Continuously manufactured magnetic polymersomes – a versatile tool (not only) for targeted cancer therapy

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Micromixer technology was used to prepare polymeric vesicles (Pluronic® L-121) dual loaded with the anticancer drug camptothecin and magnetic nanoparticles. Successful incorporation of the magnetic nanoparticles was confirmed by transmission electron microscopy. Dynamic light scattering measurements showed a relatively narrow size distribution of the hybrid polymersomes. Camptothecin polymersomes reduced the cell viability of prostate cancer cells (PC-3) measured after 72 h significantly, while drug-free polymersomes showed no cytotoxic effects. Covalent attachment of a cancer targeting peptide (bombesin) as well as a fluorescent label (Alexa Fluor® 647) to the hybrid polymersomes was performed and specific cell binding and internalization were shown by flow cytometry and confocal microscopy. Relaxometry measurements clearly demonstrated the capacity of magnetic polymersomes to generate significant T2-weighted MRI contrast and potentially allow for direct monitoring of the biodistribution of the polymersomes. Micromixer technology as an easy, fast and efficient way to manufacture hybrid polymersomes as theranostic drug delivery devices is a further step from basic research to personalized medicine.

Introduction

Cancer is still one of the major public health problems in the world. For example, cancer causes one in every four deaths in the United States.1 Although big steps towards highly potent new anticancer drugs have been made, conventional chemotherapy still causes severe adverse side effects due to nonspecific biodistribution and inefficient delivery to the tumor site. Moreover, drug resistance in tumor tissue occurs more frequently, making new approaches for a safe and efficient drug delivery necessary.

Nanomedicine has developed rapidly in the last few years to address the optimization of drug administration in terms of stability, solubility and bioavailability of therapeutic agents with engineered nanocarriers. Safety issues play a key role in the development of drug delivery systems, e.g., protection of healthy cells by encapsulation of highly toxic drugs as often applied in cancer therapy. A controlled release at the site of action and specific targeting strategies can help to ensure a safe delivery to the diseased tissue and prevent undesired adverse side effects. Recently, systems for combined diagnostic and therapeutic approaches, the so-called theranostics, have gained the interest of many researchers because of their immense potential for directly monitoring therapy.2–4 The additional imaging capability of the nanocarriers enables a non-invasive in vivo real-time evaluation of the therapeutic effects.

Liposomes are one of the oldest and best investigated drug delivery devices and have also already been studied as potential theranostic tools for cancer diagnosis and treatment.5,6 Nevertheless, the applicability of liposomal systems suffers from limitations due to their poor stability in the blood stream and little synthetic variance, whereas amphiphilic polymers allow an individual engineering of tailor-made vehicles for the biomedical application.7–12 Polymersomes, vesicles assembled from block-copolymers, offer over polymeric micelles an additional hydrophilic inner compartment and thus enable multiple loading of a water-soluble cargo in the inner lumen as well as hydrophobic components in the membrane.13,14

A critical step towards the clinical use of nanomedical drug delivery devices is the ability to scale up the manufacturing process and make drugs available for a broad portion of the population, and then also to adapt the system individually to the patients’ needs. Furthermore, biocompatibility is mandatory to get approval from the government (e.g., FDA) for a new material.

One material in which biocompatibility and applicability for pharmaceutical applications have already been shown in
numerous research articles is poly(ethylene oxide)--poly(propylene oxide) block copolymers (PEO–PPO).\textsuperscript{15,16} These commercially available polymers in a broad range of compositions in terms of molecular weight and EO/PO ratio (trademarks: Pluronic®, Poloxamer®, Lutrol®) have been extensively studied concerning their toxicology and safety and are FDA approved for numerous cosmetic and medical applications.\textsuperscript{17} PEO–PPO–PEO is a non-biodegradable polymer; nevertheless molecules below the 10–15 kDa range are usually cleared by the kidney and can be excreted in urine.\textsuperscript{18,19}

Pluronic® L-121 (EO\textsubscript{5}PO\textsubscript{68}EO\textsubscript{5}) is the most hydrophobic Pluronic\textsuperscript{®} triblock polymer which tends to form vesicle structures.\textsuperscript{20–22} The hydrophilic PEO portion, which is presented on the surface of nanocarriers, has been shown to reduce opsonization and prevent uptake by macrophages of the reticuloendothelial system (RES), allowing for prolonged circulation in the blood stream.\textsuperscript{23} The more hydrophobic PPO in the middle of the triblock polymer forms the membrane of the polymersome and can be exploited to incorporate hydrophilic components such as poorly water-soluble drugs or dyes.\textsuperscript{21}

The encapsulation of hydrophobically coated iron oxide nanoparticles into the PPO block of Pluronic\textsuperscript{®} vesicles leads to polymer hybrid composites with a broad range of potential biomedical applications. They include hyperthermia,\textsuperscript{24} magnetically guided transport to diseased tissues\textsuperscript{25} and controlled drug release in the target tissue triggered by an external alternating magnetic field.\textsuperscript{2} Encapsulated super-paramagnetic iron oxide nanoparticles (SPIIONs) provide additionally defined and enhanced contrast in magnetic resonance imaging (MRI) and in magnetic particle imaging (MPI).\textsuperscript{26–28} Magnetic polymersomes are therefore promising multifunctional theranostic tools in nanomedicine.

