DOI: 10.1002/ mabi.201800397

Full Paper

Polypeptidic Micelles Stabilized with Sodium Alginate Enhance the Activity of Encapsulated Bedaquiline

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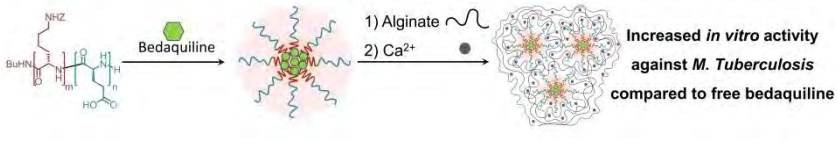
Abstract: Herein we describe the coating of polypeptidic micelles with sodium alginate as a strategy to improve the stability of micelles for drug delivery. Bedaquiline, approved in 2012 for the treatment of multidrug resistant tuberculosis, has been used as example of hydrophobic drug for the study of the loading efficiency, of the release of the encapsulated drug in different media and of the in vitro antimicrobial activity of the system. Alginate coating prevented the

burst release of the drug from micelles upon dilution and led to a sustained release in all tested media. In view of possible oral administration, the alginate coated micelles showed better stability in gastric and intestinal simulated media. Notably, the encapsulated bedaquiline showed increased in vitro activity against M. tuberculosis compared to free bedaquiline.



1. Introduction

Drugs used for the treatment of life threatening pathologies such as cancer or some infectious diseases often also carry serious undesired side effects. Moreover, the search of new active drugs is resulting quite difficult and efforts are being focused on the development of improved formulations of already known drugs.[1]

Nanotechnology can provide diverse nanocarriers to reduce the side effects by decreasing the dose to be administered primarily through enhancing drug solubility, preventing its degradation, and consequently extending its circulation time.[2]

Nanocarriers can be built from inorganic components, lipids, dendrimers and biodegradable polymers. Among them, polymers based on synthetic polypeptides have gained importance due to their biocompatibility, biodegradability and great chemical diversity of side chains which make them suitable for a broad variety of chemical modifications[3] including crosslinking groups,[4] stimuli responsive units,[5,6] and conjugation with drugs[7] or with active targeting species.[8,9] Using those strategies, synthetic polypeptides can be used as active therapeutic polymers but also as drug carriers in the form of hydrogels, vesicles, micelles or as components of polymeric nanoparticles.[8] Among them, micelles based on amphiphilic synthetic polypeptides have been reported for the encapsulation and delivery mainly of antitumoral drugs with hydrophobic nature taking advantage of their nanoscopic size suitable for the passive accumulation in tumors.[8] However, due to their supramolecular nature, micelles are prone to disassemble upon dilution or upon changes in the medium conditions, such as pH, or ionic strength.[10] The consequence is the burst release of the drug upon administration and the loss of the advantages of using a carrier. Thus, different strategies have been used for the stabilization of micelles such as covalent crosslinking [8,11] metal coordination[12] or ionic interactions.[13] However, the approach of stabilizing the micelles with a polymer coating remains unexplored. This strategy offers the opportunity for

modularity, where a variety of polymers can be tested and used to obtain different properties for the surface of the carrier while preserving the encapsulation properties of the polymeric micelle. Polysaccharides derived from natural sources possess a great potential for this purpose since they are biocompatible, biodegradable and mucoadhesive.[14] They can form hydrogels and provide different functionalization depending on the natural source, i.e. amines, carboxylic acids. In particular, alginate is an FDA-approved polysaccharide that presents good mucoadhesive properties and undergoes gelation through coordination with metal ions, typically Ca^{2+}.[15] Those metal ions are also susceptible of interaction with polyglutamate or polyaspartate segments in the hydrophilic block of an amphiphilic polypeptide.

In the work presented here we aimed at demonstrating how alginate can be used as coating to stabilize polypeptidic micelles and how it can remarkably decrease the release of a hydrophobic drug encapsulated within their hydrophobic core. As hydrophobic block we have chosen poly(benzyloxycarbonyl lysine) for its good encapsulation properties of hydrophobic drugs.[16] Bedaquiline (BQ) was selected as lipophilic drug based on its importance regarding the treatment of multidrug-resistant (MDR) tuberculosis (TB). MDR-TB is a worldwide health problem recognized by the World Health Organization (WHO) since only 54 % of MDR-TB cases are currently being successfully treated worldwide.[17,18] In this context, BQ was granted approval by U.S. Food and Drug Administration (FDA) in 2012 to be used to treat MDR-TB in a multidrug treatment regime and when other options to treat this condition using other existing drugs have been exhausted. Albeit, it is a very effective drug, BQ has been associated to serious side effects including induction of life-threatening cardiac arrhythmias.[19] The aim of a nanotechnological approach in this case is to develop a nanocarrier for antimicrobial drug delivery that could help its safer administration and could be applied to treat other multidrug resistant infections in the future.[20]

2. Experimental Section

2.1. General Methods

γ-benzyl-L-glutamic acid (H-BnE-OH), ϵ-*N*-benzyloxycarbonyl-L-lysine (H-ZK-OH) and triphosgene were purchased from Fluorochem and used as received. Bedaquiline, nile red and sodium alginate (120.000-190.000 g mol^{-1}) were obtained from Sigma-Aldrich and used as received. DMSO, DMF and THF were purchased from Acros Organic dried over 4 Å molecular sieves. Diethyl ether stabilized with BHT and hexane were purchased from Scharlab and used as received. BnE-NCA and ZK-NCA were prepared and recrystallized five times following published procedures as reported in the supplementary information.[21]

Human plasma was purchased from Sigma-Aldrich.

Dyalisis was performed in SnakeSkin™ dialysis tubing, with 3500 Da MWCO from ThermoFisher. Millex syringe driven filter unit (nylon) with a pore size of 0.45 μm were purchased from Merck Millipore.

Difco™ Middlebrook 7H9 broth was purchased from BD and supplemented with 10% ADC and 0.05% Tween® 80 (Scharlau). Resazurin sodium salt was purchased from Sigma.

2.2. Instruments and Measurements

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer. Chemical shifts are given in ppm relative to the solvent residual peak, which was used as internal reference. Coupling constants are given in Hertz. Spectra were processed with *MestReNova 10.0.2* software from MestreLab Research. FTIR spectroscopy was performed in a *Jasco* FT-IR 4100 instrument with an ATR accessory in which samples were measured without any preparation. All frequencies of characteristic bands were reported in cm^{-1}. Syringe pump additions were performed with a New Era *NE-300 Just Infusion* equipment, employing HSW syringes. Polypeptides were characterized by combining a Waters 2695 GPC with a tandem of

detectors: multiangle laser light scattering (MALLS) detector at 60 °C, viscosimeter (DV) and refractive index (RI) from Wyatt Tech. The system was equipped with one guard and two columns Phenogel Linear (2) (5 μm, 7.8 x 300 mm) from Phenomenex. HPLC-grade *N*,*N*-dimethylacetamide (DMAc), containing 0.1 M LiBr, was used as the mobile phase at a flow rate of 1.0 mL min^{-1} and 60 °C. Concentrations between 1.0 and 5.0 mg mL^{-1} were injected into the columns at an injection volume of 200 μL. ASTRA software from Wyatt Technology was used to collect and analyze the data. Dynamic Light Scattering (DLS) analysis were carried out to obtain hydrodynamic diameter and polydispersity index (PDI) using a Brookhaven 90Plus DLS instrument, by means of the Photo-Correlation (PCS) technique. All measurements were done in Milli Q water at the concentration of 0.5 mg mL^{-1} for micelles and alginate-coated particles at 25 °C. Surface potential (ζ-potential) of each nanocarrier was determined by measuring the electrophoretic mobility of a 0.01 mg mL^{-1} nanocarrier suspension in aqueous 1 mM KCl at 25 °C with a Plus Particle Size Analyzer (Brookhaven Instruments Corporation). UV-Vis spectra were measured with a Varian Cary 50 UV/Vis spectrophotometer.

