Encapsulation of low molecular weight heparin (bemiparin) into polymeric nanoparticles obtained from cationic block copolymers: properties and cell activity†

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Bemiparin (fractionated low molecular weight heparin)-loaded nanoparticles were prepared by two consecutive w/o emulsions and an inversion to an o/w emulsion for various polymer systems as controlled release formulations. New synthetic block copolymers, poly(methyl methacrylate-b-trimethyl aminoethyl methacrylate) (PMMA-b-PMAETMA), with controlled microstructure and molecular weight, were prepared by RAFT (Reversible Addition-Fragmentation chain-Transfer) polymerization creating a set of polymers with different amounts of cationic charges. For comparison, a non-biodegradable positively charged polymer, Eudragit® RS PO, and a biodegradable polymer poly(lactic-co-glycolic acid), PLGA, were used. The microstructural arrangement of MMA and MAETMA sequences in PMMA-b-PMAETMA results in self-assembled core-shell nanoparticles in water with a positively charged surface, which interacts with bemiparin. The formulations were evaluated in terms of particle size, zeta potential and morphology by scanning electron microscopy (SEM). The entrapment of bemiparin molecules was confirmed by a negatively increased zeta potential value and the detection of a sulfur signal by energy dispersive X-ray spectroscopy (EDAX). High encapsulation efficiency was reached with all the polymeric matrices, ranging from 89 to 98%. Systems prepared with synthetic block copolymers PMMA-b-PMAETMA and PLGA showed higher in vitro bemiparin release than Eudragit® RS PO systems. For each formulation, bemiparin released from nanoparticles preserved its biological activity as shown by the BaF32 cell proliferation assay in the presence of fibroblast growth factor (FGF2).

Introduction

Polymeric nanoscale drug delivery systems are attractive for the therapeutic delivery of drugs to target tissues. Heparin (HP) is a highly sulphated and negatively charged glycosaminoglycan formed by β or β (1→4) linked uronic acid (90% β-L-iduronic acid, 10% β-D-glucuronic acid) and β-D-glucosamine residues that present several biological functions including anticoagulation, and an interesting regulation role of growth factor activity, including those of fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs).† Its pharmacological activity has been demonstrated in many diseases such as asthma, allergic rhinitis, inflammatory bowel disease, vascular disorders,‡§ and has recently been shown to provide anti-angiogenic properties.δ

Unfractionated heparin (UFH) is polydisperse while low molecular weight heparins (LMWH), such as bemiparin (HIBOR®), are obtained by the chemical β-elimination depolymerization of UFH† with a molecular weight of less than 8 kDa. LMWHs contain a precisely defined disaccharide composition with a higher efficacy safety ratio due to the better defined anti-Xa/anti-IIa ratio that reduces heparin side effects, including problems of coagulation and bleeding. Heparin is administered systemically, due to the lack of absorption when administered orally and its instability at acidic pH. Additionally it has a short half-life in vivo. Research has been performed on using the biological and chemical properties of heparin to develop more efficient administration methods to decrease side effects associated with repeated HP injections, such as HP-induced thrombocytopenia, osteoporosis and alopecia. To facilitate drug permeation, evade proteolytic degradation and prolong intestinal retention time, to ultimately improve the oral bioavailability of HP, hydrogels and particulate carrier systems have been recently developed.γ

Potential pathways for the delivery of heparin include chemical conjugation,†δ encapsulation into a hydrogel,γ processing into a
polymer matrix, or binding it to a carrier by electrostatic forces. Composite materials with heparin were often investigated as hemocompatible coatings, but also gels for subcutaneous injections, micro- and nanoparticles or electrospun fiber mats were the center of attention. Most applications are targeted at the use of heparin as an anti-coagulant, but the use in cancer treatment is of increasing interest.

Nanoparticles (NPs) are attractive due to their potentially long circulation time and the possibility to be endocytosed by cells. The use of nanoparticles as drug carriers has been preferred in delivering biologics because when they are loaded with an adjuvant that aids the absorption, they can be simultaneously localized at the site of absorption with the drugs. NPs allow HP protection from degradation while the drug can be slowly released from the particles in an unaltered state. Ubrich and co-workers showed previously that LMWH can be encapsulated into a range of NPs based on either biodegradable polyε-caprolactone and PLGA or positively charged nonbiodegradable polymethacrylates, Eudragit® RS and RL microparticles. A sequential w/o and o/w emulsifying technique was employed to generate these LMWH loaded microparticles either using the polymer alone or a blend of polymers. The highest encapsulation efficiencies obtained in both studies were 39–47%. The LMWH release was limited to 20–30% when Eudragit® RS PO either alone or in combination with PCL or PLGA was used, possibly due to the strong ionic interactions between the drug and the polycationic polymer. These particles were then used for oral administration of heparin-loaded NPs in rabbits. Each formulation increased the anti-factor Xa activity. These particles displayed high bioavailability of heparin in vivo, probably by overcoming the slow absorption of the negatively charged heparin.

