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pH- and thermo-responsive microcontainers as potential drug delivery systems: Morphological characteristic, release and cytotoxicity studies

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ABSTRACT

Polymeric pH- and thermo-sensitive microcontainers (MCs) were developed as a potential drug delivery system for cancer therapy. It is well known that cancer cells exhibit notable characteristics such as acidic pH due to glycolytic cycle and higher temperature due to their higher proliferation rate. Based on these characteristics, we constructed a dual pH- and thermo-sensitive material for specific drug release on the pathological tissue. The MC's fabrication is based on a two-step procedure, in which, the first step involves the core synthesis and the second one is related to the shell formation. The core consists of poly(methyl methacrylate (PMMA), while the shell consists of PMMA, poly(isopropylacrylamide), poly(acrylic acid) and poly(divinylbenzene). Three different types of MCs were synthesized based on the seed polymerization method. The synthesized MCs were characterized structurally by Fourier transform infrared and morphologically by scanning electron microscopy. Dynamic light scattering was also used to study their behavior in aqueous solution under different pH and temperature conditions. For the loading and release study, the anthracycline drug daunorubicin (DNR) was used as a model drug, and its release properties were evaluated under different pH and thermo-conditions. Cytotoxicity studies were also carried out against MCF-7 breast cancer and 3T3 mouse embryonic fibroblast cells. According to our results, the synthesized microcontainers present desired pH and thermo behavior and can be applied in drug delivery systems. It is worth mentioning that the synthesized microcontainers which incorporated the drug DNR exhibit higher toxicity than the free drug.

1. Introduction

Stimuli responsive nanomaterials are in the epicenter of scientific research in the last few years, owing to its multiple applications in the biomedical field, including drug delivery [1–4], magnetic resonance imaging [5–8], targeted delivery and controlled release [9–12]. Among the characteristics of nanomaterials, their shape, size, surface chemistry and composition, play a crucial role in their use as appropriate drug delivery system. The spherical form, which improves the nanomaterial's circulation in the blood and the internal cavity, which can host hydrophobic entities (i.e. drugs, proteins, siRNA) with high loading capability, are two major benefits of the microcontainers (MCs). Up to date, a number of methods have been used to synthesize hollow polymeric MCs, most of which use a template, either hard such as polystyrene which removal is based on their treatment by an organic solvent increasing the process complexity [13–16] or soft such polyaniline which forms a spherical shape template through self-assembly method which removal is unnecessary [17–20]. The advantage of the template method is the mono-dispersity of the MCs and the control of the formed cavity size. Besides the template method, dynamic swelling [21–23], encapsulation of non-solvent [24–27] and polymeric micelles [28–31] can also be used to synthesize hollow MCs. Synthesis of core–shell particles with internal cavity can be achieved by various routes among which, a two-stage emulsifier-free emulsion polymerization [32–37]. In this type of polymerization, the desired monomers that are used in the second stage are polymerized in the presence of a seed, which is prepared in a previous step.

Herein, we study the behavior of a new synthetic MC under different pH and temperature conditions and its potential use as a competitive drug delivery system. The fabrication technique consists of two steps. In the first step, the core is fabricated by poly(methyl methacrylate) (PMMA) through the emulsifier-free emulsion polymerization, and in the second step, the shell is fabricated by monomers which induce the desired properties. The MC's shell consists of thermo- and pH-sensitive polymeric segments such as poly(isopropylacrylamide) (PNIPAAm) and poly(acrylic acid) (PAA) which alter their properties under specific conditions; PMMA is used for its biological compatible properties and poly(divinylbenzene) as shell cross-linker. The above sensitive monomers are broadly used, according to literature, aiming at creating multi responsive polymeric systems [38,39]. PAA is a pH-sensitive

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polymer which is broadly used in synthetic biomaterials [40–43], and PNIPAAm is a thermo-sensitive polymer which is used due to its different response in temperature changes [38,39,44]. By combining the above mentioned properties, we succeed to create a dual-responsive, pH- and thermo-sensitive MC. The selected synthetic method is simple, economic and ecological friendly in comparison to other literature described methods.

2. Experimental section

2.1. Materials

Methyl methacrylate (MMA 99%) and acrylic acid (AA 99.5%) (Acros Organics) were distilled under vacuum before used. The crosslinker divinyl benzene (DVB, 80%) (Sigma–Aldrich), potassium persulfate (KPS) (Panreac) and N-isopropylacrylamide (NIPAAm, 99%) (Sigma–Aldrich) were used as received. Daunorubicin hydrochloride (DNR) was provided by Pharmacia & Upjohn and used as received. Fetal bovine serum (FBS) was purchased from PAN Biotech GmbH. Dulbecco's modified Eagle's medium (DMEM) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by Sigma–Aldrich.