2.3. Typical Polymerization Procedure

ZK_{30}-*b*-BnE_n block co-polypeptides were prepared by successive monomer addition without intermediate purification steps using the method described by Zou et al. with minor modifications.[22] Different molecular weights and block ratios were obtained by varying the monomer to initiator ratio. In a typical polymerization procedure, **ZK NCA** (232 mg, 0.76 mmol) was dried under high vacuum and transferred to a flame-dried Schlenk flask under N_2 atmosphere where dry DMF (0.5 mL) was also added. Next, a stock solution of butyl amine in dry DMF (0.5 % v/v) (0.5 mL) was added to the monomer solution under steady N_2 flow with the N_2 outlet connected to a drying agent ($MgSO_4$). The reaction was monitored by FTIR-ATR until NCA bands at 1850, 1789 and 920 cm^{-1} disappeared (about 3 h). Then a solution of

BnE NCA (200 mg, 0.76 mmol) in 1 mL of dry DMF was transferred via cannula to the Schlenk reaction flask. The reaction was further stirred under continuous N_2 flow for 4 hours and then cooled down to 0 °C until total consumption of the monomer by FTIR-ATR. Once no monomer was detected in solution, the reaction mixture was poured into 40 mL of cold diethyl ether and centrifuged (4000 rpm, 10 min) to afford a white residue which was resuspended in fresh diethyl ether under sonication and centrifuged again. Finally, the residue obtained was dried under vacuum to afford the block copolymer as a white solid, in quantitative yield.

2.4. Deprotection of the poly(benzyl glutamate) block[16]

Benzyl protected block copolymer **ZK_{30}-*b*-BnE_m** (84 mg, 0.006 mmol) was dissolved in THF (8 mL) thereupon KOH (46 mg, 0.26 mmol) was added dissolved in the minimum amount of deionized water (50 µL). The reaction mixture was stirred overnight. The crude was poured into 20-fold excess of cold diethyl ether and cooled down to -23 °C for 1 h to allow full precipitation of the reaction product. Then, the sample was centrifuged (15 min, 4500 rpm) and the residue was dried under vacuum to afford a white solid which was further purified by dialysis (3500 MWCO) against water over 3 days. Finally, the deprotected block copolymer **ZK_{30}-*b*-E_m** was isolated as a white solid after freeze-drying.

2.5. Preparation of empty micelles

5 mg of the amphiphilic block copolypeptide were dissolved in DMSO (1 mL) and then 4 mL of a phosphate buffer solution (10 mM, pH 7.4) were added dropwise using a syringe pump with an addition rate of 0.1 mL min^{-1}. The mixture was further stirred for 2 hours and then dialyzed against water by replacing it with fresh water 5 times after intervals of 1 hour each time. The final concentration was determined by weighing the dry residue of a freeze-dried aliquot.

2.6. Determination of the critical micellar concentration (CMC)

Nile red was placed in light protected vials (59.5 pmol in each vial) by allowing to evaporate an aliquot (11.9 μL) of a 5 μM stock solution in dichloromethane. Several micellar dispersions were prepared with concentrations of micelles ranging from 1.5 10^{-3} to 1.2 mg mL^{-1}. For each concentration, micellar dispersion (595 μL) was added to a nile red aliquot to reach a final nile red concentration of 0.1 μM in each vial. The mixtures were then stirred overnight. Next, nile red fluorescence spectrum was registered from 550 to 700 nm while exciting at 560 nm. The relative emission intensity at 606 nm was plotted versus polymer concentration and CMC was determined as the concentration corresponding to the intersection point of the lower horizontal and the slope tangent.

2.7. Bedaquiline (BQ) encapsulation

A typical experiment to determine the optimal amount of BQ for encapsulation started with the preparation of a DMSO solution (0.7 mL) containing BQ (0.8-3.0 mg) and **ZK_{30}-*b*-E_{30}** (5 mg). The solution was stirred for 2 hours to ensure homogeneous mixing and then it was added dropwise onto a phosphate buffer solution (pH = 7.4, 10 mM, 4.3 mL) using a syringe pump with an addition rate of 0.1 mL min^{-1}. Once the addition was completed, the mixture was further stirred for 2 hours and then submitted to dialysis against water by replacing it with fresh water 5 times after intervals of 1 hour each time.

The morphology of the micelles was analyzed by Bright Field Transmission Electron Microscopy (BF-TEM) in a FEI Tecnai T20 microscope operating at 200 kV. To prepare the samples 10 μL of micellar solution were dropped onto a parafilm patch and a TEM grid was soaked in the drop. After 30 s, the TEM grid was drawn and the excess solution was removed with a filter paper. The TEM grid was then immersed in a drop of phosphotungstic acid. After 30 s, the excess stain solution was again removed with a filter paper, and the grid was

thoroughly washed with water, by three consecutive immersions with fresh milliQ water each time. Finally, the sample was allowed to dry overnight.

Histogram representation of the diameter distribution was obtained using ImageJ software for diameter measurement of around 100 particles and OriginLab® (OriginLab, Northampton, MA, USA) software to obtain the frequency count statistical analysis.

2.8. Sodium alginate coated micelles

Sodium alginate was used to stabilize the micelles. Two different concentrations of sodium alginate were used for this purpose, 20 %wt (**BQ-M30-L**) and 56 %wt (**BQ-M30-H**), relative to the total amount of material. Stabilization with 20 %wt sodium alginate was achieved by adding 100 µL of sodium alginate aqueous solution (10 mg mL^{-1}) to a BQ-loaded micelles (**BQ-M30**) dispersion (0.8 mg mL^{-1}, 5 mL), and stirred 5 minutes to ensure a homogenous mixture. Then, the solution was poured into 3 mL of $CaCl_2$ (10 mM) and further stirred for 5 minutes. The coated particles were washed twice with milli-Q water using centrifugation (6 min, 4 °C, 16000 rpm). Finally, the content of BQ after coating with sodium alginate was evaluated by extracting it from the capsules in DMSO and by spectrophotometric analysis as previously described. The same procedure was used for the 56 %wt coating adding 500 µL of sodium alginate aqueous solution (10 mg mL^{-1}).

The morphology of alginate-coated micelles was analyzed by Scanning Electron Microscopy using a field emission SEM Inspect F50 (FEI). For the measurements of the nanoparticles histogram representation of the diameter distribution was obtained using ImageJ software for the diameter measurement of around 100 particles and OriginLab® (OriginLab, Northampton, MA, USA) software to obtain the frequency count statistical analysis.