Inspired by these promising results, we center the attention on the application of cationic acrylic polymers of well defined microstructure, charge and low polydispersity, prepared by Reversible Addition-Fragmentation chain-Transfer (RAFT) polymerization, and for comparison the biodegradable and non-charged polymer, poly(lactic-co-glycolic acid) (PLGA) and a non-biodegradable and polycationic polymer (Eudragit® RS PO) to encapsulate bemiparin, ultra-LMWH with an average molecular weight of 3600 g mol⁻¹.

RESOMER RG 504 H is a biocompatible, neutral and biodegradable random copolymer of lactic and glycolic acids (PLGA) with equimolecular monomer composition obtained by ring-opening polymerization (Fig. 1). It is a common choice in the formulation of nanoparticles due to its biological and biochemical properties. Electrostatic forces with bemiparin are absent and the drug is packed into the polymer matrix during processing with additional driving force.

This is in contrast to Eudragit® RS PO, a non-biodegradable random polycationic synthetic polymer, widely used in the formulation of drug delivery systems and more specifically in nanoparticle formulations due to its mucooadhesive characteristics. It is based on poly(ethyl acrylate-co-methyl methacrylate-co-trimethyl aminoethyl methacrylate) poly(EA-co-MMA-co-MAETMA) (1 : 2 : 0.1) and is insoluble at acidic and physiological pH, but swells at basic pH.

The synthetic polymer was inspired by the structure of Eudragit® RS PO, which is based on methyl methacrylate (MMA) (and some ethyl acrylate) and trimethyl aminoethyl methacrylate (MAETMA), but with well defined microstructure and morphology.

Block copolymers based on PMMA-b-PMETMA were prepared by RAFT polymerization, an easy and versatile polymerization technique that allows the synthesis of macromolecules with a strictly controlled chemical structure using mild reaction conditions (Fig. 1). The composition of the cationic monomer was varied between 0.02 and 0.1 molar ratios in order to obtain well defined block copolymers with different lengths of the cationic PMAETMA segment (different charge densities). Variation of the amount of cationic charges, which will bind to HP via electrostatic forces, will modulate encapsulation efficiency and release, thus altering the biological activity.

Biological activity and stability of the prepared bemiparin loaded nanoparticles were analyzed for these copolymer systems. Nanoparticles based on Eudragit® RS PO and RESOMER RG 504 H were then used as control systems. HP has been shown to stabilize and protect growth factors, including different isoforms of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), from degradation and at the same time increase the affinity of the complex to cell receptors. In this work, the biological activity of bemiparin nanoparticles was evaluated using BaF32 cells expressing the FGF receptor 1c, which interacts with FGF2. This in vitro model provides information about the bemiparin capacity to form the ternary system (bemiparin-FGF-FGFR-1c) that triggers proliferation in these cells.
Experimental

Materials

The Low Molecular Weight Heparin (LMWH) used was bemiparin (111.3 U mg⁻¹, 5500 Da) and was kindly donated by ROVI PHARMACEUTICALS LABORATORIES (Madrid, Spain). Eudragit® RS PO (mean M₇₅: 150 000 Da) was purchased from Degussa (European Pharmacopoeia). PLGA 50:50 molar ratio (mean M₇₅: 40 000 Da) (RESOMER RG 504 H) was supplied by Boehringer Ingelheim Pharma GmbH & Co. Polyvinylalcohol used as a surfactant (PVA, M₇₅: 31 000–50 000 Da, 87–89% hydrolyzed) was purchased from Sigma. Methyl methacrylate (MMA) (Acros Organics) and [2-(methacryloyloxy)ethyl]trimethylammonium (80% purchased from Sigma. Methyl methacrylate (MMA) (Acros Organics) and [2-(methacryloyloxy)ethyl]trimethylammonium (80% purchased from Sigma-Aldrich, 99% purity, suitable for cell culture, 10% (v/v) fetal bovine serum (FBS) ( Gibco®), a 1% (v/v) penicillin–streptomycin mixture (Gibco®), and the conditioned medium was stored at −20 °C until use.

Methods

Preparation of block copolymers by RAFT polymerization.

The RAFT agent, 4-cyanopentanoic acid dithiobenzoate (CPADB), used to carry out the MMA polymerization was synthesized as reported by Y. Mitsukami and collaborators.

