Pre-formed biodegradable zwitterionic nanoparticles as tunable excipients for the formulation of therapeutics directly at the point of care

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Abstract: Polyester-based nanoparticles (NPs) are among the most adopted drug delivery systems developed so far. This is mainly due to their ability to increase the bioavailability of the loaded therapeutics, to prevent the adverse effects often associated to their use, and to eliminate the toxic excipients necessary to formulate them. In addition, these NPs are biodegradable under physiological conditions thus avoiding the polymer accumulation in the body. However, the complexity in the formulation and storage hampers the cost-effective use of these formulations reducing their availability among the patient population. In addition, the manifold drugs available on the market, characterized by different chemical structures and charges, impose the continuous optimization of different delivery systems for their efficient formulation. Therefore, tunable NPs able to encapsulate different drugs with high loading efficiencies and to modulate their release after administration are urgently needed. In this work, a method to formulate different drugs directly at the point of care using only a syringe and starting from pre-formed NPs has been developed. Highly tunable zwitterionic NPs have been synthesized *via* the combination of ring opening polymerization (ROP) and reversible addition-fragmentation chain transfer (RAFT) emulsion polymerization and, then, used to load paclitaxel, doxorubicin, or ibuprofen with high efficiency. A controlled release of such therapeutics has been achieved by tuning the characteristics of the NPs, in particular the addition of charged groups.

1. Introduction

Biodegradable polymer nanoparticles (NPs) are rapidly coming to the forefront of drug delivery, diagnosis, and other areas of biomedicine due to their versatility and the ability to degrade under physiological conditions[1–3]. In particular, they have been successfully used to formulate poorly water-soluble drugs avoiding the use of toxic surfactants, such as the cremophor EL® in the case of the novel NP-based paclitaxel formulations (*e.g.* Genexol®[4]), or to reduce the side-effects of many drugs (*e.g.* Doxil[5] and Trabectedin[6]).

Among the many types of NPs developed[6], the ones structurally composed of a polyester core and a polyethylene glycol (PEG) shell are the most studied and adopted so far[7,8]. In fact, the polyester chains provide a suitable environment for the encapsulation of hydrophobic drugs and confer biodegradability to the NPs, being amenable to hydrolysis. On the other hand, the PEG portion confers colloidal stability and, at the same time, allows the NPs to escape the body immune system and the non-specific absorption of proteins[9]. Indeed, PEG is the most commonly studied anti-fouling material[10–12] and it has been considered the golden standard in this field for a long period[13,14]. However, recent studies have shown that this polymer can induce the production of IgM antibodies that stimulate the fast clearance of the PEGylated NPs after repeated administrations[15,16]. This so-called "accelerated blood clearance effect" can reduce the efficacy of therapies that are based on these NPs. For this reason, in the recent years, valuable alternatives to PEG have been researched, such as poly(2-hydroxypropylmethacrylamide)[17,18] and zwitterionic polymers[19–21]. These latter ones represent a promising alternative to PEG thanks to the strong hydration layer that the anionic and cationic groups are able to promote[22]. Among the polyzwitterions used in the biomedical field, sulfobetaine (SB)[8,23,24], carboxybetaine (CB)[25], and phosphorylcholine (PC)[26] based polymers are the most studied for the stabilization of NPs since they are able to mimic the zwitterionic character of most cell membranes[20].

The synthesis of biodegradable zwitterionic NPs generally requires the production of block copolymers comprising a zwitterionic portion and a polyester one. These block copolymers are subsequently nanoprecipitated in water with the assistance of water-miscible organic solvents[19]. In the nanoprecipitation method, microfluidic mixing devices are generally adopted to obtain small and narrowly dispersed NPs and laborious purification steps are then used to remove the toxic organic solvent and the drug not encapsulated[27,28].

To overcome this technological complication, we have recently developed a method to formulate drugs into NPs directly at the point of care just by using a common syringe[6,29,30]. This method consists in the dissolution of an amphiphilic copolymer and the drug in a small amount of a slightly toxic water-miscible solvent (*e.g.* DMSO or ethanol). This organic mixture then undergoes

few aspiration/ejection cycles through the needle of a syringe pre-loaded with an isotonic solution (*e.g.* phosphate-buffered saline, PBS). The amphiphilic copolymers self-assemble into NPs in these reduced turbulence conditions and the drug is entrapped into the NP lipophilic core during its formation. The formulation is then directly injected without the need of further post-processing steps (*e.g.* dialysis and lyophilization). However, this procedure cannot be applied to zwitterionic copolymers because they are not soluble in DMSO or ethanol, but only in more toxic solvents (*e.g.* methanol) that cannot be injected into patients[19]. For this reason, a subsequent dialysis is necessary, thus compromising the direct translation of this protocol to the point of care.

However, the advent of the reversible addition-fragmentation chain transfer (RAFT) emulsion polymerization has allowed to produce highly tunable NPs composed of block copolymers directly in aqueous media[31,32]. To make these NPs biodegradable, oligoester-based macromonomers can be adopted to synthesize the hydrophobic portion of the copolymers and hence to drive their simultaneous self-assembly[33]. These macromonomers are produced *via* ring opening polymerization (ROP) of lactones (*e.g.* caprolactone or lactide) in the presence of a primary alcohol comprising a vinyl group (*e.g.* hydroxyethyl methacrylate, HEMA)[34,35]. In addition, these macromonomers can be provided with a charged functional moiety by exploiting the reactivity of their hydroxyl end-group[36].

We now exploit these latest advances in the synthesis of highly controllable colloids to produce biodegradable zwitterionic NPs that, with minimal effort, can be adapted for the efficient loading and controlled release of different drugs. In particular, we realized this synthesis by combining the ROP and RAFT emulsion polymerization to produce amphiphilic block copolymers self-assembled into NPs directly in water and with minimal use of organic solvents. The hydrophilic portion of these copolymers is a poly(phosphoryl choline methacrylate) (polyMPC) produced *via* RAFT polymerization. On the other hand, the hydrophobic portion is obtained by chain extending this zwitterionic macromolecular chain transfer agent (macro CTA) with an oligocaprolactone-based macromonomer produced *via* ROP. Starting from these pre-formed colloids, we developed a method for the efficient encapsulation of poorly water-soluble drugs by using only a syringe, so that the procedure could be easily performed at the point of care without the need of post-processing steps. In addition, the possibility of creating negatively and positively charged macromonomers has been investigated and used to produce NPs that can slow-down the release of drugs presenting a net charge at physiological conditions (*e.g.* doxorubicin and ibuprofen).

Without pretending of developing a drug delivery system ready to be translated into the clinic, we want to demonstrate with this work that, with minimal technological complexity, poorly water-soluble drugs can be formulated directly at the point of care using pre-formed biodegradable

nanoparticles, reducing the amount of toxic surfactant and organic solvents currently adopted in many approved products.

2. Materials and methods

2.1 Materials

ε-Caprolactone (CL, 97% MW=114.14 g/mol), stannous octoate ($Sn(Oct)_2$, MW = 405.12 g/mol), 2-hydroxyethyl methacrylate (HEMA, 97%, MW=130.14 g/mol), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPA, 97%, MW=279.38 g/mol), 4,4′-azobis(cyanovaleric acid) (ACVA, 98%, MW=280.2 g/mol), succinic anhydride (99%, MW=100.07 g/mol), Choline chloride (99%, MW = 139.62 g/mol), N,N-Dicyclohexylcarbodiimide (DCC, 99% MW=206.33 g/mol), 4-(Dimethylamino)pyridine (DMAP, 99% MW=122.17 g/mol), 2-methacryloyloxyethylphosphorylcholine (MPC, 97%, MW=295.27 g/mol), ethanol (EtOH, 99.8%), dimethyl sulfoxide (DMSO), methanol (MeOH), acetone, acetonitrile (ACN), tetrahydrofuran (THF), dichloromethane (DCM), methanol-*d4* (MeOD), chloroform-d ($CDCL_3$) were purchased from Sigma Aldrich and used as received. 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (V-50, 97%, MW=271.19 g/mol) was purchased from Acros Chemicals.