2.9. Determination of bedaquiline (BQ) encapsulation efficiency (EE) and drug loading (DL)

EE is defined here as the percentage of encapsulated drug over the amount initially added in the preparation of nanocarriers. An aliquot of the particle dispersion was withdrawn and dissolved in DMSO, in which the carriers are not stable. The concentration of BQ was then inferred from its absorbance at 333 nm using a previously obtained calibration curve in DMSO. DL is defined as the drug concentration (%wt) in the loaded carrier (**BQ-M30**, **BQ-M30-L** or **BQ-M30-H**). An aliquot of the micellar solution was withdrawn and then freeze-dried. The dry residue was weighed, dissolved in DMSO, and the concentration of BQ was obtained from the absorbance at 333 nm after interpolation in a calibration curve.

2.10. Release studies

All release studies were performed at 37 °C. Human gastric and intestinal simulated media, free of enzymes or proteins, were prepared according to published procedures and used for drug release experiments.[23]

Briefly, intestinal simulated medium was prepared weighing the corresponding amount of salts and diluted in water to reach final concentrations of KCl (0.20 mg mL^{-1}), NaCl (8 mg mL^{-1}), NaH_2PO_4 (0.24 mg mL^{-1}) and Na_2HPO_4 (0.24 mg mL^{-1}). Finally, pH was adjusted to pH 7.

Enzyme-free gastric simulated media consisted in a 2 mg mL^{-1} NaCl solution adjusted to pH 1.6.

Also 0.9 % NaCl and water (storage medium) were used for the experiments. BQ-loaded nanocarriers were mixed with the appropriate medium at a concentration of 0.1 mg mL^{-1} BQ-loaded nanocarrier and incubated at 37 °C with gentle agitation. With these conditions, bedaquiline total concentration was below 20 μg mL^{-1} and sink conditions were likely met.[24] Samples were analyzed immediately after mixing (t = 0) and after 2, 4 and 24 h at 37 °C. For the analysis, the dispersion was passed through a nylon syringe filter (0.45 μm pores) to separate the carrier (retained in the filter) from the medium. Then, 500 μL of DMSO were

passed through the filter to extract BQ still encapsulated. The concentration of BQ in DMSO was measured by spectrophotometric analysis of the absorbance at 333 nm and the amount of released drug was obtained by difference with the drug loading. Despite their small size, polypeptidic micelles were retained in the nylon syringe filters, which are known to show high protein adhesion. For validation, dispersions of empty polypeptidic micelles (M30) at different concentrations (0.1-1.0 mg mL^{-1}) were filtered and the filtrate was analyzed using Bradford colorimetric assay and fluorescamine assay methods. In all cases, if present, polypeptide was in a concentration below our limit of detection (< 0.05 mg mL^{-1}).

Release experiments in human plasma were performed with a more diluted concentration (0.05 mg mL^{-1} BQ-loaded nanocarrier) to prevent precipitation. The samples were incubated at 37 °C with gentle agitation. Time points were registered in the same manner as in other media but employing methanol as organic solvent. After filtration, the organic solution was centrifuged 10 min 14000 rpm and the supernatant was submitted to UV-Vis spectroscopy for bedaquiline quantification.

All release experiments were done in triplicate.

2.11. Bacterial strains and culture conditions

The *Mycobacterium tuberculosis* reference strain H37Rv,[25] available at Mycobacterial Genetics Group Laboratory (University of Zaragoza), was routinely grown in Middlebrook 7H9 medium supplemented with 10% ADC and 0.05% Tween® 80 at 37ºC within a BLS 3 laboratory.

2.12. In vitro antimicrobial activity assay

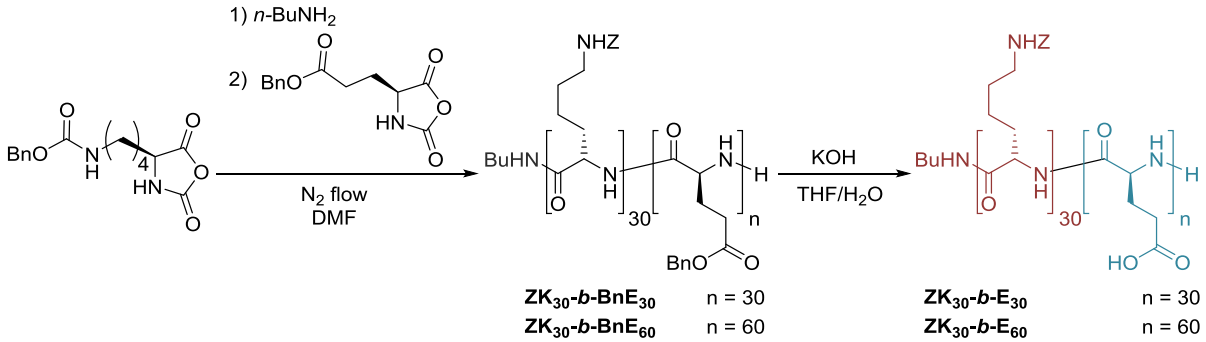
The Minimum Inhibitory Concentration (MIC) was determined using the method of Resazurin Microtiter Assay Plate (REMA).[26] In 96-well plates, a range of concentrations of the free

bedaquiline or bedaquiline-loaded nanoparticles were added in 100 µL of Middlebrook 7H9 medium supplemented with 10 % ADC and 0.5 % glycerol, in a series of two-fold dilutions spanning from 1 to 0.008 µg mL^{-1}. Empty nanoparticles were assayed as a control. Bacteria were inoculated by adding 100 µl of a suspension of 10^5 CFU mL^{-1}, as estimated by optical density and prepared from a culture in exponential growth phase. After 6 days of incubation at 37 °C, 30 µL of resazurin (0.01 % w/v) was added to each well, further incubated for 48 h at 37 °C, and assessed for color change. A change of the suspension from blue to pink indicates the reduction of resazurin to resorufin corresponding to bacterial growth. The MIC was defined as the lowest drug concentration that prevented the bacterial growth as indicated by resazurin color change.

3. Results and Discussion

3.1. Synthesis of the amphiphilic polymers

Polypeptides can be synthesized through ring opening polymerization (ROP) with the *N*-carboxyanhydride (NCA) of the corresponding aminoacids. The synthesis of the NCAs requires that the functional groups in the side chain of the aminoacids are protected. Therefore, we followed reported procedures for the synthesis of γ-benzyl-L-glutamic acid NCA (**BnE-NCA**) and ϵ-N-benzyloxycarbonyl-L-lysine NCA (**ZK-NCA**) as monomers for the ROP, as these protecting groups would allow for the selective deprotection of the polyglutamate block after ROP to achieve the targeted amphiphilic polymers (**Scheme 1**). Provided the sensitivity of polymerization reactions to the purity of the monomers we paid special attention to this parameter and recrystallized 5 times each monomer prior to ROP reactions.



Scheme 1. Synthesis of **ZK_{30}-*b*-BnE_n** polypeptides.

The synthesis of block copolymers with controlled degree of polymerization of each block requires working in living polymerization conditions, that is, chain termination and chain transfer reactions do not take place and the rate of initiation reaction is much faster than the rate of propagation. Under living polymerization conditions, block copolymers can be synthesized through the consecutive addition of monomers: first, one monomer is added to the initiator for polymerization and when the reaction mixture is depleted of monomer the polymer chains can be used as macroinitiators for the polymerization of the second monomer.