Table 1 Block copolymer system composition and mean molecular weight distribution measured by NMR and SEC respectively, for the polymerization of MAETMA in the presence of a PMMA₉₀macroRAFT agent in DMSO at 70 °C [macroRAFT] = 0.1 g mol⁻¹, [VS01] = 0.02 g mol⁻¹, [MAETMA] = 10, 5 or 2.5 g mol⁻¹. F is defined as the molar composition percentage of each segment in the block copolymer system

<table>
<thead>
<tr>
<th>[MAETMA]</th>
<th>[PMMA macroRAFT]</th>
<th>% Conversion after 6 h</th>
<th>M₇₅th (measured by SEC)</th>
<th>FPMMA/FMAETMA (calculated by NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[100] : [1]</td>
<td>67</td>
<td>69 000</td>
<td>66 000</td>
<td>1.40</td>
</tr>
<tr>
<td>[50] : [1]</td>
<td>60</td>
<td>61 200</td>
<td>64 000</td>
<td>1.41</td>
</tr>
<tr>
<td>[25] : [1]</td>
<td>56</td>
<td>57 900</td>
<td>56 000</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* Unit composition of the block copolymer systems were calculated by ¹H-NMR using the 0.3–1.2 ppm integration peak for PMMA + PMAETMA and 4.2–4.4 ppm integration peak for PMAETMA.
apparatus equipped with an isocratic pump serial 200 connected to a differential refractometric detector (serial 200a). Two Resipore columns (Varian) were conditioned at 70 °C and used to elute the samples (1 mg mL⁻¹ concentration) at 0.3 mL min⁻¹ HPLC-grade N,N'-dimethylformamide (DMF) supplemented with 0.1% v/v LiBr. Calibration of SEC was carried out with monodisperse standard poly(methyl methacrylate) samples in the range of 2.9 × 10⁴ to 480 × 10⁵ Da obtained from Polymer Laboratories with a sample injection volume of 20 µL.

3H- and 13C-NMR spectra were recorded on a Mercury 400BB spectrometer, operating at 400 and 133.3 MHz, respectively. The spectra were recorded by dissolving the corresponding sample in deuterated dimethylsulfoxide (DMSO-d₆), chloroform (CDCl₃) or deuterium oxide (D₂O).

Copolymerisation reactions were performed inside the NMR equipment to determine the kinetic parameters. These experiments were carried out at 70 °C with a pulse sequence of 7 µs equivalent to a 90° tip angle, a 60 s delay time, a spinning rate of 7 Hz, and one acquisition (FID), nₜ = 1, for each datum.

**Nanoparticle preparation.** The preparation of nanoparticles was carried out by the emulsion technique previously described and modified as follows: 1 mL of the aqueous bemiparin solution (2% w/v) was first emulsified during the addition of 10 mL of each polymer solution in ethyl acetate (1% w/v). The resulting water-in-oil emulsion was thereafter mixed by sonication for 1 min with a 2% PVA aqueous solution (40 mL), involving the inversion to an o/w emulsion. After evaporation of ethyl acetate under reduced pressure, the nanoparticles were isolated by centrifugation (12 000 rpm for 20 min at 25 °C). After three cycles (washing with deionized water and then centrifugation) to remove the PVA, the supernatants from the previous centrifugation steps were pooled for free drug measurement and drug entrapment efficiency evaluation. After the final centrifugation, nanoparticles were resuspended in water as a colloidal suspension and freeze-dried.

**Size distribution, zeta potential and morphology.** The core-shell morphology of nanoparticles was analysed by scanning electron microscopy (SEM) using a Philips XL 30 ESEM apparatus at an accelerating voltage of 15 keV. It was equipped with a field emission Hitachi SU800 apparatus. The samples were prepared by deposition of the corresponding nanoparticle suspension (0.01 mg mL⁻¹) over small glass disks (13 mm diameter and 1 mm thickness), and the solvent (H₂O) was evaporated at room temperature for 24 h. All the samples were coated with chrome prior to examination by SEM. Surface chemical characteristics were analyzed by Energy Dispersive X-ray analysis (EDAX). The evidence of bemiparin encapsulation and surface chemical characteristics were detected by energy dispersive X-ray analysis (EDX). Zeta potential and particle size distribution were evaluated with a Zetasizer NanoZS (Malvern Instruments, UK) equipped with a He–Ne laser beam with a wavelength of 633 nm and a scattering angle of 173°. The zeta potential measurements were performed in disposable folded capillary cells (DTS1060, Malvern Instruments) and particle-size measurements in square polystyrene cuvettes (DTS0012, Malvern Instruments) with 0.01 mg mL⁻¹ aqueous solutions at 25 °C.

Hydrodynamic diameter (Dₚ) and polydispersity index were determined by dynamic light scattering (DLS). The intensity of light scattered was used to calculate the mean hydrodynamic diameter (2-average mean), based on the Stokes–Einstein equation, assuming the particle to be spherical. The nanoparticle suspension (0.01 mg mL⁻¹) was prepared in a 25 mM HEPES buffer. For each sample, the statistical average and standard deviation (SD) of data were calculated from at least five measurements.