2.2 Synthesis of oligoester-based macromonomers

Biodegradable oligoester-based macromonomers were synthesized *via* ROP of caprolactone (CL) using HEMA as initiator and $Sn(Oct)_2$ as catalyst according to a previously published protocol[33]. We synthesized two macromonomers (*i.e.* HEMA-CL_3 and HEMA-CL_5, with the subscript referring to the average CL degree of polymerization) with different molecular weights by setting the HEMA to $Sn(Oct)_2$ ratio equal to 200 and the monomer to initiator molar ratio (q) equal to 3 and 5, respectively. As an example, for HEMA-CL_5, 10.52 g of CL (92 mmol) and 10 mg of Na_2SO_4 were weighted in a round-bottom flask and heated to 125 °C in a constant temperature oil bath. 2.40 g of HEMA (18 mmol, CL/HEMA = 5 mol/mol) and 37.35 mg of $Sn(Oct)_2$ (92 μmol) were mixed together and then injected into the pre-heated mixture. The mixture was left to react under stirring for 2.5 h. Macromonomer conversion (X) and degree of polymerization (q) were characterized *via* ^{1}H NMR ($CDCl_3$) on a Bruker Spectrometer (400 MHz) according to a previously published protocol[33] and reported in **Table S1**.

Two negatively charged macromonomers (HEMA-QCL_3 and HEMA-QCL_5) were synthesized *via* succinylation of HEMA-CL_3 and HEMA-CL_5, respectively. As an example, for

HEMA-QCL_5, 6 g of HEMA-CL_5 (8.6 mmol) were mixed with 1.03 g of succinic anhydride (10 mmol) in a round bottom flask and heated to 90 °C overnight under stirring. The product was then dissolved in dichloromethane and washed with brine (1:1 vol/vol) to eliminate the unreacted succinic anhydride. The solvent was removed under reduced pressure and, then, the macromonomers were dried under vacuum. An aliquot was withdrawn for ^{1}H NMR (in $CDCl_3$) characterization.

Two positively charged macromonomers (HEMA-CCL_3 and HEMA-CCL_5) were synthesized through DCC-mediated esterification of HEMA-QCL_3 and HEMA-QCL_5 with choline chloride. As an example, for the preparation of HEMA-CCL_5, 3 g of HEMA-QCL_5 (3.7 mmol) were added to 0.7 g of choline chloride (5 mmol, choline chloride/HEMA-QCL_5=1.3 mol/mol), 0.091 g of DMAP (0.7 mmol) and 50 mL of anhydrous acetonitrile. 1.2 g of DCC (4.5 mmol) was dissolved in 22 mL of anhydrous acetonitrile and, then, added dropwise using a syringe pump (model NE-300, New Era Pump, US). The temperature was maintained below 5 ± 1 °C over a period of 1 h and then left overnight at room temperature under continuous stirring. The mixture was then filtered three times to remove the dicyclohexylurea, and then acetonitrile was removed under vacuum. The product was dissolved in chloroform and washed twice with brine (1:1 vol/vol). Finally, the solvent was removed under reduced pressure and the product dried in a vacuum oven at 35 °C overnight. An aliquot was withdrawn for ^{1}H NMR (in $CDCl_3$) characterization.

The number-average molecular weight (*Mn*) and dispersity (*Đ*) of all the synthesized macromonomers (**Table S1**) were evaluated *via* gel permeation chromatography (GPC) with a Jasco Apparatus. The samples were dissolved in THF at 4 mg mL^{-1} and filtered through a PTFE 0.45 μm pore-size membrane before injection. The separation was performed at a flow rate of 0.5 mL min^{-1} at 35 °C and with three Superchrom PLgel 5 μm columns (0.5-1000 kDa). All the values reported were determined from differential refractive index data and were relative to poly(styrene) standards (from 580 to 197 300 g/mol, Polymer Laboratories).

2.3 Synthesis of the zwitterionic macro CTA

A zwitterionic macro CTA (25MPC, being 25 the target degree of polymerization) was synthesized *via* RAFT solution polymerization of MPC using CPA as RAFT agent and ACVA as initiator. The monomer to CPA molar ratio and the ACVA to CPA molar ratio were set to 25 and 1/3, respectively. Briefly, 2.23 g of MPC (7.6 mmol), 85 mg of CPA (0.3 mmol), and 28 mg of ACVA (0.1 mmol) were dissolved in a mixture of 5 mL of ethanol and 5 ml of 3 mM acetic buffer (acetic acid/sodium acetate pH = 4.5). The solution was poured in a 25 ml round bottom flask and purged with nitrogen for 20 min. The mixture was heated to 65 °C and left to react for 24 h under constant stirring. The product was purified by double precipitation in acetone, dried under vacuum, and stored at −20 °C. An aliquot

of the reaction mixture was withdrawn before the purification and analyzed *via* ^{1}H NMR (methanol-*d4*) to calculate the monomer conversion (*X*) and degree of polymerization (*n*). The number-average molecular weight (*Mn*) and dispersity (*Đ*) were evaluated *via* aqueous GPC with a Jasco apparatus. The sample was dissolved at 4 mg mL^{-1} in 0.05 M Na_2SO_4 / acetonitrile (80/20 v/v) mixture and filtered through a 0.45 μm pore-size nylon membrane. The separation was performed at a flow rate of 0.5 mL min^{-1} at 35 °C with three Suprema columns (particle size 10 μm and pore sizes of 100, 1000, and 3000 Å, Polymer Standards Service). All the values reported were determined from differential refractive index data and were relative to polyethylene glycol standards (from 238 to 884000 g/mol, Polymer Laboratories).

2.4 Synthesis of biodegradable zwitterionic nanoparticles *via* RAFT emulsion polymerization

The NPs were synthesized *via* RAFT emulsion polymerization of the oligoester-based macromonomers using 25MPC as macro CTA and ACVA as initiator with a total latex concentration equal to 10 wt.%. The ACVA to 25MPC molar ratio was kept constant to 1/3, while the macromonomer to 25MPC mole ratio (*p*) was set to 15, 30 or 60. As an example, for the NPs composed of the block copolymer 25MPC-30CL5 (target degree of polymerization for HEMA-CL_5, *p*, equal to 30), 0.132 g of 25MPC, 0.361g of HEMA-CL_5 and 1.6 mg of ACVA were dissolved in 7 ml of 3 mM acetic buffer (pH = 4.5) and 3 ml of ethanol. The mixture was purged with nitrogen for 30 minutes and placed in an oil bath at 65 °C for 24 h. The conversion and degree of polymerization (*p*) were determined *via* ^{1}H NMR (in MeOD). The nanoparticles were purified *via* dialysis (3.5 kDa molecular weight cut-off, regenerated cellulose, Spectrum Laboratories) against distilled water for 2 days. The 25MPC-30CL5, 25MPC-30QCL5, and 25MPC-30CCL5-based nanoparticles were used to evaluate the ability of our drug delivery carriers to withstand a freeze-drying step. In brief, 1 mL of 20 mg mL^{-1} NP suspension was mixed with 10 mg of glucose (1 wt.%) and then rapidly frozen with liquid nitrogen. The mixtures were freeze-dried for 24 hours at -50 °C and at a pressure of 0.15 mbar with a Telstar LyoQuest. The lyophilized NPs were then re-constituted by directly adding 1 mL of distilled water. The NP volume average diameter (*Dv*), polydispersity index (*PdI*), and surface charge (ζ) were evaluated *via* dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS, at a scattering angle of 173°. All the reported values are an average of three independent measurements.

