pH-Responsive Polymeric Nanoparticles as Drug Delivery Systems

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Abstract
Micelles are excellent devices to be used as controlled drug delivery systems since they exhibit the ability to protect the drug or encapsulated substance from the routes of degradation until they reach the site of action; moreover, they can pass through biological barriers and reach intracellular compartments. In addition, when a drug is administered and released from the dosage form, the kinetic behavior of the drug depends largely on their chemical structure. However, when the drug is immersed at the core of a NP, the physicochemical properties that actually affect the distribution of the drug in the body are those from the latter. As a result, this approach controls drug release, diminishing side effects and increasing therapeutic rates. In the present work novel smart micelles have been prepared and tested as drug delivery systems.

ABBREVIATIONS
NP: Nanoparticles; HEMA: 2-Hydroxyethyl Methacrylate; DMA: N,N-Dimethylaminoethyl Methacrylate; DEA: N,N-Diethylaminoethyl Methacrylate; FMA: Furfuryl Methacrylate; RMN: Nuclear Magnetic Resonance; GPC: Gel Permeation Chromatography; DMF: N,N-Dimethylformamide; ATRP: Atom Transfer Radical Polymerization; CC: Click Chemistry; CMC: Critical Micelle Concentration; DLS: Dynamic Light Scattering; PDI: Polydispersity Index; Dh: Hydrodynamic Diameter.

INTRODUCTION
Amphiphilic block-copolymers constitute a special type of copolymers, formed by two incompatible blocks, linked to each other. They are of special interest because they could self-assemble into nanoparticles (NPs) and they are useful in many fields such as, cosmetic, drug delivery, biosensors, gene therapy, and more [1-4]. It is also known that the administration of lipophilic drugs mainly administered rectally or by inhalation, is associated with a number of drawbacks, depending on the route of administration. In addition to some inherent disadvantages, such as the need of reiterative dose administration at certain intervals; monitoring drug concentrations so that they are within the therapeutic range; and the administration of those drugs is also a challenge as they have a marked variability in their kinetic behavior [5]. Consequently, new methods for the administration of lipophilic drugs are needed.

In the last few years, the use of smart functionalized systems has reached major attention because they are able to protect the drug from the degradative environment and to go across biological barriers, reaching intracellular compartments. On these systems, the distribution of the therapeutic molecule does not depend on its chemical structure but on the physical-chemical properties of the micelles; in addition, the drug release can be triggered from a smart system by means of a change in a particular property of the media, let’s say temperature, ionic strength, or pH. Hence, not only is the drug distribution controlled but the adverse effects are also reduced, so enhancing the therapeutic results [6]. Even though the use of nanocarriers as drug-delivery systems offers many advantages, some drawbacks need addressing to assess the reliability of the delivery systems, such as the evaluation of the interactions between nanocarriers and biological systems [7].

The variety of pH gradients that can be found in the body has notably attracted the interest of researchers, being one of the first and most evaluated stimuli [6]. Thus, it is possible to design NPs sensitive to, for example, the acidic environment of solid tumors. In this context, once they get in contact with the harm tissue and in response to the low pH, they will deliver the drug improving the therapeutic results.

However, the performance of the self-assembled micelles might be limited by suffering from low structural stability and tending to be disrupted upon large dilution (below its critical micelle concentration), for example in blood circulation, accelerating the premature drug release with consequent serious side effects. This drawback can be avoided by the stabilization of...
the NP in the shell or the core thereof.

The overall aim of the present work is the preparation of aqueous dispersions of organic stabilized NPs, formed by self-assembly of tailor-made block copolymers, which may be used as smart and vectorizable, controlled drug delivery systems capable of responding under the right stimulus.