Zeta potential analysis was carried out using laser doppler electrophoresis (LDE) with 20 runs per measurement. The zeta potentials were automatically calculated from the electrophoretic mobility using Smoluchowski’s approximation:

\[
U_E = 2\pi f/3\eta \rightarrow z = U_E/\eta e
\]

This approximation is generally applied when the measurements are carried out in aqueous media and considers that Henry’s function takes a value of 3/2. Where \( U_E = \) electrophoretical mobility, \( z = \) zeta potential, \( \eta = \) dielectric constant, \( \eta = \) viscosity, and \( f/ka = \) Henry’s function.

**Differential Scanning Calorimetry (DSC).** Glass transition temperatures (\( T_g \)) were measured by DSC with a Perkin Elmer DSC7 interfaced to a thermal analysis data system TAC 7/DX. The dry samples (10–15 mg) were placed in aluminium pans and heated from –20 to 180 °C at a constant rate of 10 °C min⁻¹. \( T_g \) was taken as the midpoint of the heat capacity transition.

**Bemiparin encapsulation efficiency.** The drug encapsulate efficiency (EE) was determined by high performance liquid chromatography (HPLC) using a SHIMADZU SIL-20 equipment with UV-Vis detection at 242 nm. The mobile phase was 1% (w/v) acetic acid in milli-Q water and measurements were carried out at 37 °C and 1 mL min⁻¹. The amount of non-entrapped drug recovered in the external aqueous phase after centrifugation was quantified using a calibration curve obtained from different solutions of known concentration, by integration of the peak at 2.3 min ± 0.15 min. The percentage of EE was defined as:

\[
\%EE = ([\text{bemiparin}]_o - [\text{bemiparin}])/[\text{bemiparin}]_o \times 100
\]

where [bemiparin]₀ is the total added bemiparin amount and [bemiparin] is the non-encapsulated bemiparin amount.

**Bemiparin release.** Drug release from NPs was evaluated in PBS (saline phosphate buffer: pH 7.4, 0.01 M; NaCl: 0.15 mol L⁻¹). For this purpose, 10 mg of loaded bemiparin NPs were suspended in 5 mL of PBS and incubated at 37 °C under gentle magnetic stirring. At various time intervals, 0.5 mL sample was withdrawn and replaced by 0.5 mL of fresh buffer. These aliquots of 0.5 mL were centrifuged at 12 000 rpm for 15 min and supernatants were analysed by HPLC (SHIMADZU SIL-20) at 242 nm to determine the amount of bemiparin released. The pellet was placed in the original nanoparticle suspension with the fresh buffer replaced. A calibration curve of bemiparin was obtained previously from solutions of known concentration in the same medium, by measuring the integration of a peak at 2.3 min ± 0.15 min.
**BaF32 cell proliferation assay.** The BaF32 cell proliferation assay was used to determine the activity of the bemiparin released from the polymer systems. The readout of this assay was cell proliferation which indicated the biological activity of free and encapsulated bemiparin by the formation of ternary complexes on the cell surface. BaF32 cells were maintained in an RPMI 1640 medium containing 10% (v/v) FBS, 10% (v/v) WEHI-3BD conditioned medium, and a 1% (v/v) penicillin–streptomycin mixture. WEHI-3BD cells were maintained in an RPMI 1640 medium supplemented with 2 g L\(^{-1}\) sodium bicarbonate, 10% (v/v) FBS, and a 1% (v/v) penicillin–streptomycin mixture, and the conditioned medium was collected three times per week and stored at \(-20^\circ\text{C}\) until it was required. For the mitogenic assays, the BaF32 cells were transferred into IL-3 depleted medium for 24 h prior to experimentation and seeded into 96-well plates at a density of \(8 \times 10^4\) cells per well in the presence of free bemiparin or encapsulated bemiparin at different concentrations and with and without FGF2 (0.03 nM). Cells in the presence of a medium without any treatment were used as a negative control. Cells were incubated for 72 h in 5% CO\(_2\) at 37 \(^\circ\text{C}\), and the number of viable cells was assessed using the MTS assay. The MTS reagent was added to the cell cultures 6 h prior to measurement of the absorbance at 490 nm. Cell proliferation was assayed by measuring the increase of absorbance, which corresponded to the cell number. To compare the difference between the free and encapsulated bemiparin, analysis of variance (ANOVA) of the results was carried out using \(p < 0.05\) significance level.