2.5 Synthesis of the zwitterionic block copolymers in solution and subsequent self-assembly

For a direct comparison of the methodology reported herein, similar amphiphilic block copolymers were also synthesized *via* the RAFT solution polymerization of HEMA-CLq using the zwitterionic

hydrophilic block (25MPC) as macro CTA and ACVA as initiator. The ACVA to 25MPC molar ratio was set equal to 1/3, while the macromonomer to 25MPC molar ratio (*p*) was selected as 15, 30 and 60. As an example, for the 25MPC-30CL5, 0.159 g of 25MPC, 0.435 g of HEMA-CL_5, and 1.93 mg of ACVA were dissolved in 3 mL of methanol. The solution was purged with nitrogen for 20 minutes and then placed in an oil bath at 65 °C for 24 h. The copolymer was then precipitated twice in a ten-fold excess of diethyl ether, dried under vacuum, and stored at −20 °C. An aliquot of the mixture was analyzed before and after the purification *via* ^{1}H NMR.

The NPs were produced through the self-assembly of the synthesized amphiphilic block copolymers. More in details, 60 mg of copolymer were dissolved in 0.27 mL of methanol. The organic phase was then aspired and ejected 3 times from a syringe pre-loaded with 3 mL of PBS and equipped with a 21 G, 38 mm needle. The NP size distribution was evaluated *via* DLS.

2.6 Paclitaxel loading and release tests

The loading and release tests of PTX were performed on the neutral NPs (25MPC-30CL5). Four different tests were conducted by changing the NP concentration, the amount of DMSO used to dissolve the drug, and the NP production procedure. In the first two tests, the NPs were diluted to a concentration of 60 mg/mL and all the other experimental conditions were kept constant except for the amount of organic solvent used. Briefly, in the first experiment, 6 mg of PTX were dissolved in 30 µL of DMSO. After the complete dissolution of the drug, the solution was aspired by a syringe with a needle pre-filled with 2 mL of NPs dispersed in distilled water. The mixture was ejected and aspired three times. In the second test, 6 mg of PTX was directly mixed with 2 mL of NPs dispersed in distilled water, without solubilizing it in DMSO. After centrifugation at 5000 rpm for 10 minutes, the supernatant was collected. In the third test, instead, the NPs were obtained starting from the amphiphilic copolymers. Briefly, 10 mg of the copolymer were dissolved in 200 µL of a methanol/DMSO mixture (75/25 vol.%). Then, the solution was added to 0.5 mg of PTX to obtain the complete dissolution of the drug and the copolymer. After the complete mixing, the NPs were obtained and at the same time loaded with the drug by aspiring the solution in a syringe with a needle pre-filled with 3 mL of distilled water, with the mixture being ejected and aspired three times. In the last case, the NPs had a concentration equal to 3.33 mg/mL as in the latter experiment. Briefly, 0.5 mg of PTX were mixed with 200 µL of a methanol/DMSO mixture (75/25 vol.%). The solution was aspired by a syringe with a needle pre-filled with 3 mL of NPs dispersed in distilled water. The mixture was ejected and aspired three times. In all cases, the NPs were filtered with a 0.45 µm syringe filter and the loading efficiency was evaluated by taking aliquots of 0.1 mL of the formulation and analyzing it via HPLC. The NP suspensions were then loaded in dialysis cassettes (Spectra/Por,

molecular weight cut-off 3.5 kDa) and dialyzed against PBS for 24 h. The buffer was changed every 2 h to ensure proper sink conditions. The release profile was investigated by taking aliquots of 0.1 mL of the formulation in the dialysis cassette at predetermined time points (1, 2, 4, 6 and 24 hours).

The PTX amount in the stored aliquots was determined by HPLC-UV at 230 nm to investigate drug loading, release and concentration. The methods employed were illustrated in our previous publications[29,37]. The analytical reference standard powder of paclitaxel and the internal standard (IS) used, IDN5390 were generously provided by Indena SPA, Settala (MI), Italy. Briefly, 0.1 mL of NPs solution were spiked with 5 µg of IS and extracted with 0.5 ml of acetonitrile. After vortexing for 10 s, the samples were centrifuged at 13,000 rpm for 10 min. The organic phase was separated and dried under nitrogen, and the residues were dissolved with 250 µl of mobile phase. 50 µL of the reconstitute samples was injected in the HPLC system. Empty NPs samples were used to prepare the calibration curve by the addition of PTX at different concentrations.

2.7 Doxorubicin loading and release tests

The loading of Doxorubicin (Dox) was performed directly on pre-formed NPs structurally composed of either 25MPC-30CL5 or 25MPC-30QCL5 to investigate the impact of the NP net charge. Briefly, 2 mg of Dox were dissolved in 0.8 mL of DMSO. After the complete dissolution of the drug, the mixture was aspired with a syringe pre-filled with 2 mL of 20 mg mL^{-1} NP suspension in distilled water. The mixture was ejected and aspired three times through the needle. The NPs were then filtered with a 0.45 μm nylon filter and an aliquot was withdrawn to evaluate the drug loading efficiency. The same procedure was repeated in the absence of the polymer, with the aim of testing the syringe method and the release rate also for the free drug. The release of Dox from the NPs and as a free drug was investigated via dialysis against PBS. Briefly, 2 mL of loaded NPs were put inside a dialysis cassette (Slide-A-Lyzer™ Dialysis Cassettes, molecular weight cut-off 3.5 kDa) and immersed in 200 mL of PBS, changed every 2 h to ensure proper sink conditions. The release profile was investigated by taking aliquots of 1 ml from the release medium at predetermined time points (1, 2, 3, 4, 8 and 24 h) and immediately analyzed via UV spectrophotometer. In order to be able to evaluate the Dox content incorporated in the NPs, the creation of a new HPLC method became necessary. The analyses were carried out on a HPLC (Waters Associates, Milford, MA, USA, model 2487 Variable Wavelength Detector) with a RP C18 (Merck Millipore®) analytical reverse phase column (250 mm×4 mm, 5 µm), maintained at 40 °C. The mobile phase consisting of a mixture of acetonitrile:water and H_3PO_4, 31:69 (v/v), was eluted with a flow rate of 1 mL min^{-1}. Before the first injection, the column was conditioned for 30 min. 50 µL of the sample were injected and analyzed at a wavelength equal to 380

nm and the run time was 10 minutes. Five standard concentrations were obtained by diluting the first stock solution of Dox in DMSO (1006 µg/ml) and a calibration curve was then obtained. A linear regression analysis through the five standard concentrations (1.006, 10.06, 50.3, 100.6, 1006 µg/ml) was done and the correlation coefficient was obtained equal to 0.9999.

2.8 Ibuprofen Loading and Release

The loading and release test of Ibuprofen was performed both on the positively charged NPs (25MPC-30CCL$_5$) and on the neutral NPs (25MPC-30CL5). In both cases 3 mg of Ibuprofen (5% w/w) were mixed with 0.12 ml of DMSO (3.8% v/v). After the complete dissolution of the drug, the solution was aspired by a syringe pre-filled with 3 mL of 20 mg mL^{-1} NP suspension in distilled water. The mixture was ejected and aspired three times. The NPs were filtered with a 0.45 µm nylon filter in both cases and the loading efficiency was evaluated by taking 0.1 mL aliquots of the formulation and analyzing them via HPLC. The NP suspension was then dialyzed against PBS for 24 h in a dialysis cassette (Spectra/Por, molecular weight cut-off 3.5 kDa). To ensure proper sink conditions, the PBS was changed every 2 h. The release profile was investigated by taking aliquots of 100 µl from the cassette at predetermined time points (1, 2, 3, 4, 5, 6, 8 and 24 h) and analyzing them *via* HPLC. The same procedure but without NPs was applied to measure the release of the free drug. The analyses were carried out *via* HPLC (Jasco 2000 Series equipped with a variable wavelength detector) with a reversed phase C18 column (Restek, 250 mm in length, 4 mm internal diameter and 5 µm particle size) maintained at 40 °C. The separation was performed at 1 mL/min in isocratic conditions with a mobile phase consisting of a mixture of 50/50 vol/vol ACN/20 mM phosphate buffer at pH 6. The detection was carried out at 240 nm. The Ibuprofen concentration was obtained from a calibration curve relating the peak area (mAU min) to the concentration obtained from the analysis of 6 samples at known concentration. The correlation coefficient of the linear regression of the experimental data was equal to 0.994.