ROP of NCAs can be run under living polymerization conditions using a variety of initiators including transition metal complexes, thiols, silazanes, amines and ammonium salts with tetrafluroborate or chloride anions. Most often, the ROP needs to be run under high vacuum and/or low temperature to optimize the conditions needed for a living polymerization for block copolymers or high molecular weight polymers.[27] Based on the reports we tested the ROP of **BnE NCA** initiated by butyl amine under vacuum conditions at room temperature and evaluated the polymerization based on gel permeation chromatography, ^{1}H NMR and MALDI ToF mass spectrometry of the obtained polymers. MALDI spectra showed peaks due to chains grown by the desired normal amine mechanism (NAM), which is initiated by the amine, and peaks due to competitive activated monomer mechanism (AMM), in which the amine deprotonates the –NH– of one NCA monomer which then acts as initiator (Figure S9).

Lowering the temperature of the reaction to 0 ºC, decreased the relative intensity of the AMM peaks but the increased reaction times led to the appearance of a peak series due to formylation, which is a termination reaction due to the solvent. In view of the results, we tested the same reaction conducted under a continuous flow of nitrogen gas as reported by Zou *et al*.[22] The flow of nitrogen gas allows the removal of carbon dioxide released during ROP which results in an increase of the overall reaction rate. With these conditions formylation of the growing polymeric chains was prevented and peaks due to AMM were minimized (Figure S9).

We then applied those conditions to the sequential block copolymerization of **ZK NCA** and **BnE NCA** to obtain the targeted block copolymers as shown in Scheme 1. We used ^{1}H NMR, and GPC as main tools for the characterization of the **ZK_{30}-*b*-BnE_n** polypeptides in terms of degree of polymerization, ratio between blocks and polydispersity (**Table 1**).

Table 1. Characterization data of the prepared block copolymers.

Polymer[a)]	**$M_{n,expected}$ [$\times10^{-4}$ Da]**	**$M_{n,NMR}$**[b)] **[$\times10^{-4}$ Da]**	**$Ratio_{NMR}$**[b)]	**$M_{n,GPC}$**[c)] **[$\times10^{-4}$ Da]**	**PDI**[c)]	**Yield [%]**
ZK_{30}-*b*-BnE_{30}	1.45	1.76	1:37:36	1.97	1.17	> 99
ZK_{30}-*b*-BnE_{60}	2.11	2.06	1:30:50	2.62	1.16	97

[a)] The polymerization reactions were carried out with a flow of N_2 in DMF at 25 °C with [n-Bu-NH_2]$_o$ = 0.025 M [ZK NCA]$_o$ = 0.76 M. [b)] ^{1}H NMR spectroscopy. [c)] GPC-MALS, 0.1 M LiBr in DMAc at 60 °C.

As expected, the ratio between both blocks was essentially preserved as shown by ^{1}H NMR spectroscopy (**Figure 1**), only slightly shifted compared with the theoretical one based on the feed. GPC analyses yielded monomodal molecular weight distributions with a remarkable reproducibility among batches as verified by GPC trace analysis (Figure 1).[28]

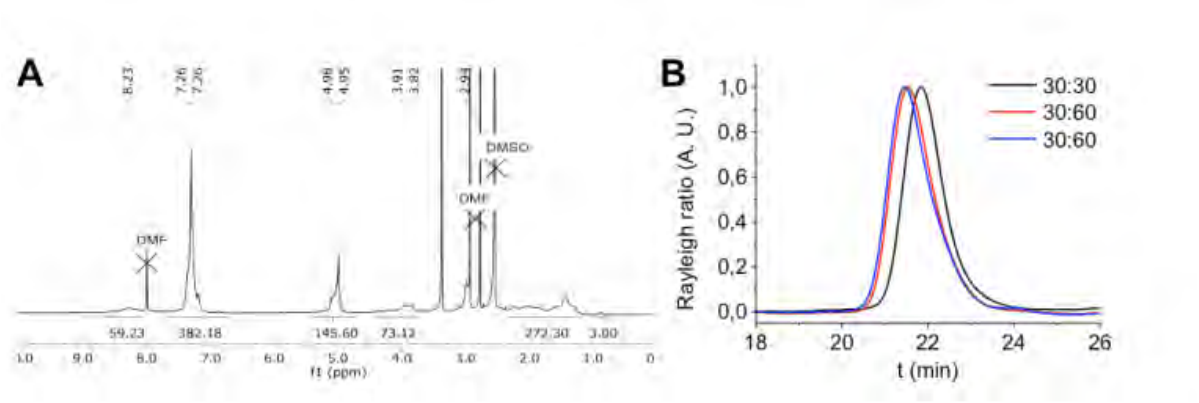


Figure 1. A) ^{1}H NMR spectrum of **ZK$_{30}$-*b*-BnE$_{30}$** in DMSO-d^6 and integrals used for the calculation of $M_{n,NMR}$ and the ratio between blocks from Table 1. B) GPC profiles (Rayleigh ratio signals) of copolymerization of **BnE NCA** and **ZK NCA** with different DP: black (30:30) $M_{n,GPC} = 1.97 \times 10^4$ Da, PDI = 1.17; red (30:60) $M_{n,GPC} = 2.62 \times 10^4$ Da, PDI = 1.16; blue (30:60) $M_{n,GPC} = 2.64 \times 10^4$ Da, PDI = 1.14.

The amphiphilic structures were obtained by deprotection of the benzyl ester moieties by basic hydrolysis using KOH in THF[16] with subsequent purification by dialysis. This purification step enabled us to perfectly remove the reagents and the organic solvent and was suitable for both polymer lengths. The amphiphilic structures were characterized by ^{1}H NMR, yet just the hydrophilic signals were detected in deuterium oxide, due to the lack of solubility of the hydrophobic part (Figure S8). Other basic conditions for the deprotection step afforded similar results based on yield and purity.[29]

3.2. Self-assembly behavior of the amphiphilic polymers

ZK$_{30}$-*b*-E$_{60}$ and **ZK$_{30}$-*b*-E$_{30}$** are amphiphilic block copolymers designed to self-assemble in aqueous solution so that the contact of the hydrophobic block, ZK$_{30}$, with the aqueous environment is minimized. The morphology of the self-assembled structures primarily depends on the nature of the blocks, on the total length and on the internal structure of the block copolymer, meaning by this the number and relative lengths of the blocks. Taking into

account those parameters and previous reports, we expected to obtain micelles from the synthesized amphiphilic copolymers.[16]

Based on the solubility of **ZK$_{30}$-*b*-E$_{60}$** and **ZK$_{30}$-*b*-E$_{30}$** we selected the nanoprecipitation method as the most suitable for the formation of self-assembled structures (Figure S10). It consists of dissolving the block copolymer in an organic solvent in which both blocks are soluble thereupon adding an aqueous solution in which one of the blocks is not soluble. During this process, water diffuses into the organic phase, leading to the aggregation of the hydrophobic chains and the self-assembly of the amphiphilic block copolymers. Finally, the organic solvent can be removed by evaporation or dialysis.

In our studies on the self-assembly of **ZK$_{30}$-*b*-E$_{60}$** and **ZK$_{30}$-*b*-E$_{30}$** we used DMSO as organic solvent as the polymers were more readily solubilized in it than in THF and insoluble in other organic solvents. A phosphate buffer was selected as non-solvent for the hydrophobic block. Once the addition of the aqueous solution was completed, DMSO was removed by dialysis (Figure S10).

This method allowed the formation of micelles in a reproducible manner with both polymers. Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter of the obtained micelles (**Table 2**), which were found to be larger for **ZK$_{30}$-*b*-E$_{60}$** than for **ZK$_{30}$-*b*-E$_{30}$**. The hydrodynamic diameter between 70 and 90 nm of the micelles **M30** obtained from **ZK$_{30}$-*b*-E$_{30}$** was consistent with other reported micelles from polypeptides with similar degree of polymerization.[12] However the **M60** produced from **ZK$_{30}$-*b*-E$_{60}$** were larger than expected from the difference in the polymer length.