**Results and discussion**

**Synthesis of block copolymers**

RAFT polymerization is a versatile way to access block copolymers, especially considering the robustness of the process in the presence of many functional groups.\(^{26,32-34}\) Inspired by the structure of Eudragit® RS PO, the block copolymers were based on PMMA segments while the second block was obtained from trimethyl aminooethyl methacrylate. Controlled molecular weight PMMA was synthesized using CPADB as the RAFT agent,\(^{35}\) which followed the expected living polymerization (ESI). The polymerization of the second monomer, MAETMA, in the presence of a poly(methylmethacrylate) macroRAFT agent leads to chain extension and therefore to the formation of an amphiphilic block copolymer PMMA-\(b\)-PMAETMA. In an ideal RAFT polymerization, the molecular weight of the block copolymer and the ratio of both blocks can theoretically be predicted using the ratio of concentration of monomers, the thiocarbonylthio derivative concentration and the monomer conversion of the polymerization.\(^{36}\)

PMMA with \(M_\text{n}(\text{GPC})\) of 55 000 g mol\(^{-1}\) (PDI = 1.24) was initially prepared and was employed as a macroRAFT agent in DMSO at 70 \(^\circ\text{C}\) using three different ratios of MAETMA to thiocarbonylthio functionalities (100 : 1, 50 : 1 and 25 : 1) for chain extension. Aliquots were taken at two and six hours and the conversion was analysed by \(^1\text{H-NMR}\) (Fig. 2a). The rate of polymerization increased with increasing monomer concentration. The molecular weight distribution of the resulting block copolymers increased linearly with conversion (Fig. 2b). The polydispersity index ranged between 1.40 and 1.45, which
indicated a narrow molecular weight distribution of the resulting block copolymers (Table 1), and therefore this suggests that the transfer of the radical to the macroRAFT agent was successful. For further confirmation of the formation of block copolymers, the polymers were purified using dialysis against water to remove unreacted MAETMA. The polymers obtained after a polymerization time of 6 hours were purified, freeze-dried and analysed by $^1$H-NMR to determine the composition (Fig. 3).

Table 1 summarizes the characteristics of the AB diblock copolymers prepared, their targeted and measured molar compositions, and $M_n$ and polydispersity index.

Preparation of nanoparticles (NPs)

Bemiparin is subject to degradation if not protected by a drug delivery system. Therefore different drug delivery systems were investigated. PLGA does not bind to bemiparin and encapsulation of bemiparin occurs only via packing during the emulsion process. Eudragit® RS PO with its positive charges binds to the negatively charged bemiparin. The cationic charges provided by the MAETMA units are randomly distributed along the non-degradable P(EA-MMA-MAETMA) random copolymer chain; therefore the polymer cannot be dissolved in an aqueous solution. Block copolymers based on the same building blocks listed in Table 1 can in contrast form stable micellar nano-aggregated systems, which in physiological fluids can slowly disaggregate to give small micelles that in a period of time would be resolved.

Amphiphilic block copolymers are known to undergo self-organization into micelles, which would be suitable as drug carrier systems. Light scattering studies not only confirm the formation of micellar aggregates but also reveal their sizes. All block copolymers prepared were analysed using DLS after dispersion of the block copolymers in DMF and in water. Since the particle size can be influenced by the concentration, all the samples were measured at a constant concentration of 3 mg mL$^{-1}$ at 25 °C. All the block copolymers showed a tendency to form multimicellar aggregates depending on the solvent. DLS of the copolymer samples after a fine dispersion in water shows that the average size of aggregates in water increases with the increasing of the length of the PMAETMA block (Table 2). However, the average size indicates the relatively high tendency toward the formation of multimicellar aggregates. When DMF is used as the dispersion medium, the hydrodynamic diameter ($D_h$) is much smaller than that in water, indicating that the block copolymer has a clear tendency toward the formation of a unimicellar system. Block copolymers were soluble in ethyl acetate where micelle formation was absent.

The block copolymer PMMA-$b$-PMAETMA was soluble in ethyl acetate for any composition and concentration interval used in this work and when a diluted solution was added to water at high stirring, a self-organised micelle dispersion was formed. Depicted in Fig. 4a, the micelle nanoparticles have a core–shell organisation with the more hydrophobic MMA blocks in the core and the hydrophilic MAETMA sequences in the shell. According to the light scattering results, these micelles are not stable enough in water and tend to aggregate in multimicellar nanoparticles with a clear charged hydrophilic surface (Table 3).

![Fig. 4 Nanoparticle formation scheme prepared by an emulsion technique using the block copolymers obtained via RAFT. (a) Preparation of non-loaded polymeric nanoparticles and (b) preparation of loaded nanoparticles with bemiparin. A TEM image of the NP system 86/14 PMMA-$b$-PMAETMA obtained with a field emission Hitachi SU8000 apparatus is added.](image-url)

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Hydrodynamic diameter ($D_h$) of block copolymer systems measured by DLS with a Zetasizer NanoZS (Malvern Instruments, UK) using different solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Sample Composition</td>
</tr>
<tr>
<td>86/14</td>
<td>PMMA$<em>{970}$-$b$-PMAETMA$</em>{158}$</td>
</tr>
<tr>
<td>92/8</td>
<td>PMMA$<em>{970}$-$b$-PMAETMA$</em>{84}$</td>
</tr>
<tr>
<td>98/2</td>
<td>PMMA$<em>{970}$-$b$-PMAETMA$</em>{20}$</td>
</tr>
</tbody>
</table>
Table 3  Mean hydrodynamic diameters (nm) and zeta potential values (mV) of the nanoparticle systems measured by DLS with a Zetasizer NanoZS (Malvern Instruments, UK) after precipitation from ethyl acetate solutions