2.9 Cytotoxicity Analysis

The cytotoxicity of 25MPC-30CL5, 25MPC-30QCL5, and 25MPC-30CCL5 was evaluated *in vitro* in the case of the ovarian cancer cell line IGROV1. This choice derived from the deep knowledge of this model[38–40], allowing to standardize the cell growth and make extremely reproducible the effects of the treatments with different antiproliferative agents. The cells were seeded in six-well plates (Costar) at the concentration of 20000 cells/mL. 72 h after the seeding, the cells were incubated with 0.4 and 0.08 mg/mL of NPs. After 72 h of exposure, the cells were detached using 0.5 mL of trypsin, re-suspended in 2 mL of PBS and counted using a Coulter Counter ZM (Coulter Electronics, UK).

The procedure was applied three times for each concentration and the average number of cells was considered and expressed as a percentage of the untreated cells (control) at the same time.

3. Results and Discussion

3.1 Synthesis of the biodegradable zwitterionic nanoparticles

The RAFT emulsion polymerization is a powerful tool to obtain highly tunable and narrowly dispersed nanoparticles directly in aqueous media[31,41–43]. In this work, we combined this process with the ROP to obtain biodegradable zwitterionic NPs with different size and charge (1a in **Figure 1**). The detailed synthesis approach for these biodegradable NPs is reported in **Scheme S1** and **Scheme S2**. In particular, we have synthesized two oligocaprolactone-based macromonomers *via* ROP targeting a degree of polymerization (*q*) equal to 3 and 5, respectively (**Figure S1**, HEMA-CL_3 and HEMA-CL_5 in **Table S1**). These macromonomers were subsequently used to chain-extend a zwitterionic macromolecular RAFT agent (**Figure S2,** 25MPC in **Table S1**) directly in a water/ethanol mixture to produce NPs that are structurally composed of amphiphilic block copolymers (**Figure S3**, 25MPC-p$CL_{3\text{-}5}$ with p=15, 30 and 60 in **Table S2**). The hydrophilic MPC portions of the block copolymers are expected to be located on the NP surface while the lipophilic lateral oligoester chains are confined in the NP core. A similar NP structure can be obtained synthesizing these zwitterionic block copolymers via RAFT solution polymerization and then by self-assembling them directly in water *via* nanoprecipitation[19] (1b and 2b in **Figure 1**). The drawback, in this latter case, is the use of a toxic solvent like methanol, necessary to dissolve the zwitterionic block in the nanoprecipitation step.

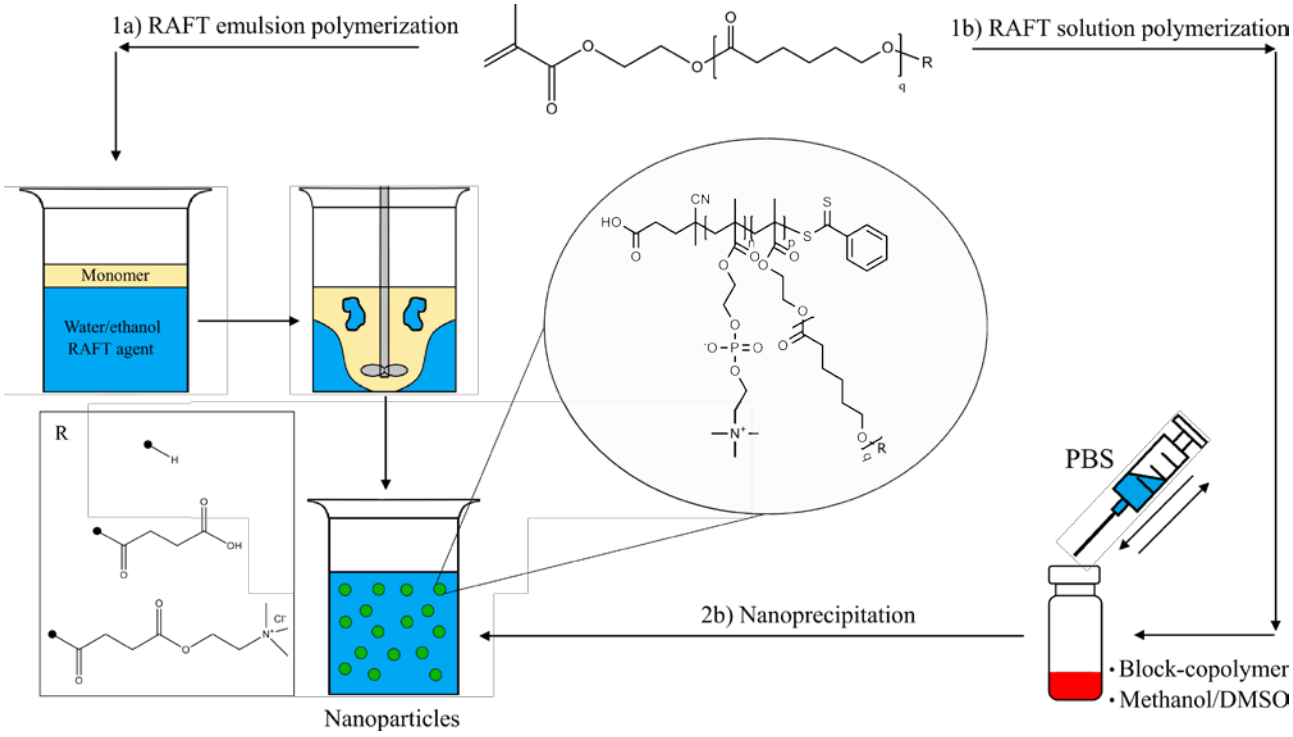

Figure 1. Different strategies to synthesize zwitterionic NPs. In the first case (**1a**), the final colloid is obtained *via* RAFT emulsion polymerization of a PCL-based macromonomer in the presence of a zwitterionic RAFT agent. In the second case, the amphiphilic block copolymer is obtained first *via* RAFT solution polymerization (**1b**) and, then, it is dissolved in a methanol/DMSO mixture. The organic mixture is then aspired and ejected several times through the needle of a syringe pre-filled with an isotonic solution (**2b**). In this latter case the NPs are obtained via the self-assembly of the block copolymer.