ζ-potential value was found negative for both preparations, as expected from the hydrophilic block consisting of a polycarboxylic acid under measuring conditions, well above its pK_a.

Table 2. Characterization results for the carriers formed by **ZK_{30}-*b*-E_m**.

Micelle	Polymer	D_H[a] [nm]	PDI[b]	CMC[c] [mg mL^{-1}]	ζ-potential [mV]
M30	**ZK_{30}-*b*-E_{30}**	80.0 ± 8.0	0.12 ± 0.05	0.03	-36.8 ± 2.2
M60	**ZK_{30}-*b*-E_{60}**	194.3 ± 7.1	0.13 ± 0.01	0.18	-50.6 ± 2.6

[a] Hydrodynamic diameter as determined by dynamic light scattering (DLS). [b] Polydispersity index. [c] Critical micellar concentration.

Finally, the stability of micelles against dilution was assessed by measuring the critical micellar concentration through the encapsulation of nile red dye at different dilutions. The lower value obtained for **ZK_{30}-*b*-E_{30}** indicated that its aggregates would be stable in a broader concentration range than **M60** which is key for their application in drug delivery (Table 2 and Figure S11).

Provided the higher stability toward dilution of **M30** aggregates compared to **M60,** we decided to focus our encapsulation studies on **M30**.

3.3. Encapsulation of bedaquiline (BQ)

BQ is an effective antimicrobial drug which is indicated for the treatment of MDR–TB.[19] Unfortunately, treatment with BQ is expensive[30] and has also been associated to important side effects, which makes advisable the use of alternative drug delivery strategies, so that bioavailability could be increased and dosing reduced.[17,20] Taking advantage of the hydrophobic nature of BQ, its encapsulation in a drug carrier is the strategy of choice as any chemical modification of the drug is avoided and its solubility and bioavailability might be enhanced.

Due to the scarce solubility of BQ in water, for its encapsulation we first applied our method of preparation of **M30** (Figure S10), in which BQ could be introduced after dissolving it in

DMSO with the polymer. However, adding the buffer dropwise into a BQ and polymer solution in DMSO led to precipitation of BQ. In consequence, we decided to modify the method by adding the solution in DMSO into the phosphate buffer (Figure S12). Different initial BQ/**ZK$_{30}$-*b*-E$_{30}$** mass ratios (*R*) between 0.15 and 0.60 (Table S1) were tested to obtain BQ-loaded micelles. The encapsulation efficiency increased with *R* until it reached a maximum of 60 % for *R* = 0.30. However, at that ratio BQ partially precipitated, making difficult the purification of the loaded micelles. For higher values of *R* the encapsulation efficiency (EE) decreased as precipitation increased and the loaded micelles became less stable. Therefore, a ratio *R* = 0.24 was selected as optimal considering the stability of the resulting loaded micelles, **BQ-M30**, as well as the obtained encapsulation efficiency (58.3 ± 10.0 %) and drug loading (19.2 ± 3.0 %) (**Table 3**). With that ratio between the BQ and the polymer in the feed (*R* = 0.24) we prepared all the batches used in this work in scales in the range 50-100 mg of starting polymer.

Table 3. Characterization results for bedaquiline loaded carriers.

Carrier[a)]	**D_H**[b)] **[nm]**	**PDI**	**Diameter [nm]**	**ζ-potential [mV]**	**DL**[c)] **[%]**	**EE [%]**
BQ-M30	47.5 ± 3.1	0.125 ± 0.04	18.2 ± 3.5[d]	-23.6 ± 1.8	19.2 ± 3.0	58.3 ± 10.0
BQ-M30-L	435.8 ± 17.0	0.245 ± 0.03	228.6 ± 30.4[e]	-25.2 ± 2.2	17.3 ± 0.6	NA
BQ-M30-H	406.0 ± 28.3	0.238 ± 0.01	243.6 ± 34.5[e]	-28.0 ± 1.3	16.5 ± 2.1	NA

[a)] BQ-M30: bedaquiline-loaded polypeptidic micelles. BQ-M30-L: bedaquiline-loaded polypeptidic micelles coated with alginate (20 %wt). BQ-M30-H: bedaquiline-loaded polypeptidic micelles coated with alginate (56 %wt). [b)] The hydrodynamic diameter (DH) was obtained from DLS measurements. [c)] Drug loading is the concentration (%wt) of bedaquiline in the loaded carrier (BQ-M30, BQ-M30-L or BQ-M30-H). [d)] The diameter of the particles was measured in TEM images. [e)] The diameter of the particles was measured in SEM images.

In literature, bedaquiline encapsulation and vehiculization has been reported only very recently with nanoemulsion-based nanocarriers [31] and polymeric nanoparticles.[24] Our work

is the first successful attempt to encapsulate bedaquiline in polymeric micelles. DL and EE percentages obtained in our work are quite comparable with those obtained with chitosan coated nanoparticles (i.e. 28 % DL and 70 % EE) and herein reported DL of bedaquiline in polymeric micelles is much higher than the one obtained with lipid nanoparticles (i.e. 2.8 %).[31]

3.4. Stabilization of the micelles with alginate

Polymeric micelles have been extensively used for drug delivery purposes and now there is increasing evidence that they need to be stabilized to prevent premature disintegration and to improve their performance.[10] Covalent crosslinking of the core of the micelle is the approach followed most often for such stabilization, which involves a higher synthetic complexity.

Herein we propose a supramolecular coating with sodium alginate, a naturally occurring polysaccharide, which is already approved by FDA along with calcium alginate and alginic acid. Sodium alginate is known to form hydrogels upon addition of $CaCl_2$. Our hypothesis was that the interaction between the polysaccharide and the polyglutamic segments through coordination of calcium ions could stabilize the micelles surface. Thus, loaded particles were coated through ionotropic gelation process, by the consecutive treatment of the micelles with sodium alginate and $CaCl_2$ to afford the desired nanogel coating (**Figure 2**).

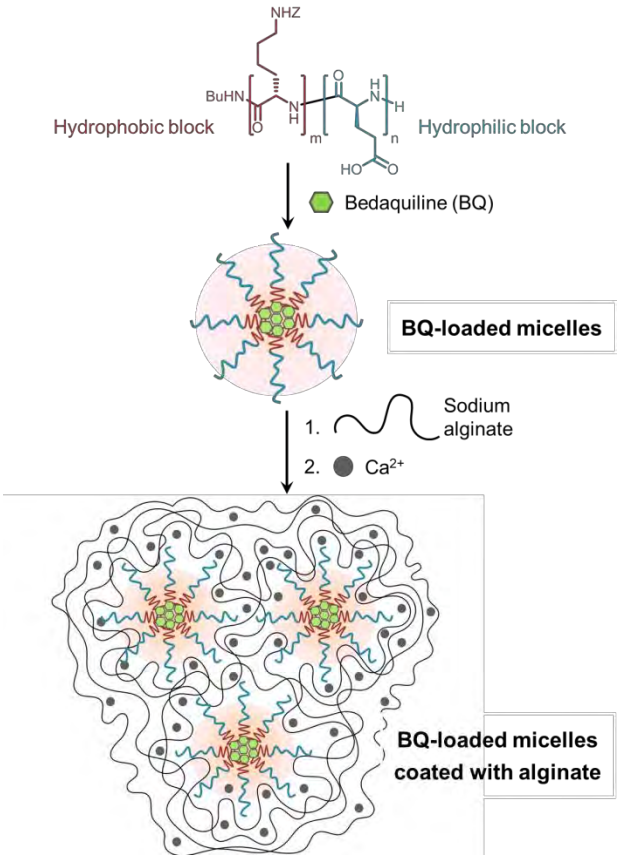


Figure 2. Block copolypeptide for the encapsulation of bedaquiline in the hydrophobic core of the micelle. Following drug encapsulation, the micelles are coated with alginate for stabilization.