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_h$ (nm) ± SD</th>
<th>ZP (mV) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>98/2 NPs</td>
<td>202 ± 25</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>92/8 NPs</td>
<td>165 ± 20</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>86/14 NPs</td>
<td>123 ± 14</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Eudragit® RS PO NPs</td>
<td>284 ± 42</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>PLGA NPs</td>
<td>121 ± 5</td>
<td>-14 ± 1</td>
</tr>
<tr>
<td>Bemiparin loaded 98/2 NPs</td>
<td>105 ± 12</td>
<td>-28 ± 1</td>
</tr>
<tr>
<td>Bemiparin loaded 92/8 NPs</td>
<td>144 ± 21</td>
<td>-14 ± 1</td>
</tr>
<tr>
<td>Bemiparin loaded 86/14 NPs</td>
<td>198 ± 17</td>
<td>-10 ± 3</td>
</tr>
<tr>
<td>Bemiparin loaded Eudragit® RS PO NPs</td>
<td>142 ± 20</td>
<td>-32 ± 2</td>
</tr>
<tr>
<td>Bemiparin loaded PLGA NPs</td>
<td>117 ± 13</td>
<td>-37 ± 2</td>
</tr>
</tbody>
</table>

The addition of bemiparin was carried out by a two step emulsion process as shown in Fig. 4b. The first step was the preparation of bemiparin loaded micelles by adding a diluted solution of bemiparin in water to the solution of the block copolymer in ethyl acetate under vigorous stirring. This results in the formation of very small micelles with the hydrophilic ionized bemiparin in the core of the micelle, whereas in the outer part are concentrated the more hydrophobic copolymer sequences. With this arrangement, the formation of bridges is easy by ionic interactions of the sulphate groups of bemiparin and the ammonium ions of the PMMA-b-PMAETMA block copolymer, with this characteristic distribution of micro-domains. In a second step, this water in oil emulsion is added to a diluted solution of poly(vinyl alcohol) (PVA) in water, and under these conditions a phase inversion is produced, in such a way that the more hydrophobic components (PMMA segments) are concentrated in the core, and the hydrophilic components (bemiparin + PMAETMA sequences) are distributed in the shell phase. The consequence is the formation of nanoaggregates, which are stabilised by the complexation between the ionic sulphate groups of bemiparin and the ammonium ions of the copolymer entities (Fig. 4b). The PVA acts as an o/w emulsifying agent to avoid aggregation of nanoparticles.

The nanoparticle formation with the two commercial polymers was carried out in a similar manner; however the PLGA system does not form core–shell micelles by phase inversion as EUDRAGIT and PMMA-b-PMAETMA systems. In the case of PLGA, nanoparticles were formed by double emulsion instead.

Particle size and zeta potential (ZP) measurements by DLS

The ZP has been used as an index of the NP stability in the suspension. It can greatly affect the particle stability in suspension through the electronic repulsion between particles. As a result, a higher absolute value of the ZP indicates a more stable suspension, and a lower value implicates colloid instability, which could lead to aggregation of NPs. Particles prepared with the cationic systems without bemiparin exhibited strongly positive zeta potential values in water due to the presence of the ammonium charge group. PLGA NPs showed a small negative ZP probably due to the carboxylic end groups of the copolymer chain. When bemiparin was encapsulated in the nanoparticle the zeta potential value became more negative, which can be attributed to the presence of bemiparin on the NP surface. Encapsulated bemiparin not only neutralised the positive charges of Eudragit® RS PO and the RAFT made block copolymers, but also provided a negative ZP to the loaded NPs. The non-neutralised sulphate groups provided stability to the NP shell. Table 3 shows the block copolymer composition influence on the ZP values obtained. The higher cationic segment length and the smaller ZP absolute value are due to a higher charge neutralization between bemiparin and the corresponding cationic block copolymers. It means a higher $D_h$ of the NP system. When all the particles in suspension have a large negative or positive ZP then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low ZP values then the aggregation is favoured and the $D_h$ obtained is higher.

Size and morphology characterization of the NP systems by SEM

The morphology of NP systems was determined using Scanning Electron Microscopy (SEM) and it was predominantly spherical (Fig. 5). Evidence for the encapsulation of bemiparin was also provided by EDAX analysis, by means of which the sulfur band in the NP systems was observed (Fig. 6), and by Fourier Transform Infrared (FTIR) spectroscopy (ESI†). Non-loaded NPs did not give a detectable signal of S in the corresponding EDAX. Logically, the
contribution of the S signal is associated with the initiator linked at the end of the copolymer chains, and according to the high molecular weight, the contribution is very small.