However, independently from the production route, the NP size (*D*) can be finely tuned by playing with the stoichiometry of the ROP and RAFT polymerization. In particular, *D* can be modulated by varying the length (*q*) and the number (*p*) of the lipophilic lateral chains, as schematically sketched in **Figure 2a**, according to eq(1)[19].

$$D = \frac{6 \cdot \mathrm{p} \cdot (MW_{HEMA} + \mathrm{q} \cdot MW_{CL})}{N_{Avo} \cdot A_{Cov} \cdot \rho_{b\_lipo}} \quad (1)$$

where N_{Avo} is the Avogadro number, A_{cov} the surface area that a single hydrophilic chain can cover, ρ_{b_lipo} the density of a single lipophilic block, and MW_{HEMA} and MW_{CL} are the molecular weight of HEMA and of a caprolactone unit, respectively. It is evident from eq(1) that *D* is only a function of the copolymer microstructure and it is not remarkably influenced by the processing parameters (*e.g.* stirring rate, concentration). This is confirmed in **Figure 2b**, where we show that the volume average size *Dv* of the NPs obtained from the RAFT emulsion polymerization of HEMA-CL_3 and HEMA-CL_5 shares a linear trend with *p* similar to the *Dv* of the NPs produced *via* nanoprecipitation (data from ref.[19]). In addition, the slope of the linear trends increases with *q*, in agreement with eq(1). The modulation of p and q enables also the tuning of the degradation time. In fact, we demonstrated with

similar copolymers as those reported in this work that the rate of hydrolysis of the ester bonds and hence the NP dissolution rate is strictly related to the distribution of the hydrophobic units in the copolymer chains. In particular, for NPs with large q and small p the degradation is accelerated. Therefore, the modular block copolymers achievable through the combination of ROP and RAFT allow a strict control over the physico-chemical properties of the corresponding NPs[19,21].

A major difference between the nanoprecipitation and the RAFT emulsion polymerization lies in the polydispersity index and latex concentration for the produced NPs. With the RAFT emulsion polymerization, we were able to obtain NPs with PdI in the range 0.13-0.14, with a concentration of 10 wt.%. On the other hand, following the self-assembly route, only poor concentrations of 0.3 wt.% and PdI = 0.190 were obtained (data from ref.[19]). These results were not unexpected. In fact, in the nanoprecipitation, the quality of the NPs strongly depends on the ability of the pre-formed block copolymers to self-assemble in water. This ability is in turn mainly affected by the polymer chain mobility and diffusivity[44,45]. In contrast, in the RAFT emulsion polymerization, the quality of the NPs is mainly dictated by the ability of the lipophilic monomer to migrate into the growing NPs during the nucleation and growth phases of the process. Therefore, as long as the pre-formed block copolymers are bigger compared to the single macromolecular units, the diffusive resistance is higher in the nanoprecipitation step, thus hampering an ideal self-assembly.

Once we established the possibility of obtaining biodegradable zwitterionic NPs with controllable size *via* RAFT emulsion polymerization, we next proved the possibility of tuning the NP net charge by acting on the macromonomer functionalization. Towards this aim, we synthesized ionizable negatively charged macromonomers *via* succinylation of HEMA-CLq (**Figure S4**, HEMA-QCL_3 and HEMA-QCL_5 in **Table S1**) and positively charged macromonomers *via* DCC-mediated esterification of the HEMA-QCLq with choline chloride (**Figure S5**, HEMA-CCL_3 and HEMA-CCL_5 in **Table S1**). We performed the RAFT emulsion polymerization of these macromonomers in the presence of 25MPC to generate zwitterionic NPs with different size and charge (**Figure S6-7**, 25MPC-pQCL_{3-5} and 25MPC-pCCL_{3-5} with p=15, 30 and 60 in **Table S2**) and spherical morphology similar to their neutral counterparts (**Figure S8**).

In all the cases, high monomer conversions (>85%) and degree of polymerization (*p*) close to the target were achieved. In addition, the linear dependency of *Dv* with *p* is preserved also in the case of charged macromonomers, as confirmed in **Figure 2c-d**.

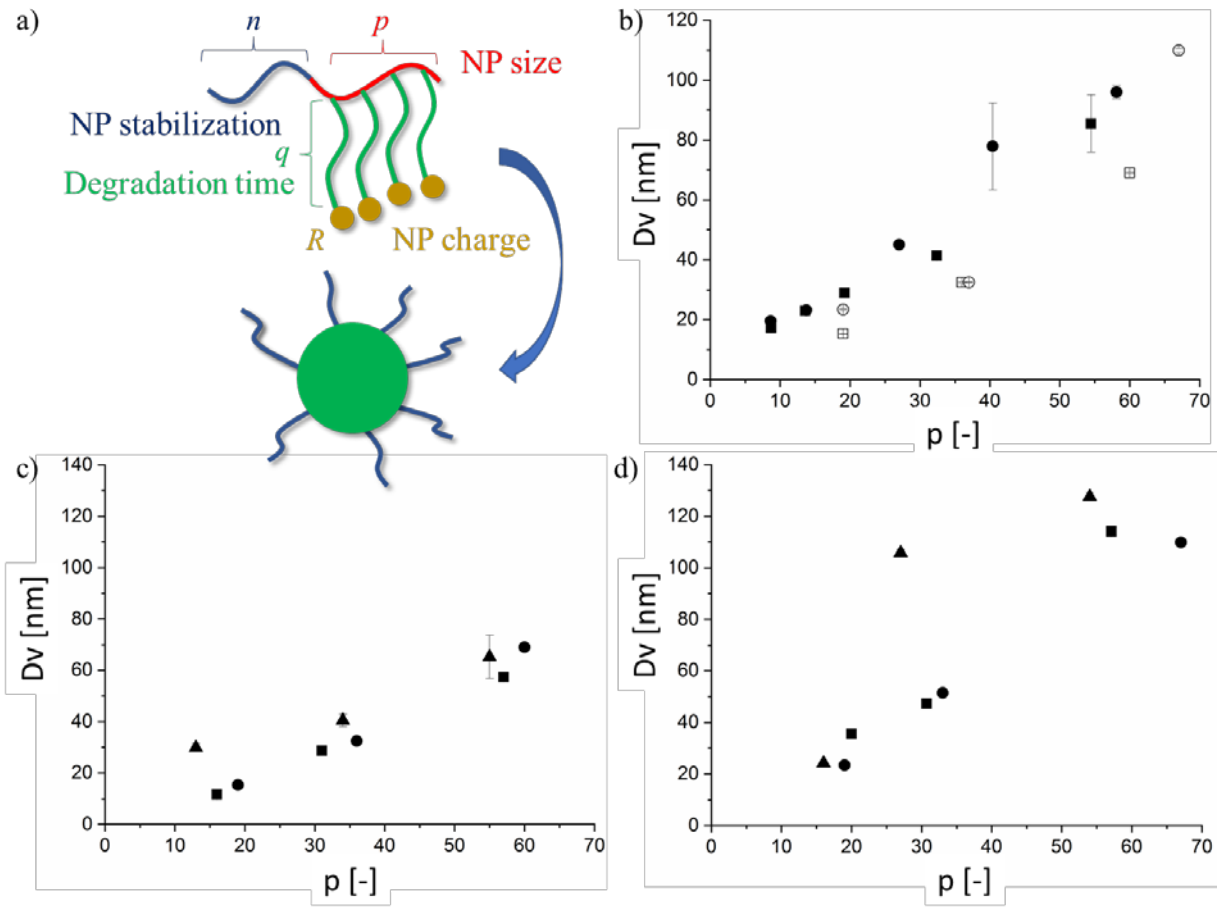

Figure 2. (a) Structure of the block copolymer and its self-assembly behavior. (b) Volume average diameter *Dv* as a function of p for NPs produced at two different values of q, namely q=3 and 5, as obtained from the nanoprecipitation (self-assembly) of the corresponding block copolymers (■ for HEMA-CL_3, ● for HEMA-CL_5) or *via* RAFT emulsion polymerization (□ for HEMA-CL_3, ○ for HEMA-CL_5). (c) *Dv* as a function of p for neutral (●), negatively (■) and positively (▲) charged NPs obtained from HEMA-CL_3 based macromonomers. (d) *Dv* as a function of p for neutral (●), negatively (■) and positively (▲) charged NPs obtained from HEMA-CL_5 based macromonomers.

However, the NPs based on these charged macromonomers present an overall higher NP *Dv* compared to the NPs obtained with the neutral macromonomers. This behavior can be ascribed to a partial water swelling of these colloids caused by the charged groups present in the NP core. Interestingly, while the positively charged NPs present a high net positive ζ-potential, the difference between the negatively charged and neutral NPs is less marked. This can be ascribed to the use of negatively charged species (*i.e.* CPA and ACVA) as chain transfer agent and radical initiator during the synthesis and to the incomplete ionization of the carboxylic acid groups in the core of the negatively charged NPs.