MATERIALS AND METHODS

All chemicals used were purchased from Aldrich Chemical Co. HEMA, DEA, DMA, and FMA were passed through a basic alumina column and distilled before use. IR spectra were recorded on a Jasco FT/IR 4200 spectrometer equipped with ATR. NMR spectra were recorded at the CITIUS Service (University of Seville) at 300 K on either a Bruker Advance AV-500 or a Bruker AMX-500. GPC analyses were performed using a Waters apparatus equipped with a Waters 2414 refractive index detector and two Syrager HR columns (7.8 x 300 mm) linked in series, thermostatted at 40°C, and using DMF as the mobile phase at a flow rate of 0.5 mL/min. Molecular weights were estimated against methyl methacrylate standards. The average hydrodynamic diameter (D), size distribution (polydispersity index, PDI), and Z-potential of the samples were determined with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25°C, with a particle size analysis range of 0.6 nm to 6 µm. The morphology and distribution of the NPs were characterized by scanning electron microscopy (SEM) using a field emission HITACHI S5200 microscope operating at 5 kV, also used at the CITIUS Service (University of Seville). Measurement of absorbance of UV and visible light were conducted with an Agilent 8453 UV–visible spectrophotometer (Palo Alto, USA), equipped with diode array detection (DAD), and the data were the result of at least three measurements.

RESULTS AND DISCUSSION

Synthesis

The synthesis of the amphiphilic block-copolymer and the crosslinker used in the present work has been published elsewhere [8,9]. The monomers were selected so that the polymers would contain: (a) pH-sensitive units (DMA and DEA) on both, the hydrophobic and the hydrophilic segments leading to pH-sensitive polymers; (b) furan rings in the lipophilic block (FMA units), able to take part in Diels-Alder reaction; (c) hydroxyl functional groups in the hydrophilic block (HEMA units), which would be available for further vectorization with the appropriate marker agents if required. Hence, the hydrophilic block was made up of the hydrophilic monomers HEMA and DMA and the hydrophobic block contained DEA and FMA (Figure 1).

Characterization data of the block-copolymer

M_n = 34,700; M_w = 45,100; M_w/M_n = 1.3. Experimental copolymer composition (determined by 1H NMR): (DMA_{31%}-HEMA_{19%})-block-(DEA_{45%}-FMA_{5%}). IR, ν, (cm⁻¹): 3420 (O-H), 2964 (¼C-H st, furan), 2932 (¼-C-H st), 1722 (C=O st), 1453, 1383 (γ ring skeleton furan), 1264, 1240 (C-O st ester), 1141

Figure 1 General structure of synthesized methacrylate-based block-copolymers and cross-linker.
(C-N st amine). $^1$H NMR (DMSO-d$_6$,500 MHz), δ (ppm): 7.67-6.47 (m, 3 H, furan); 4.98 (s, 2H, COOC$_{\text{H2-furan}, \text{FMA}}$); 4.82 (s, 1 H, OH, HEMA); 4.51-3.32 (m, 4H, COOC$_{\text{H2}, \text{DMA and DEA}}$); 3.91-3.82 (m, 6H, COOCH$_2$, DMA, DEA, HEMA); 3.59 (bs, 2H, COOCH$_{\text{H2}, \text{HEMA}}$); 2.26-2.11 (m, 10 H, N(CH$_3$)$_2$, DMA and N(CH$_2$CH$_3$)$_2$, DEA); 2.03-1.62 (m, 8 H, -CH$_2$CH$_3$, DMA, HEMA, FMA, DEA); 1.06-0.58 (m, 18 H, -CH$_2$C(CH$_3$)$_2$, DMA, HEMA, FMA, DEA, N(CH$_2$CH$_3$)$_2$, DEA).

Micellar dispersions

For the preparation of the micellar dispersions, the polymer was dissolved in THF and then, the organic solution was added drop wise into double-distilled water and gently stirred for 72 hours. The micelle formation took place, leading to the typical soft bluish appearance of this type of suspensions usually found when light is scattered from nanosized particle suspensions, suggesting the NP formation (Figure 2). By Dynamic Light Scattering (DLS) it was found that quasi monodisperse systems were achieved at 0.25 mg/mL polymer concentration (PDI = 0.12; $D_h = 205$ nm; Figure 2), whereas at concentrations above 0.45 mg/mL, the micelles were clustered resulting in polydisperse systems. Representative SEM images of the prepared samples under neutral pH conditions are displayed in (Figure 3), which confirmed the presence of nanosized structures.

Building on the experience in both, formation of micelles stabilized by steric agents [8] and studies of the Diels-Alder reactions onto polymers functionalized with furfuryl groups [9], the stabilization of the nanoparticles by crosslinking their core via Diels Alder reactions was carried out (Figure 4). The NPs were stable for months, in a wide range of pH (from pH 3.0 to pH 8.0) and under dilution assays.