**Encapsulation efficiency (EE)**

The amount of bemiparin entrapped within the polymeric nanoparticles was determined by measuring the amount of the drug released to the external aqueous solution recovered after centrifugation and washing the nanoparticles. Summarized in Table 4, the entrapment efficiency within polymeric nanoparticles was affected by the nature of the polymer. When PMMA-b-PMAETMA and Eudragit® RS PO polymers were used, the encapsulation efficiency of bemiparin was higher than that observed for a PLGA polymer. Owing to the polyanionic nature of bemiparin, ionic bonds between the drug and the quaternary ammonium groups of the polymers led to an increase of bemiparin immobilization compared to the PLGA polymer. Considering the %EE, the mean charge density of bemiparin and the corresponding charge density of the polymer systems, the bemiparin encapsulation percentage and the charge ratio of the NP systems were calculated. These results corroborated the results obtained by ZP, the higher cationic segment length (PMAETMA) and the higher neutralization of the bemiparin charges (Table 4). It is noticeable that the amount of loaded bemiparin is very similar for all the NP systems analysed (Table 4).

**DSC measurements**

PMMA-b-PMAETMA block copolymers showed a unique thermal transition ($T_g$) between 86 and 110 °C, depending on the composition. This transition can be attributed to the $T_g$ of the MMA segment. The systems showed a decrease of $T_g$ value as the $F_{PMAETMA}$ was increased in the polymer. This feature can be due to the plasticization effect of the MAETMA segments in the polymer system. However, when loaded NPs were analysed, two different glass transition temperatures were observed, which belong to the MAETMA and MMA segments respectively. It can be explained as the result of the interaction of the ionic drug bemiparin that increases the hydrophilic segment character and causes a nanodomain segregation of both blocks. In that case, the $T_g$ values approach those of the corresponding homopolymers (Table 5).

**Bemiparin release**

Fig. 7 illustrates the in vitro release profiles at pH 7.4 obtained for each formulation of encapsulated bemiparin. All the

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**Table 4** % Encapsulation Efficiency (EE) of bemiparin loaded NP systems measured by HPLC-UV. Charge densities, $\overline{\rho}$ and $\overline{\rho'}$, are apparent relative densities.

<table>
<thead>
<tr>
<th>NP system</th>
<th>EE-% ± SD</th>
<th>mg bemiparin encapsulated</th>
<th>Wt-% bemiparin encapsulated into the NPs</th>
<th>$\overline{\rho}$ (bemiparin density charge) $\times 10^{\overline{3}}$</th>
<th>$\overline{\rho'}$ (polymer density charge) $\times 10^{\overline{3}}$</th>
<th>$\overline{\rho'}$/$\overline{\rho}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA NPs</td>
<td>89 ± 3</td>
<td>178</td>
<td>15.1</td>
<td>1.53</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>86/14 NPs</td>
<td>98 ± 2</td>
<td>196</td>
<td>16.4</td>
<td>1.68</td>
<td>2.39</td>
<td>0.70</td>
</tr>
<tr>
<td>92/8 NPs</td>
<td>96 ± 3</td>
<td>192</td>
<td>16.1</td>
<td>1.65</td>
<td>1.31</td>
<td>1.26</td>
</tr>
<tr>
<td>98/2 NPs</td>
<td>95 ± 4</td>
<td>190</td>
<td>16.0</td>
<td>1.63</td>
<td>0.36</td>
<td>4.53</td>
</tr>
<tr>
<td>Eudragit® RS PO NPs</td>
<td>94 ± 2</td>
<td>188</td>
<td>15.8</td>
<td>1.61</td>
<td>0.33</td>
<td>4.88</td>
</tr>
</tbody>
</table>

$^a$ Bemiparin encapsulated = [(mg bemiparin)$_b$ x %EE)/100. $^b$ Wt % bemiparin encapsulated into NPs = [(mg bemiparin encapsulated) x 100]/[(mg bemiparin encapsulated) + (mg polymer)]. $^c$ $\overline{\rho}$ = [(g bemiparin)/$M\rho$(bemiparin)] x no. of disaccharide units x 2.7 (negative density bemiparin/disaccharide). $^d$ $\overline{\rho'}$ = [(g polymer)/$M\rho$(PMMA-b-PMAETMA)] x no. of [MAETMA units].
formulations showed an initial burst release; however the influence of the chemical structure of the polymers on the release profile was evident at longer time points at pH 7.4. Eudragit® RS PO NPs released the least amount of bemiparin over the 70 day analysis period which was probably due to ionic interactions between the ammonium groups of the polymer and the sulphate groups of bemiparin. This polymer system is hydrophobic as the random distribution of MAETMA along the polymer chain does not allow phase segregation to occur and PMMA sequences are dominant. This makes water diffusion more difficult, and therefore the bemiparin release.