Overall, the combination of the ROP and RAFT emulsion polymerization allows the production of narrowly dispersed biodegradable zwitterionic nanoparticles with tunable net charge at high concentration. In addition, we expect that the fine tuning of *q* and *p* can be used also in this case

to decouple the NP size, block copolymer MW, and NP degradation time in a similar way to what shown for the NPs obtained *via* nanoprecipitation[19,46].

3.2 Drug formulation and release

The loading of therapeutics into polymer NPs can be generally carried out during or after their formation. In general, it is preferable to avoid loading a drug during the NP formation through RAFT emulsion polymerization due to the harsh environment of the reaction (*i.e.* high temperature) and the presence of radicals that may degrade the active principle. On the other hand, the simultaneous NP formation and drug loading can be achieved *via* nanoprecipitation. In this case, the therapeutic is co-dissolved with an amphiphilic block copolymer in a water-soluble organic solvent. The viscosity of the resulting organic phase must be kept low enough to allow the correct self-assembly of these polymeric surfactants into NPs and, at the same time, the loading of the drug inside the forming NP core. On the other hand, the amount of drug in the formulation must be high enough to meet the therapeutic requirements (*i.e.* dose and drug concentration). In addition, the organic phase should be able to dissolve the drug and the amount of block copolymer necessary to formulate it. For this reason, in the nanoprecipitation method, there is an intrinsic trade-off between the achievable drug concentration and the limitation in the organic solvent content in the final formulation. Out of this trade-off, it is not possible to obtain a good NP quality (*i.e.* $Dv < 200$ nm and low PdI values) because of the diffusive limitations during the NP production step. Additional problems arise when the drug and the polymeric surfactant are not soluble in the same water miscible organic solvent or one of the two is soluble only in highly toxic organic solvents (*e.g.* methanol for the zwitterionic-based block copolymers). In these cases, this formulation procedure cannot be directly translated at the point of care since laborious purification and storage processes are required. For example, a dialysis must be performed before injection to remove the organic solvent and this purification step cannot be easily performed by the end-user.

In order to address the issues related to the insolubility of the zwitterionic block copolymers in safe solvents, we have directly synthesized these NPs in an aqueous medium and the loading of the drug has been carried out in a second step (**Figure 3a**). The procedure involves only the use of a syringe and a needle, similarly to the nanoprecipitation protocol. However, in this case the water miscible organic solvent is used only to dissolve the drug and to transport it into the lipophilic core of the pre-formed NPs. This allows loading a higher amount of drug with a lower volume of organic solvent, since it is no longer necessary to dissolve any high MW polymeric surfactant with the drug. The maximum drug concentration and minimum organic concentration are now only dictated by the

maximum solubility of the drug in the adopted organic solvent. However, the drug should be able to counter-diffuse in the NP core to achieve a good encapsulation efficiency and its sustained release.

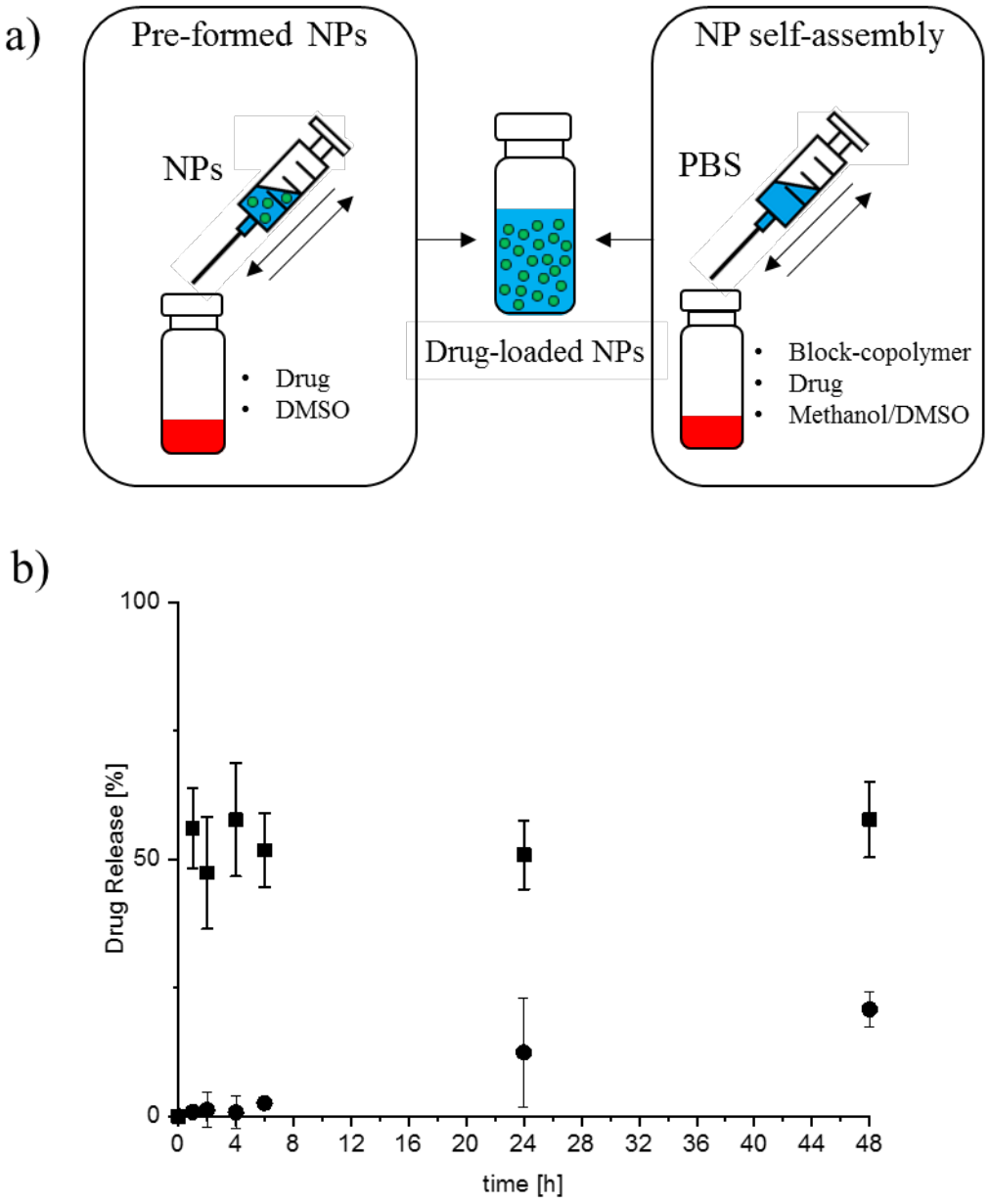

Figure 3. a) Loading of the drug with the two different syringe-based protocols starting from pre-formed NPs or dissolved block copolymers. b) PTX release from the NPs obtained via the nanoprecipitation protocol (■, entry 1 in **Table 1**) or from the pre-formed NPs (●, entry 2 in **Table 1**).

In order to demonstrate this approach, we have formulated paclitaxel (PTX), a lipophilic anticancer therapeutic that it is generally administered intravenously with the aid of ethanol and a surfactant (i.e. cremophor EL in the Taxol® formulation[47,48]), into NPs with the two different syringe

protocols described above. We adopted the NPs structurally composed of the block copolymer 25MPC-30CL5 for this test (**Table 1**).