Different sodium alginate quantities were tested to stabilize the micellar formulation, a lower amount of 20 %wt (**BQ-M30-L**) and a higher amount of 56 %wt (**BQ-M30-H**), so that its effect in the structure of the assembly and on the final properties for release could be assessed. Both alginate amounts provided stable colloidal solutions. Interestingly, the pellets formed by **BQ-M30-L** and **BQ-M30-H** upon centrifugation could be readily redispersed, which facilitated the purification of the particles. Non-stabilized micelles **M30** and **BQ-M30**, on the contrary, did not afford a pellet after centrifugation and had forced us to use dialysis for purification.

3.5. Nanocarriers characterization

Bedaquiline-loaded micelles before and after stabilization with alginate were submitted to physicochemical characterization to determine their physical characteristics (mainly size and surface potential).

The comparison between the hydrodynamic diameter distribution of BQ-loaded micelles before and after their stabilization with sodium alginate is reported in **Figure 3** and Table 3.

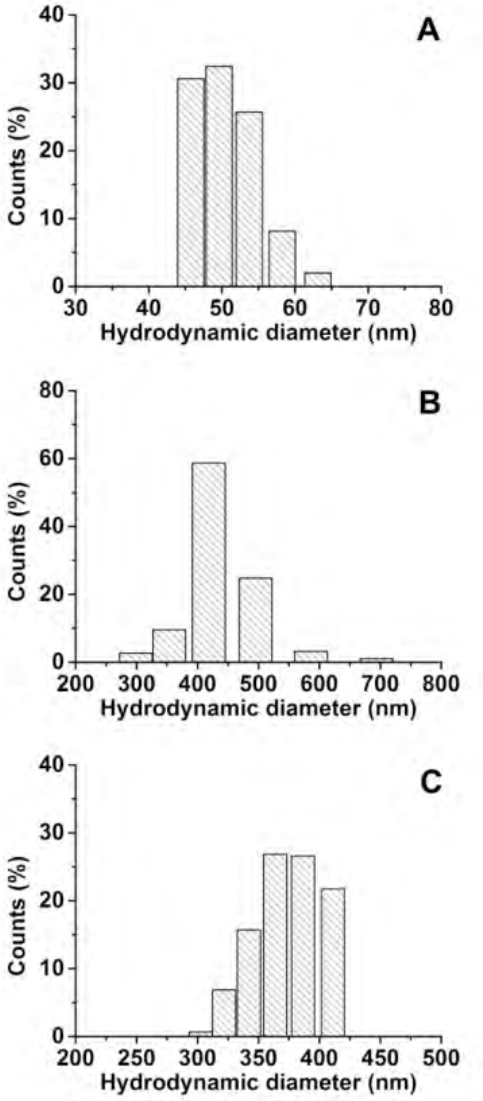


Figure 3. DLS data for the BQ-loaded nanocarriers: **BQ-M30** (A), **BQ-M30-L** (B) and **BQ-M30-H** (C).

The structural characterization of the loaded micelles, **BQ-M30**, indicated that BQ could act as a scaffold that triggers the formation of the micelle around it.[16] Indeed, loaded micelles were around 50 nm diameter by DLS, smaller than the non-loaded ones which were about 80 nm. This shift can be attributed to the hydrophobic forces that improved the stacking of ZK chains around the aromatic drug (Figure 3A).[8,16]

The particles observed after stabilization with alginate were much larger according to DLS measurements, with a hydrodynamic diameter around 300-500 nm for both alginate concentrations. ζ -potential of the capsules remained negative as expected from the presence of negatively charged carboxylate groups in the alginate coating.

BQ-M30 could be imaged using BF-TEM (**Figure 4**A and B), which revealed a particle size around 20 nm (Figure 4C), consistent with the DLS measurements (Table 3). **BQ-M30-H** and **BQ-M30-L** could be studied by SEM (Figure 4D and E and Figure S13). In both cases also the frequency analysis of the size distribution is reported (Figure 4F and Figure S13C).

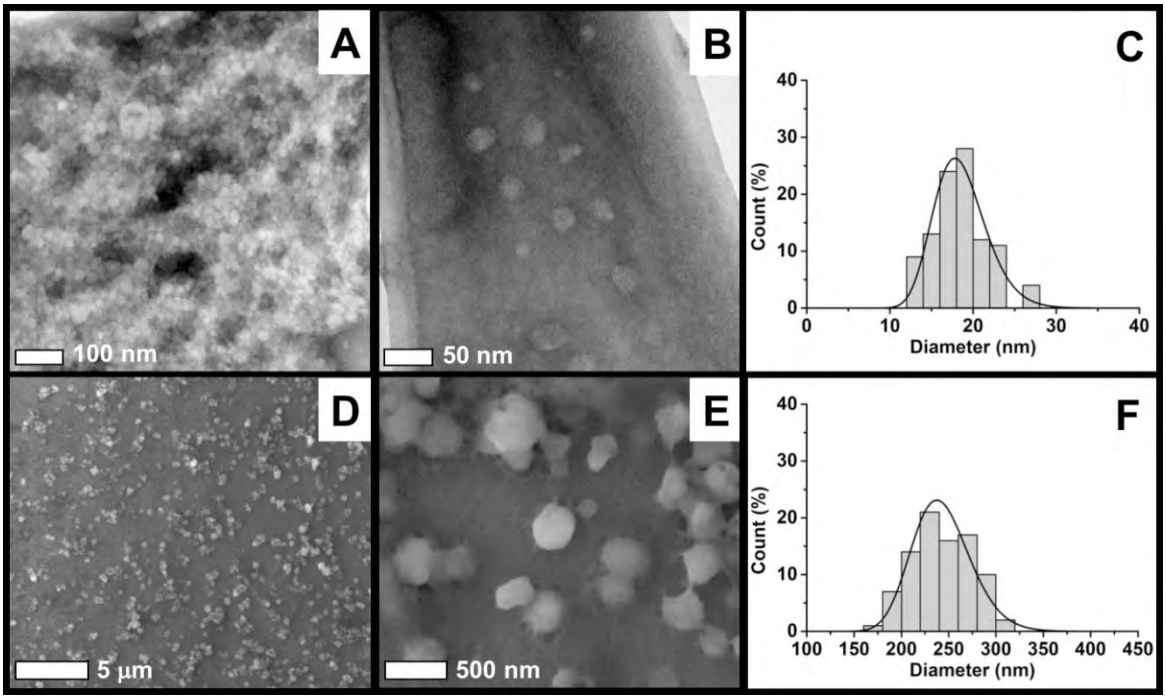


Figure 4. BF-TEM micrographs of bedaquiline loaded micelles **BQ-M30** (A and B) and histogram of the preparation (C). SEM micrographs of bedaquiline-loaded alginate coated

particles using 56 % wt alginate **BQ-M30-H** (D and E). Histogram obtained from SEM images of **BQ-M30-H** (F).

