Supporting Information.

Validation of smart nanoparticles as controlled drug delivery systems. Loading and pH-dependent release of pilocarpine.

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Materials

All the chemicals used were purchased from Sigma-Aldrich and used as received. The buffer solution at pH 7.00 (25°C) for loading and release assays was a solution of potassium dihydrogen phosphate/disodium hydrogen phosphate (SKU 1094070500, Certipur®) with pH variations with temperature of ± 0.09 from 5 ºC to 50 ºC. The buffer phosphate solution at pH 5.5 and at 0.2 M concentration used in release assays was freshly prepared at 25 ºC.

General Methods

The average diameter, 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 ºC, with a particle size analysis range of 0.6 nm to 6 µm. The intensity of the scattered light (expressed in kilo counts per second) was measured by dynamic light scattering (DLS). The instrument was provided with 4 mW He–Ne laser (λ = 633 nm), digital correlator ZEN3600, and non-invasive backscatter (NIBS®) technology. Measurements were carried out at a scattering angle of 173º to the incident beam, and data analyzed using CONTIN algorithms (Malvern Instruments). Data for each dispersion were collected from at least three runs. The Z-Average size or Z-Average mean obtained from DLS is a parameter also known as the cumulants mean. It is the primary and most stable parameter produced by the technique. This mean is calculated from the intensity weighted distribution, leading to the statement that the Z-Average size is the harmonic intensity-weighted arithmetic average particle diameter. The hydrodynamic size measured by Dynamic Light Scattering (DLS) is defined as the size of a hypothetical hard sphere.
that diffuses in the same fashion as that of the particle being measured. In practice though, particles or macromolecules in solution are non-spherical, dynamic (tumbling), and solvated. Because of this, the diameter calculated from the diffusional properties of the particle will be indicative of the apparent size of the dynamic hydrated/solvated particle and hence, the term “Hydrodynamic diameter”. The hydrodynamic diameter, or Stokes diameter, therefore is that of a sphere that has the same translational diffusion coefficient as the particle being measured, assuming a hydration layer surrounding the particle or molecule. The hydrodynamic diameter is measured using the Stokes-Einstein equation (eqn 1):

\[ D_H = \frac{kT}{3\pi\eta D} \]  

(1)

where \( D_H \) = hydrodynamic diameter, \( k \) = Boltzmann’s constant, \( T \)= absolute temperature, \( \eta \) = viscosity, and \( D \) = diffusion coefficient.

The zeta potential (\( \zeta \)) was calculated from the electrophoretic mobility (\( \mu \)) and then applying the Henry equation. The Smoluchowski approximation \( \zeta = \eta \mu / \varepsilon \), where \( \kappa \alpha >> 1 \) (where \( \eta \) is the solution viscosity, \( \varepsilon \) is the dielectric constant of the medium, and \( \kappa \) and \( \alpha \) are the Debye–Hückel parameter and the particle radius, respectively) was used. The electrophoretic mobility (\( \mu \)) was obtained by performing an electrophoresis experiment on the sample and measuring the velocity of the particles using Laser Doppler Velocimetry (LDV). Data acquisitions were performed using ZetaSizer Nano software.

The morphology and distribution of the NPs were characterized by scanning electron microscopy (SEM) using a HITACHI S5200 field-emission microscope operating at 5 kV. Before SEM observations, the dispersions were deposited and allowed to dry on a monocrystalline silicon
support treated with oxygen plasma for 80 s to make it more hydrophilic. DLS and SEM measurements were all performed at the CITIUS Service (University of Seville). Measurement of UV and visible light absorbance was performed with an Agilent 8453 UV–visible spectrophotometer (Palo Alto, USA), equipped with diode array detection (DAD); the data were the result of at least three measurements.

**Preparation of micellar dispersions**

For the preparation of the micellar dispersions, the amphiphilic block-copolymer [(DMA$_{25\%}$-HEMA$_{25\%}$)-block-(DEA$_{45\%}$-FMA$_{5\%}$)] was dissolved in THF, and then the solution was added dropwise into double-distilled water and stirred for 72 hours. The dispersion turned into a soft bluish solution – the typical appearance of nanosized particle suspensions. Dynamic Light Scattering (DLS) revealed that quasi-monodisperse systems were achieved at 0.25 mg/mL polymer concentration (PdI = 0.12; $D_h = 205$ nm).

To obtain stabilized nanoparticles by Diels-Alder reactions, the polymer concentration was started at 0.25 mg/mL, and the amount of the cross-linking agent added (1,8-dimaleimide-3,6-dioxaoctane: DMDOO) was set so that either 10% or 20% of furan rings in the NP core would react with maleimide rings of DMDOO, leading to degrees of cross-linking of 10% and 20%, respectively. DLS and SEM analyses were performed to assess the nanoparticle stability.

**Pilocarpine-loading assays**

The encapsulation studies of the drug pilocarpine (Figure S1) were conducted by means of UV–Vis, DLS, and scanning electron microscopy.
Figure S1. Chemical structure of pilocarpine

**Pilocarpine calibration curve**

Prior to the analysis, a calibration curve of pilocarpine was made with pilocarpine standard aqueous solutions (5–100 µg/mL) at 215 nm (Figure S2). For the calibration, a stock solution of pilocarpine at 500 µg/mL concentration in double-distilled water was prepared. By dilution, ten solutions were prepared with the following concentrations: 100, 75, 50, 30, 25, 20, 15, 10, and 5 µg/mL.

Figure S2. Calibration curves of pilocarpine at 215 nm (UV-Vis spectroscopy) at 25 °C
Loading assays

The micellar dispersions (5 mL) in phosphate-buffered saline at pH 7.0 were mixed with aqueous pilocarpine solution (5 mL, pilocarpine concentration 0.2 mg/mL; final pilocarpine concentrations = 0.1 mg/mL; final polymer concentration = 0.125 mg/mL). The absorbance of the loaded NP dispersions was measured at the maximum UV absorption of pilocarpine (215 nm) and at time intervals from 0 days to 6 days. The original micellar dispersion was used as blank.

Release assays

For each trial, a mini-dialysis tube (1 kDa cut-off, GE Healthcare) was used; 2 mL of the selected pilocarpine-loaded micellar dispersion (polymer concentration: 0.125 mg/mL) was added and the tube was introduced into a beaker containing 10 mL of phosphate-buffered saline at either pH 5.5 or pH 7.0. The system was stirred at 37 °C, and from time to time 0.2 mL of the incubated solution was removed and replaced with an equal volume of the same acidic or neutral buffered solution. To determine the release profiles, absorbance at 215 nm was measured. All drug release experiments investigated by UV-vis spectroscopy were done in triplicate.

The percent of drug release was determined using equation (2):

\[
\text{Drug release (\%)} = \frac{m_{\text{entrapped}(0)} - m_{\text{residual}(t)}}{m_{\text{entrapped}(0)}} \times 100
\]  

(3)

where \(m_{\text{entrapped}(0)}\) is the weight of initial entrapped pilocarpine into the NPs; \(m_{\text{residual}(t)}\) is the weight of residual pilocarpine at time “t” into the nanocarriers.