Nanocarriers

The loading capacity of lipophilic molecules by those systems has been validated in a previous work [8]. Thus, pyrene was the molecule of choice because this hydrocarbon displays an ensemble of fluorescence monomer emission peaks in the range from 375 nm to 405 nm. It is essential to highlight the exquisite sensitivity of pyrene to the polarity of the microenvironment and consequently, once this molecule was immersed into a hydrophobic environment such as the core of the prepared NPs a boost in the fluorescence emission would be expected. It was found a marked increment in the fluorescence emission providing evidence that pyrene was successfully imbibed into the micelles. Once confirmed the capacity of the NPs to load lipophilic molecules, some assays were carried out with pilocarpine.

Pilocarpine is a therapeutic molecule with low water solubility, clinically used as co-drug in glaucoma and xerostomia as well as in the treatment of head and neck cancer; it is also prescript against Sjögren’s syndrome. Salivary gland hypofunction, commonly developed during radiation therapy to the head and neck cancer and in patients with Sjögren syndrome [10], leads to diminished secretions and the acidification of saliva. The later characteristic would not only affect the regular homeostasis of the oral cavity, leading to specific changes in the salivary bacterial profiles [11], but also the demineralization of tooth enamel, with the consequent increment in the risk for caries [12]. In this context, pilocarpine is used to reduce the severity of xerostomia and salivary dysfunction since this drug can stimulate salivary tissues. Hence, the incorporation of pilocarpine into pH-sensitive NPs allows the release of the drug under those acidic environments to exert its therapeutic activity.

A bunch of experimental caption/delivery assays of pilocarpine at several pH was conducted in order to corroborate the use of those NPs as pH-responsive drug delivery systems. The incorporation of the drug into the NPs was carried out by the drop-wise addition of a solution of pilocarpine in double-distilled water (5 mL, concentration 800 µg/mL) to the dispersions and the mixtures were gently stirred for several hours at 25°C. The incorporation of pilocarpine into the micelles was followed by UV spectroscopy at 215 nm, using the corresponding non-loaded micelle solution as a blank for each sample, showing that the drug was promptly loaded into the NPs in the first hours. Once the systems kept steady, the uptake capacity was determined and found to be between 89 and 93%.
**Figure 3** Selected SEM images of the NP systems: (A) non-crosslinked NP; (B) 10% core cross-linked NP.

**Figure 4** Stabilized NP.

**Figure 5** Release of pilocarpine vs. time at pH 3.0.
The last part of the present work was focus on evaluating the release of pilocarpine from the NPs in response to a trigger stimulus. Thus, the loaded nanoparticles were immersed into an acidic solution and the drug release was quantified by UV spectroscopy. It was found that the therapeutic molecule was released under acidic pH at different rates depending on the system (Figure 5). For example, the non-cross-linked NPs displayed the slowest release rates within the dispersions studied (43% in 48 hours). Intuitively, the results might be unexpected. However, looking inside the chemical reaction on stabilized NPs, it was assumed that the non-cross-linked NPs present higher-density cores. Those ones in which the NPs have been stabilized, need to fit a highly-volume cross-linker in the core, leading to loosed-core NPs, and hence, allowing the higher permeability to the aqueous media. It was also found that the degree of cross-linking exerted a great impact at the drug release, displaying quicker release those systems in which the degree of cross-linking was higher. To exemplify the this statement, it was found that the release of the drug from the NPs stabilized with a 10% of core cross-linked was of 68% in 48 hours whilst the 20% core cross-linked NPs liberated the 91% of their load in 48 hours.

CONCLUSION

To sum up, this work sets up a method to provide stable stimulus-response NPs in biomedicine and pharmacy by total synthesis in order to achieve the formation of smart DDS. The self-assembly of the new polymer HEMA\textsubscript{45}\textsuperscript{a},-DMA\textsubscript{15}\textsuperscript{b},-FMA\textsubscript{45}\textsuperscript{c},-DEA\textsubscript{45}\textsuperscript{d} into nanoparticles was verified as well as their stabilization by means of core cross-linking. The NP was stable at acid pH, for a long time and in a wide range of temperatures. The incorporation of lipophilic molecules into the NPs such as the therapeutic agent pilocarpine, or the fluorescent molecule pirene, was also confirmed by several techniques. The prepared NPs behave as smart systems capable to control the drug release under the appropriate stimulus.

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