) preparing KBr tablets, and ^{1}H-NMR at 25 °C in a Bruker 500 MHz Advance NMR instrument (Milano, Italy) using DMSO-d_6 as solvent.

2.3. Synthesis of N,N′-bis(acryloyl)cystamine (BAC)

BAC was prepared as previously reported [9]. Briefly, a cystamine dihydrochloride aqueous solution (11.6 g, 0.05 mol in 50 mL of water) was poured into a 250 mL three-neck flask equipped with a thermometer and two 50 mL dropping funnels. Then, an acryloyl chloride (13.6 g, 0.15 mol) solution in dichloromethane (10 mL) and a NaOH aqueous solution (8 g, 0.2 mol in 20 mL water) were added dropwise at 0–5 °C simultaneously. Reaction was carried out at room temperature for 16 h. The obtained BAC was purified by recrystallization from ethyl acetate. The yield of reaction was *ca.* 73%.

2.4. Preparation of DEX-SS and FITC-DEX-SS nanogels

DEX-SS nanogels were prepared by precipitation polymerization as follows. DEXMA (0.2 g), AEMA (100 mg) and BAC (0.8 g) were dissolved in 25 mL of dry DMSO/CH_3CN (4:6 v/v) mixture in a 100 mL round-bottom flask. Then, AIBN (100 mg) was added. The flask was gently stirred (55 rpm) in an oil bath. The temperature was increased from 20 to 60 °C within 2 h and then kept at 60 °C for 24 h. The obtained particles were filtered, washed with ethanol (100 mL), acetone (100 mL) and diethyl ether (100 mL). Finally, the particles were dried under vacuum overnight at 40 °C. Aliquots of dry nanogels (50 mg) were suspended in distilled water (2 ml), transferred in dialysis tubes and dialyzed against distilled water. After 24 h, the release medium was analyzed by HPLC to verify the absence of any trace of DMSO according to literature data [30].

For cell uptake experiments, fluorescent nanogels were prepared following the same procedure, but adding in the reaction feed 0.02 g of FITC-DEX.

Nanogels size distribution was characterized using a 90 Plus Particle Size Analyzer DLS equipment (Brookhaven Instruments Corporation, New York, USA) at 25 °C. The autocorrelation function was measured at 90° and the laser beam operated at 658 nm. The polydispersity index (PDI) was estimated applying the inverse Laplace transformation and the Contin method [31]. PDI values $\leq$0.3 indicate narrow size distribution of nanogel population. All analyses were done in triplicate. Morphological analysis was carried out using SEM (Leo stereoscan S420; Leica Microsystems, Wetzlar, Germany). The nanogels were lightly sprinkling on a double adhesive tape, which was stuck on aluminum stub. The stubs were coated with gold to thickness of about 300 Å using a sputter coater and then viewed.

2.5. pH and redox-triggered destabilization of DEX-SS nanogels

The stimuli-triggered destabilization of DEX-SS was evaluated by DLS measuring the size changes of nanogels in response to pH, 10 mM GSH or both [32]. Briefly, four different DEX-SS dispersions (1.0 mg mL^{-1}) were prepared in phosphate buffer pH 7.4, in acetate buffer pH 5.0, and in 10 mM GSH solution at pH 5.0 and 7.4. The samples were incubated at 37 °C and stirred (250 rpm) for 24 h, and the size changes of DEX-SS nanogels were measured by DLS analysis.