As long as methanol is necessary to dissolve the zwitterionic block copolymer in the nanoprecipitation step, we used the same amount of this solvent also in the new syringe protocol in order to test the two formulations under the same conditions, although it is not necessary in the case of pre-formed NPs. The NPs obtained from the self-assembly of the corresponding block copolymers (entry 1, **Table 1**) were more polydisperse and with a lower PTX encapsulation efficiency compared to the ones obtained directly *via* RAFT emulsion polymerization and subsequently loaded with the drug (entry 2, **Table 1**). Given these promising results, we further investigated the possibility of eliminating methanol and increasing the drug concentration in the case of pre-formed NPs (entry 3, **Table 1**). The use of a little amount of DMSO to solubilize the drug allows increasing the final drug concentration from 0.17 to 3 mg/ml without altering the NP *Dv* and PdI, and with a drug loading >99%. At the same time, it is also possible to decrease the DMSO concentration from 4.7 to 1.5 vol.% and, in turn, to reduce considerably the overall toxicity of the formulation. However, it is not possible to eliminate the organic solvent (entry 4, **Table 1**) due to its role in the transport of the drug inside the NP core. If no organic solvent is used, large drug aggregates are formed and the encapsulation efficiency is considerably reduced (27.8%).

Table 1. PTX-loaded NPs composed of the block copolymer 25MPC-30CL5. The drug-loaded NPs are obtained *via* the syringe method starting from the dry block copolymer or the pre-formed NPs.

Entry [-]	Formulation Method [-]	Dv [nm]	PdI [-]	C_{NPs} [mg/ml]	C_{DMSO} [vol.%]	$C_{methanol.}$ [vol.%]	C_{drug} [mg/ml]	Drug Loading [%]
1	Self-assembly	79	0.21	3.3	4.7	1.5	0.17	75.9
2	Pre-formed NPs	60	0.061	3.3	4.7	1.5	0.17	>99
3	Pre-formed NPs	60	0.061	60	1.5	0	3	>99
4	Pre-formed NPs	60	0.061	60	0	0	3	27.8

Once proven that higher drug loadings without the need of toxic solvents are achievable with the pre-formed NPs, we compared the drug release kinetic against PBS and at 37 °C for these formulations (entry 1 and 2, **Table 1**). As visible from **Figure 3b**, the drug is released faster from the NPs obtained *via* the nanoprecipitation of the block copolymer. This can be explained considering the broad size distribution of these NPs after the PTX loading (PdI = 0.21), which testifies the formation of aggregates. In addition, a 50% burst PTX release occurring within the first hour is a clear evidence that the drug was poorly encapsulated in the NPs with this loading strategy. On the

other hand, the PTX is efficiently retained when loaded into pre-formed NPs, as testified by the absence of any initial burst release. In addition, the low PdI (*i.e.* 0.061) indicates that aggregation and clustering were prevented. The achievement of a sustained release enables the maintenance of the drug concentration within a designed therapeutic window for a prolonged time, which is an important aspect to increase the therapeutic index of the formulation.

This proves that it is possible to formulate lipophilic drugs using pre-formed NPs achieving high drug concentration. When compared to Taxol®, the most common PTX formulation used in clinics, this formulation allows to reduce the amount of organic solvent and at the same time it avoids the use of cremophor EL, a toxic surfactant that can cause hypersensitivity reactions[49]. For example, in the case of a common PTX dose of 175 mg/m^2 for a 75 kg patient and for a PTX concentration of 1 mg/mL, 27 ml of ethanol at a concentration of 9.3 vol.% and 28.8 g of cremophor EL should be administered with the Taxol® formulation. On the other hand, with our formulation, only 1.6 mL of DMSO at concentration of 0.5 vol.% and 6.7 g of the biodegradable zwitterionic NPs are required.

Once proved the possibility of formulating lipophilic drugs using a syringe and starting from pre-formed NPs, we have focused on the loading of therapeutics that are charged at physiological conditions. For example, doxorubicin (Dox), a positively charged anticancer therapeutic (isoelectric point = 8.4) that is generally administered intravenously[50–52], has been formulated with both neutral and negatively charged NPs (entry 1-2, **Table 2**) and the release against PBS at 37 °C from these novel formulations has been compared to the free drug (**Figure 4**). The latter was formulated using the same syringe method as it was used for loading into NPs, but with no polymer. In this way, it was possible to obtain a proper evidence of the effect of the NP formulation in modifying the release of the drug. The advantages in using polymer NPs to formulate the drug are evident. In particular, the NPs prepared from the neutral macromonomer are able to slow down the release of Dox compared to the free drug. Then, with the NPs prepared starting from the negatively charged macromonomer, we were able to further improve the drug retention for at least 24 h, thus paving the way to the maintenance of the drug concentration within a suitable therapeutic window for prolonged time.
It is worth underlying that in the case of charged molecules, a certain portion of the drug could also be retained on the NP surface, but this acts in the direction of mediating the drug release, in line with the scope of the formulation.

Table 2 Doxorubicin-loaded nanoparticles obtained via the syringe method starting from the pre-formed NPs.

Entry	Block copolymer	Dv [nm]	PdI [-]	ζ [mV]	C_{NPs} [mg/ml]	C_{DMSO} [%]	C_{drug} [mg/ml]	Drug Loading [%]
1	25MPC-30CL5	60	0.061	-19	20	3.8	0.96	>99

2	25MPC-30QCL5	89	0.18	-26	20	3.8	0.96	>99

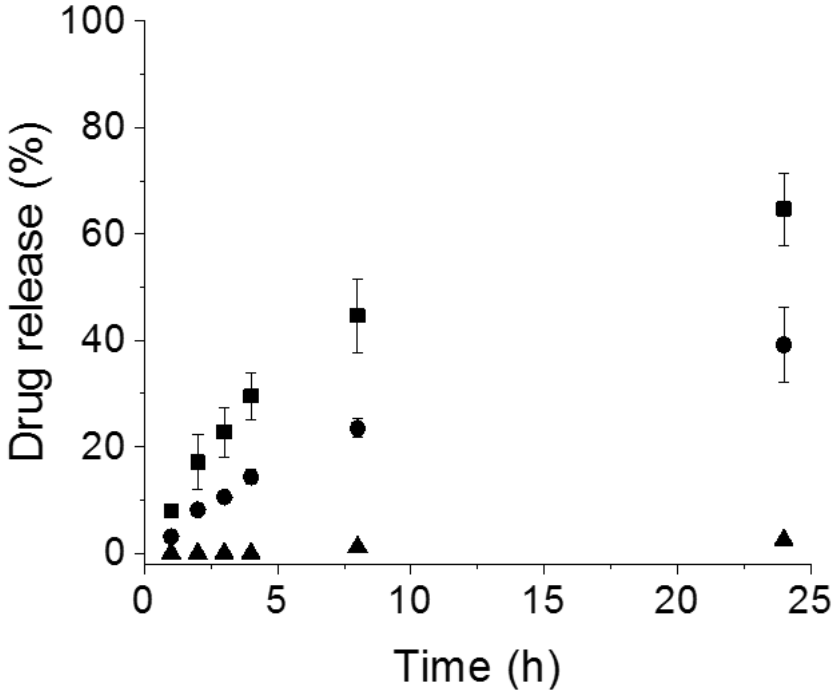

Figure 4: Release of Dox as free drug (■) or from neutral NPs (entry 1, **Table 2**) (●) and negatively charged NPs (entry 2, **Table 2**) (▲) obtained *via* RAFT emulsion polymerization.

In a similar manner, the loading of a negatively charged drug (*e.g.* ibuprofen, a nonsteroidal anti-inflammatory drug with an isoelectric point = 4.5 that it is generally administered orally[53,54]) and its release against PBS at 37 °C has been tested formulating ibuprofen with NPs made with both the neutral and positively charged macromonomers (entry 1-2, **Table 3**, **Figure 5**). In this case, the difference in terms of release rate between the free drug and the NPs produced from the neutral macromonomer is not statistically significant. On the other hand, the positively charged NPs are able to slow down the release of ibuprofen thanks to the electrostatic interactions between the carboxylic acid of the drug and the quaternary ammonium group of choline.