BQ-M30-H and **BQ-M30-L** appeared as spheres with diameters between 200 and 300 nm significantly larger than the starting BQ-loaded micelles **BQ-M30**. It is remarkable that the size of the carriers seemed to be independent of the amount of alginate we added. The higher amount of alginate could contribute to a denser rather than thicker coating or could just be eliminated in the purification. The results were consistent with the DLS estimations of the hydrodynamic diameter if we take into account that the polymeric particles are likely to contract in the measuring conditions. The size and the morphology of the isolated particles were consistent among different sample preparations.

3.6. Drug release in controlled media

The potential of the nanocarriers for BQ administration was assessed by measuring the profile of the release of bedaquiline from nanocarriers at 37 °C under conditions relevant to intravenous and oral administration.

To explore the behavior of our nanocarriers for intravenous administration, we first evaluated the effect of dilution for each carrier in water and in a NaCl solution in water (0.9 %wt) respectively as storage and vehicle media for that administration route (**Figure 5A**).

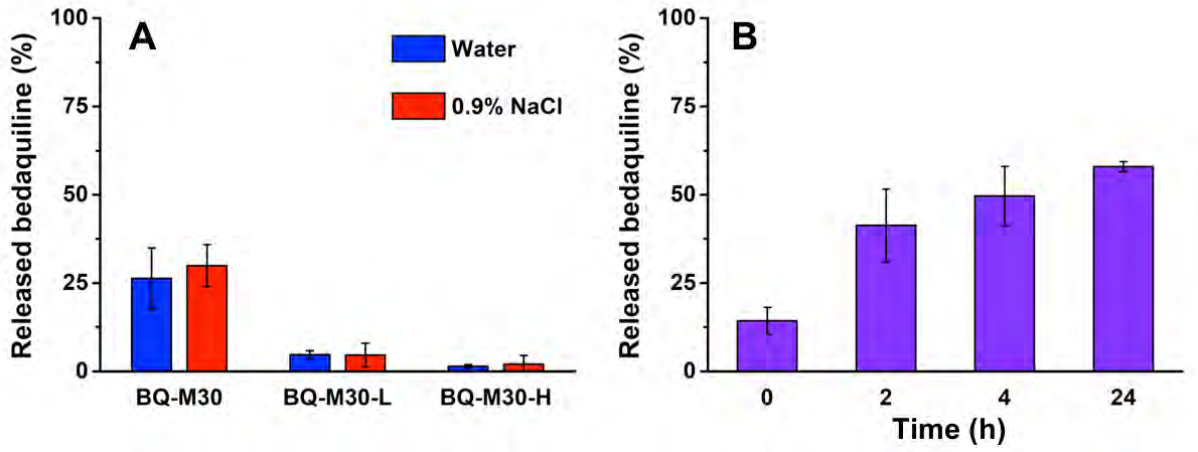


Figure 5. A) Released bedaquiline immediately after 1:10 dilution of the loaded carriers in water and in NaCl (0.9 %wt). B) Release profile in human plasma at 37 ºC of the bedaquiline encapsulated in micelles coated with alginate at the highest content, **BQ-M30-H.**

In intravenous administration the formulation would be injected short or immediately after dilution in the administration medium. Thus, we measured the bedaquiline released from the nanocarriers immediately after a ten-fold dilution in each medium, from 1.0-3.0 mg mL^{-1} to 0.1-0.3 mg mL^{-1} in polymer concentration, depending on the batch. That concentration is close to the measured CMC of the polymer, 0.03 mg mL^{-1} (Table 2). In these conditions **BQ-M30** showed a burst release of 26 % and 30 % respectively in water and NaCl solution, which was consequence of the instability of the micelles upon dilution, even though the final concentration was still above the CMC (Table 2). As expected, coating the micelles with alginate provided the desired effect and notably reduced the amount of released BQ below 5 % for both **BQ-M30-H** and **BQ-M30-L** (Figure 5A). When comparing the alginate coated micelles at the two percentages of polysaccharide, we expected lower values for **BQ-M30-H** compared to **BQ-M30-L** if we consider the denser alginate shell that could be hypothesized for **BQ-M30-H**. Indeed, **BQ-M30-H** provided a lower BQ release upon dilution, below 2 % in either media, although the difference with **BQ-M30-L** fell within the error limits.

Blood and plasma are very complex media in which the large amount of proteins and biomolecules present could adsorb to any injected nanoparticle. Interactions are mostly

hydrophobic or ionic and will probably accelerate the release of an encapsulated drug or, in the case of micelles, seriously compromise the integrity of the carrier.[32] From the presented carriers, **BQ-M30-H** was expected to be the most stable in protein-containing media because the larger amount of alginate would assure that no bare micelles are present in the sample. Thus, we performed a series of experiments to obtain the release profile in plasma of the BQ encapsulated in **BQ-M30-H** (Figure 5B). The amount of BQ initially released was remarkably low (14.3 % ± 3.8) taking into account the complexity of the medium. Following that, we observed a sustained release of BQ reaching 58.0 % ± 1.4 after 24 h. This result indicates **BQ-M30-H** can be a very good candidate for intravenous administration to extend the circulation time of BQ and increase its probability to reach infected cells.

Sodium alginate is known also for its mucoadhesive properties that make it a very good candidate for the development of drug delivery systems for gastrointestinal applications.[15] To evaluate the future possibility to use these nanocarriers for oral administration, we selected gastric simulated (GS) medium and intestinal simulated (IS) medium (both of them enzyme-free) to study the stability of the drug loading and the corresponding release profiles of the encapsulated BQ in these media (**Figure 6**).

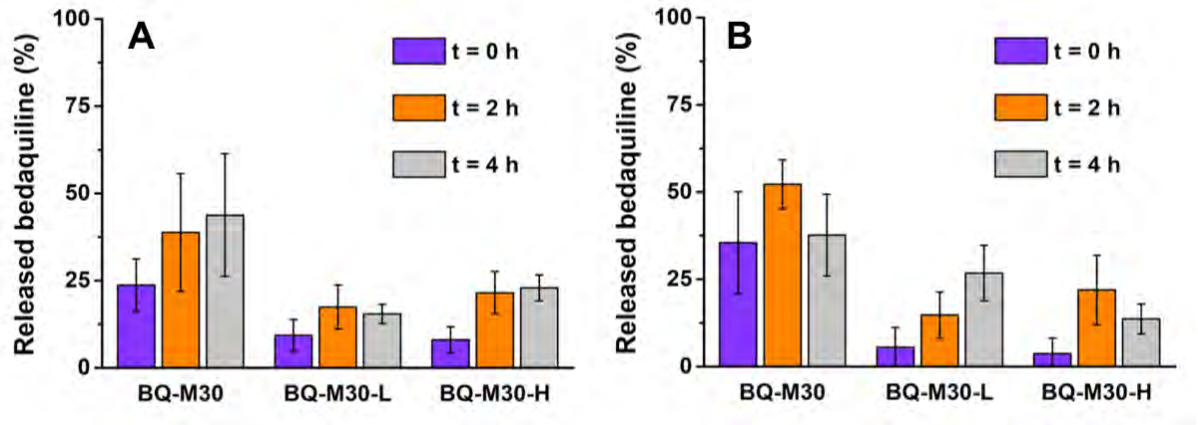


Figure 6. Release profile at 37 °C of bedaquiline encapsulated in micelles before and after alginate coating in A) human gastric simulated medium and B) intestinal simulated medium.