2.2. Instrumentation

Scanning electron microscopy (SEM) images were obtained on an FEI Inspect microscope with W (Tungsten) filament operating at 25 kV, and transmission electron microscopy (TEM) images were obtained on FEI CM20 microscope operating at 200 kV, equipped with a Gatan GIF200 Energy Filter utilized for EF-TEM elemental mapping. Fourier transform infrared (FT-IR) spectra were obtained by a Perkin Elmer Spectrum 100 Spectrometer; the spectra were scanned over the range of 4000–400 cm^{-1}. Dynamic light scattering (DLS) measurements were performed on a Malvern Instruments Zetasizer Nano Series, with a multipurpose titrator; each measurement represents the average value of 3 measurements, with 11 to 15 runs for each measurement. UV–visible absorption spectra in the wavelength range of 200–800 nm were obtained on a Jasco V-650 spectrometer. An ultrasonic bath was used for sonication (Elma Sonic, S 30H).

2.3. Synthesis of PMMA seeds

PMMA seeds were synthesized *via* the emulsifier-free emulsion polymerization method [45]. For the synthesis, 1 mL of MMA was added to 10 mL deionized water, and the mixture remained at 70 °C under N_2 flow for 30 min. After the above treatment, 20 mg of KPS aqueous solution was added, and the mixture became milky white. The mixture was kept overnight under N_2 flow, and after the completion of the reaction, it was cooled to room temperature and collected by centrifugation (3× 8000 rpm).

2.4. Synthesis of PMMA@P(MMA-co-AA-co-NIPAAM-co-DVB) microcontainers (1)

For the shell fabrication according to a typical procedure, 0.5 g of the above synthesized seeds was dispersed in 70 mL deionized water in a glass flask. 2 mL of ethanol was added, aiming at improving the dispersion of the mixture, and the reaction was left for stirring under nitrogen atmosphere for 2 h. In the mixture of the swollen seeds, 1 mL of MMA, 100 μL of AA, 50 mg of NIPAAm and 500 μL of DVB were added dropwise. The mixture was left under nitrogen for another 30 min and after that 60 mg of the initiator KPS in 1 mL aqueous solution was added. The reaction was left for 24 h under nitrogen atmosphere, and the product was collected by centrifugation (7000 rpm × 3). A similar procedure was followed for the synthesis of two other analogues PMMA@P(MMA-*co*-NIPAAm-*co*-DVB) **(2)** and PMMA@P(MMA-*co*-AA-*co*-DVB) **(3)**. The quantities and ratios of substances are summarized in Table 1.

Table 1
Quantities used in the synthetic analogues.

Samples	Seeds (g)	MMA (mg)	AA (mg)	NIPAAm (mg)	DVB (mg)	H_2O (mL)	KPS (mg)
1	0.5	940	105	50	0.5	70	60
2	0.5	940		135	0.5	70	60
3	0.5	940	224		0.5	70	60

The internal cavity was formed during the synthesis of the shell and increased further after gentle agitation in $CHCl_3$ for 10 min.

2.5. Daunorubicin loading and release

MCs were suspended in phosphate buffer saline (PBS, pH 7.4) as described elsewhere in a final concentration 1 mg/mL [46], and then DNR was added and the resulting suspension remained under stirring for three days at 10 °C. Unloaded DNR was removed by centrifugation, and its concentration was determined by UV absorption spectroscopy, monitoring DNR absorbance at 482 nm, based on a standard curve of DNR. The encapsulation efficiency (EE) was calculated according to the following formula:

$$\mathbf{EE}\ (\%) = (\mathbf{weight\ of\ loaded\ drug}/\mathbf{weight\ of\ drug\ in\ feed})\ \mathbf{x\ 100}$$

The release profile of DNR-loaded MCs was explored under acidic (citrate buffer, pH 5.5) and physiological (phosphate buffer, pH 7.4) pH conditions and at two different incubation temperatures (25 and 45 °C). Aliquots were withdrawn at given time intervals and centrifuged. The DNR concentration was determined in the supernatant by measuring the absorbance at 482 nm. Results are expressed as the mean value from two independent experiments of the released DNR (%) ± standard deviation.

2.6. Cell culture

Human breast adenocarcinoma (MCF-7) and mouse embryonic fibroblast (3T3) cells were maintained in high glucose DMEM, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.6.1. In vitro cytotoxicity studies

Cytotoxicity was evaluated by the MTT assay on MCF-7 and 3T3 cells [47]. Briefly, cells were seeded at a density of 8×10^3 cells per well in 96-well, flat-bottomed microplates and grown in 100 μL of completed growth medium 24 h prior to the incubation with the MCs, as described previously [48]. Stock solutions of the empty, DNR-containing MCs and free DNR were dissolved in ddH_2O at a concentration of 1 mg/mL and 300 μM, respectively. Subsequently, equal volume (100 μL) containing the appropriate concentration of DNR (0.02 to 60 μM) or MCs (0.2 to 561.2 μg/mL) was added to each well. After 24 h incubation, cells were washed once with PBS, and the medium was replaced with 100 μL per well of MTT solution (1 mg/mL), for a further 4 h incubation. The precipitated MTT formazan crystals were solubilized in isopropanol, and the absorbance was measured at 540 nm (reference filter 620 nm) using a microplate reader (Sirio S, SEAC Radim group). Measurements were converted to % viability compared to control experiments in which MCs had not been added. Results are expressed as the mean value, of three measurements, of the absorption at 540 nm ± standard deviation.