Table 3. Ibuprofen-loaded nanoparticles obtained via the syringe method starting from the pre-formed NPs.

Entry	Block copolymer	Dv [nm]	PdI [-]	ζ [mV]	C_{NPs} [mg/ml]	C_{DMSO} [%]	C_{drug} [mg/ml]	Drug Loading [%]
1	25MPC-30CL5	60	0.061	-19	15	3	0.8	>99
2	25MPC-30CCL5	105.3	0.184	27.8	15	3	0.8	>99

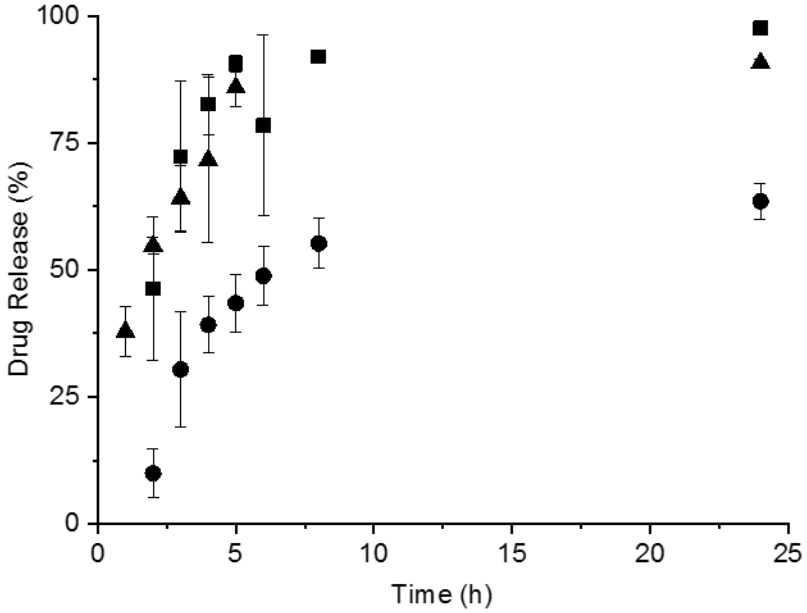

Figure 5: Ibuprofen release as free drug (▲), from neutral NPs (entry 1, **Table 3**) (■) and negatively charged NPs (entry 2, **Table 3**) (●) obtained *via* RAFT emulsion polymerization.

Therefore, our modular pre-formed NPs are able to efficiently load and control the release of different drugs, either neutral or charged at physiological pH, thus representing versatile excipients for systemic administration.

To provide a preliminary indication on the biocompatibility of these pre-formed zwitterionic NPs, the antiproliferative effect of 25MPC-30CL5, 25MPC-30QCL5, and 25MPC-30CCL5, representatives of neutral, positive and negative NPs, respectively, was analyzed on the ovarian cancer cell line IGROV1. The results are reported in **Figure S9** and show that the growth inhibition induced by the treatment is dose-dependent and low NP concentrations are well tolerated. The proliferation of the cells after 72 h exposure is strongly reduced only in the case of positively-charged NPs. This was not surprising. In fact, it is well known that cationic compounds can interact with the cell membrane leading to damages that could compromise the viability[55]. However, these data are not conclusive and should be combined with information on body accumulation and biodistribution[56].

A typical problem encountered in the translation of a promising formulation from the bench to the clinic is a suitable storage, which ideally should grant a sufficiently long shelf life. In fact, the storage in water can lead to a premature degradation of these colloids especially after long periods. With our NPs, we envision the possibility of freeze-drying the carrier, then reconstitute, and load it with the payload directly at the point of care. This is made possible by the simple loading procedure

and by the fact that no post-processing steps (*e.g.* dialysis) are required. For this reason, we have investigated the possibility of freeze-drying our NPs and, then, to reconstitute them before their end-use. In order to confirm this behavior, we froze our NPs in liquid nitrogen with 1% w/w glucose as cryoprotectant and dried them under vacuum at -50 °C and at a pressure of 0.15 mbar[57]. All of the samples could be immediately re-constituted preserving the NP size and low polydispersity (**Table S3**).

Overall, we have demonstrated that we can formulate drugs directly at the point of care by just using a common syringe and that we can modulate the release of different therapeutics by changing the physiochemical characteristics of our modular zwitterionic NPs.

Conclusions

In this work, we have synthesized modular biodegradable zwitterionic NPs with tunable size and surface charge *via* the combination of ROP and RAFT emulsion polymerization. These polymeric colloids are useful for the formulation of the manifold drugs with different properties that are populating the market by changing only minimally the excipient. With this respect, a protocol to formulate therapeutics directly at the point of care using a common syringe and starting from these pre-formed NPs has been developed and found effective for the loading and sustained release of paclitaxel, doxorubicin, and ibuprofen. In particular, it is possible to produce a novel PTX formulation with a very small amount of DMSO and without using the toxic surfactant cremophor EL adopted in the currently approved Taxol® formulation. In addition, we have showed that it is possible to control the release of therapeutics that are charged at physiological conditions (*e.g.* doxorubicin, ibuprofen) by varying the charge of the macromonomers adopted in the NP production. For the clinical translation, we envision the storage of these NPs in a lyophilized state and their re-constitution and drug encapsulation just before the final use. In fact, we demonstrated that these colloids could be easily re-dispersed in water after lyophilization, preserving their size distribution. Owing to these promising results, the NPs developed in this work can be potentially used as universal excipients to formulate poorly water-soluble or charged drugs avoiding the common post-processing steps that hamper the cost-effective use of these re-formulations and that reduce their availability among the patient population.

Supporting Information: electronic supplementary material is available at the publisher's website and reports the ^{1}H NMR spectra and characterization of the macromonomers and polymers reported

in this work, the characterization of the nanoparticles, the biocompatibility analysis and the evidence of the nanoparticle redispersibility after freeze-drying.

Conflict of Interests: The authors declare no competing interests for this work.

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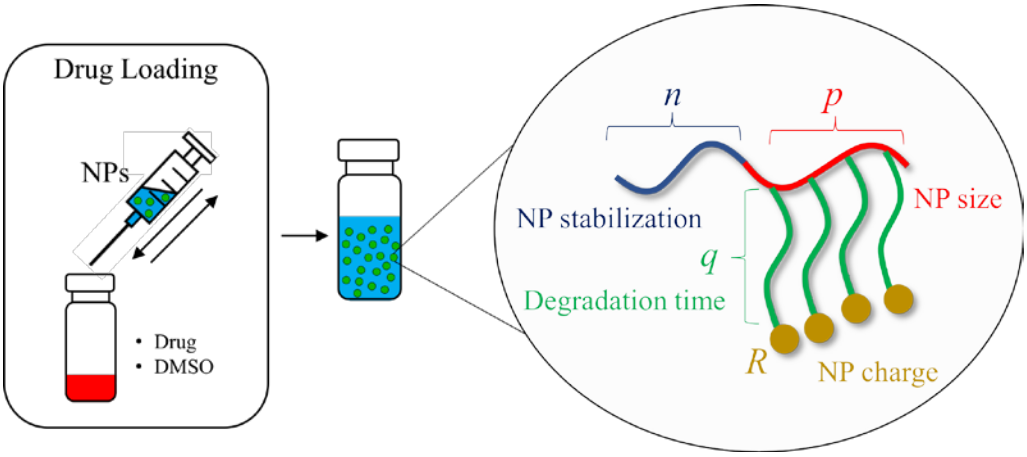

This work reports modular zwitterionic nanoparticles as universal excipients to formulate poorly water-soluble drugs directly at the point of care.