We planned the experiments on a 4 h long basis taking into account that upon oral administration the carrier is expected to leave the stomach and enter in contact with intestinal environment (small bowel) during this period of time.[33] It is desired that in either medium the amount of released BQ is minimal so that the loaded carriers can be retained integer in the intestine for a sustained release of the encapsulated drug and its absorption.

The main feature of the GS medium is the strongly acidic pH. **BQ-M30** exhibited a strong initial burst release similar in magnitude to that previously observed in water. **BQ-M30** partially aggregated for the next data points (Figure 6) and we had a high dispersion in our results. The pH of GS medium was well below the pK_a of the carboxylic acids of the outer polyglutamate block, which led to aggregation of the micelles after their outer part became hydrophobic due to protonation of the carboxylic groups. Alginate coated carriers exhibited better stability as shown by the much lower amount of released BQ and also by the lower standard deviation of the repetitions. Even though the carboxylate groups of alginate probably became protonated, the carriers remained soluble in the medium due to the polar polysaccharide chains.

Intestinal simulated medium was mainly characterized by a neutral pH and the presence of phosphate ions. The stability of the alginate coated carriers in this medium could have been compromised due to the phosphate ions in the medium sequestering the Ca^{2+} cations that keep the carrier pieces together to form insoluble calcium phosphate. However, to our satisfaction **BQ-M30-H** and **BQ-M30-L** showed a good performance with less than 25 % of released BQ in this medium after 4 h, a promising behavior for possible future study of oral administration in animal models.

3.7. In vitro antimicrobial activity

We used the well-established REMA assay,[26] to determine the in vitro antimicrobial activity of the encapsulated BQ against *M. tuberculosis* H37Rv and then we compared the minimum inhibitory concentration (MIC) obtained to that of free bedaquiline (**Table 4**). The MIC of empty carriers was studied as well, to ensure that within the range of concentrations used in the assay, the antibacterial effect was only due to the antimicrobial drug and not to the nanocarrier itself. The MIC for the empty carrier was found to be above 250 μg mL^{-1} in all cases. As shown in Table 4, not only bedaquiline was still very active against *M. tuberculosis* after encapsulation in any of the carriers but the activity seems to be increased 2 times. These results were reproduced using different batches of capsules.

Table 4. MIC values for free and encapsulated bedaquiline. All experiments were done in triplicate.

Sample	**MIC of bedaquiline free or encapsulated [μg mL^{-1}]**	**Corresponding concentration of nanocarrier [μg mL^{-1}]**
BQ (free drug)	0.03 - 0.06	-
BQ-M30	0.015 – 0.03	0.08 – 0.17
BQ-M30-L	0.015 – 0.03	0.09 – 0.18
BQ-M30-H	0.015 – 0.03	0.09 – 0.19

At the concentrations used in the assay, the empty carriers do not show any antibacterial activity. However, it seems to enhance the activity of bedaquiline. This could be due to an increase in the solubility of bedaquiline and consequent higher bioavailability. But another hypothesis even more interesting is that the mucoadhesive nature of the polymer and the alginate could provide the particles with the ability to get stuck to bacterial surface, hence delivering more efficiently BQ closer to its target.

Provided that other carriers enhancing the solubility of BQ did not lead to an increased activity of the encapsulated BQ,[31] we are currently planning future experiments to elucidate the origin of this increased activity.

4. Conclusions

In this work we have shown that a coating with sodium alginate is a good strategy to stabilize polypeptidic micelles and improve their properties regarding release profiles. This strategy does not affect the core of the carrier or the loaded drug and might be compatible with more complex polymer structures for controlled drug detachment. The synthesis of the micelles has been tested to a 100 mg scale without any particular adaptation.

Taking altogether, our results on the release of encapsulated BQ from the alginate-coated polypeptidic micelles show alginate coating is a promising strategy to overcome the limitations of polymeric micelles in terms of stability. Coating the micelles with sodium alginate preserved the properties of the micelles for encapsulation while notably reducing the initial burst release in all tested media. Chosen media were relevant for oral and intravenous administration. In particular, **BQ-M30-H** showed a 15-fold decrease of released BQ compared to micelles **BQ-M30** and, furthermore, a sustained release in a complex medium such as human plasma. Release of BQ from alginate-coated carriers was also reduced in gastric and intestinal simulated media to values well below 25 %. This means that, thanks to sodium alginate mucoadhesive properties, BQ would be probably slowly released from the nanocarrier and well absorbed in the intestine. In this way, the treatment can benefit from the advantages of the use of a nanotechnology based drug delivery system.

Remarkably the formulations of encapsulated bedaquiline showed a lower MIC compared to free BQ. This interesting result open the way for further investigations on the role of this specific nanocarrier on drug delivery and antimicrobial action.

To conclude, our approach allows the utilization of easily accessible polypeptides for encapsulation and scaling of the self-assembly process. In the near future, we plan to elucidate the causes for the improved activity of the released BQ in the presence of the polypeptide and

we also plan to adapt the synthetic process to other polysaccharides suitable for other routes of administration.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

The following files are available free of charge: brief description (PDF): The methods of synthesis and the characterization of the monomers and the polymers by NMR measurements are provided in the supporting information. The methods for the preparation of the micelles and bedaquiline micelles are also described in the supporting information along with data of the optimization of the amount of bedaquiline for encapsulation and SEM images for **BQ-M30-L**.

Appendix/Nomenclature/Abbreviations

Acknowledgements: Authors would like to acknowledge Ministerio de la Economía y Competitividad del Gobierno de España for the public funding through Proyectos I+D+i – Programa Estatal de Fomento de la Investigación Científica y Técnica de Excelencia, Subprograma Estatal de Generación de Conocimiento (project CTQ2015-66869-P) and through a Ramón y Cajal fellowship (RYC-2013-12570). Ministerio de Educación Cultura y Deporte is acknowledged for a FPU predoctoral fellowship (HSC). We are grateful for the public funding from Fondo Social de la DGA (grupos DGA). We also want to acknowledge Dr. Rodrigo Fernández-Pacheco and Dr. Scott G. Mitchell for their technical support in TEM and SEM experiments respectively and the Advanced Microscopy Laboratory (LMA) of Universidad de Zaragoza. Dr. Eugenio Vispe and the ISQCH Service for Chromatography and spectroscopy are acknowledged for the GPC measurements. We are grateful to Íñigo Echániz and M. Carmen Gámez for their technical and administrative support.

Received: Month XX, XXXX; Revised: Month XX, XXXX; Published online:

((For PPP, use “Accepted: Month XX, XXXX” instead of “Published online”)); DOI: 10.1002/marc.((insert number)) ((or ppap., mabi., macp., mame., mren., mats.))

Keywords: polypeptides, drug delivery, bedaquiline, ring opening polymerization, alginate

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Bedaquiline shows higher activity against *Micobacterium Tuberculosis* after encapsulation in polypeptidic micelles. The micelles are obtained from an amphiphilic block polypeptide and coated with an alginate gel for stabilization. It is shown that this is a good strategy to stabilize polypeptidic micelles and improve their properties regarding release profiles.

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Polypeptidic Micelles Stabilized with Sodium Alginate enhance the Activity of Encapsulated Bedaquiline