At pH 7.4 the highest bemiparin release (nearly 100%) occurred in PLGA NPs due to the amorphous structure and non-ionic character of the PLGA polymer. Drug release from PLGA NPs was mainly controlled by the diffusion of bemiparin throughout the flexible chains, and/or erosion mechanisms of PLGA, as it is a biodegradable polymer. In the first three weeks, the bemiparin release profile in a PLGA NP system did not show a significant difference compared to the block copolymer systems; however after 25 days the bemiparin release increased probably due to the higher erosion of PLGA by biodegradation. Total release of bemiparin was confirmed by dissolution of the NPs in ethyl acetate and analysis of the bemiparin content by HPLC-UV and therefore the bemiparin release.

Bemiparin release from the cationic block copolymers was found to be controlled by the three-dimensional network structure produced by ionic interactions following water diffusion into the nanoparticles. This may explain the significant retardation of bemiparin release from the Eudragit® RS PO system.

NPs prepared with PMMA-b-PMAETMA copolymers showed a higher bemiparin release compared to Eudragit® RS PO NPs, due to the microstructure of the copolymers. These block copolymers formed a self-organised system with a core of PMMA blocks and a shell of the cationic PMAETMA complexed with bemiparin (see Fig. 4b). The latter nanodomain is very hydrophilic and might allow better water diffusion compared to the Eudragit® RS PO.

NP block copolymer systems showed a slight influence of the copolymer composition on the bemiparin release profile at pH 7.4, since NPs prepared from the 86/14 copolymer presented a slower release than NPs prepared using the copolymers with lower content on MAETMA blocks (92/8 and 98/2).

**Cell proliferation assay**

The Baf32 cells provide a measure of the activity of the ternary complexes formed between bemiparin, FGF2 and FGF receptor 1c. The formation of active ternary complexes induces cell proliferation which was measured by the MTS assay. The extent of proliferation is modulated by the concentration of FGF2 and bemiparin as well as the activity of the bemiparin. As bemiparin was not chemically modified in each of the polymer systems, this assay provides a measure of the availability of bemiparin in the different polymer systems. FGF2 or bemiparin alone can induce the proliferation of the cells; however the presence of both in an active ternary complex enhances proliferation. This was observed when the Baf32 cells were exposed to free bemiparin in the concentration range of 5–60 nM in the presence or absence of FGF2 (Fig. 8). There was, however, no dose-dependent increase in the proliferation of the cells in the presence of increasing concentrations of bemiparin. Bemiparin encapsulated in each of the polymer systems was also analysed by the Baf32 cell assay where cells were exposed to different concentrations of the polymer systems based on the amount of encapsulated bemiparin between 5 and 60 nM. Bemiparin encapsulated in PLGA NPs showed dose-dependent increase in cell proliferation with increasing concentrations of encapsulated bemiparin. The highest dose of bemiparin encapsulated in PLGA NPs was equally as active as free bemiparin (p < 0.05). This may be due to 40% release of bemiparin from PLGA within 72 h (Fig. 7). Bemiparin encapsulated in Eudragit® RS PO was significantly less active than free bemiparin (p < 0.05) except at the highest concentration used and is likely to be attributed to the low release of bemiparin at 72 h of approximately 10%. When PMMA-b-PMAETMA block copolymers were used to encapsulate bemiparin, the systems were significantly less active than free bemiparin (p < 0.05) probably due to the ionic retention of bemiparin with the cationic group of the polymer carrier. The 98/2 PMMA-b-PMAETMA system became dose-
dependent as the PLGA system because at pH 7.4 the bemiparin release in that system was close to 40% at 72 h (Fig. 7).

**Conclusion**

RAFT polymerisation was shown to be a suitable method to prepare amphiphilic block copolymers of PMMA-b-PMAETMA. These amphiphilic block copolymers were used as carriers for the controlled delivery of bemiparin due to their micellar self-assembled nanoparticles loaded with bemiparin. Loaded NPs with a mean size between 100 and 200 nm, depending on the block copolymer composition, were obtained with a predominantly spherical morphology. Eudragit® RS PO was used as a structural and chemical model with positively charged functions but different microstructures. PLGA was taken as a biodegradable and biocompatible model very commonly used in drug delivery. RAFT block copolymers showed similar profile plots for a PLGA system and higher drug release than Eudragit® RS PO. The encapsulated-bemiparin in the PLGA and 98/2 block copolymer systems showed a dose dependent behavior in cell proliferation, which was not appreciated in either, Eudragit® RS PO NP system or non-encapsulated bemiparin. The encapsulation with the block copolymer system offers a modular release way for the protection and application of bemiparin as an activating agent of the function of growth factors.

**References**