Here we report the manufacturing of theranostic magnetic polymersomes in a continuous microfluidic system, using biocompatible, commercially available, FDA approved polymers. Our aim was to incorporate the anticancer drug camptothecin into the polymersomes’ membrane together with the hydrophobic SPIIONs and specifically deliver the drug to cancer cells. The specific delivery was enabled through covalent attachment of the targeting peptide bombesin and tested in prostate cancer cells. Furthermore, the polymersomes were made into theranostic agents by adding magnetic iron oxide nanoparticles to allow for diagnostic MR imaging.

**Results and discussion**

**Preparation and characterization of loaded polymersomes**

Empty as well as hybrid camptothecin and magnetite loaded Pluronic® polymersomes were prepared continuously in a micromixing device (Scheme 1). Microfluidic systems gained popularity in the last few years because of their high throughput and a good control over size compared to older methods such as the rehydration technique or nanoprecipitation methods which often require post-preparation steps (e.g., extrusion) to obtain samples of narrow size distribution. Even though microfluidic systems for polymersome preparation have already been described, those devices generate giant vesicles in the micrometer range.\textsuperscript{29–31} The here-presented micromixer device enables control over size and shape of polymeric assemblies even below 100 nanometers as published recently.\textsuperscript{32}

*In situ* loading of the polymersomes with the anticancer drug camptothecin as well as with magnetic nanoparticles was performed simply by adding the cargo to the starting polymer solution and resulted in homogeneous iridescent polymersome

![Scheme 1](image_url)

Continuous preparation of loaded magnetic polymersomes: the starting polymer solution (PEO–PPO–PEO in tetrahydrofuran) is diluted with water, the selective solvent for the PEO block, and induces polymersome self-assembly. The microstructured mixing device is a stainless steel caterpillar micromixer with twelve mixing steps and a mixing channel with an inner volume of 10 µL. Hydrophobic agents were loaded *in situ* by simply adding the cargo (magnetic nanoparticles or drug molecules) to the starting polymer solution prior to mixing. Due to the hydrophobicity of those compounds incorporation in the hydrophobic part of the vesicle membrane occurs. Prior carboxylation of the end-groups of the polymer enables further surface functionalization and conjugation to specific targeting moieties.
Drug-loaded polymersomes

Drug-loaded polymersomes

Drug load, determined by a HPLC assay, was 2.4% and 2.6% of the polymer weight for single and dual loaded polymersomes, respectively. This conforms to an encapsulation efficiency of close to 100% (96% and 100% as determined by HPLC analysis).

A very high concentration of 100 μg mL⁻¹ camptothecin in a 0.4 wt% polymer formulation was achieved, which corresponds to 70 μmol camptothecin per gram polymer and is 70 fold higher than drug loading in other formulations such as Pluronic® F-127 presented previously by Cortesi et al. as well as Barreiro-Iglesias and co-workers.³³,³⁴

Transmission electron microscopy (TEM) shows the morphology of the hybrid polymersomes (Fig. 2 and 3). The Pluronic® copolymers have almost no contrast under TEM, due to the very low attenuation of the electron beam. Therefore hybrid polymersomes are largely present as isolated clusters of iron oxide nanoparticles. Furthermore, the morphology is partly destroyed by the sample preparation as samples were dried on a solid carbon-coated grid (Fig. 2).

Cryogenic TEM (cryo-TEM) imaging, however, preserved the size and morphology of the polymer assemblies in the hydrated state and allowed for hybrid polymersomes imaging as they are present in water. Cryo-TEM measurements confirmed the dynamic light scattering results and showed polymersome diameters of about 160 nm. Furthermore static light scattering measurements indicated the vesicular structure with an Rₓ to Rₜ ratio of 1 (data not shown). Moreover, the iron oxide nanoparticles are distributed rather uniformly (even when the grid is

| Table 1 Characteristics of the Pluronic® polymersomes |
|-----------------|---|---|---|---|
| Polymer | Dₜₐ [nm] | μ₂(90°) | ζ-Potential [mV] | Fe₃O₄ [% (w/w)] | CPTb [% (w/w)] |
| Empty polymersomes |
| L121 | 126 | 0.14 | −19 ± 2 | — | — |
| L121-COOH | 150 | 0.11 | −44 ± 3 | — | — |
| Magnetic polymersomes |
| L121 | 120 | 0.07 | −19 ± 3 | 4.1 | — |
| L121 | 125 | 0.04 | −17 ± 3 | 7.1 | — |
| L121 | 122 | 0.03 | −19 ± 2 | 17.4 | — |
| L121-COOH | 146 | 0.05 | −45 ± 3 | 7.4 | — |
| Drug-loaded polymersomes |
| L121 | 126 | 0.12 | −17 ± 2 | — | 2.4 |
| L121 | 122 | 0.06 | −18 ± 3 | 7.1 | 2.6 |

ₐ Average hydrodynamic diameters of the polymersomes were determined by angle-dependent dynamic light scattering. The second cumulant μ₂ can be attributed to the polydispersity of the sample (cumulant method,³³,³⁴ normalized μ₂ = 0.05 at 90° scattering angle, corresponding to a polydispersity in radius of approximately 25%). b CPT – camptothecin.

Fig. 1 Camptothecin-polymersomes (right) and dual loaded polymersomes with camptothecin and iron oxide nanoparticles before (center) and after (left) magnet separation.

Fig. 2 Transmission electron microscopy image of Pluronic® L121-COOH polymersomes loaded with magnetic nanoparticles (7.4% iron oxide).
tilted), which also confirms a vesicular morphology. A micellar structure where nanoparticles are present in the internal volume instead of the membrane would show a higher contrast in the centre of the sphere.

Based on the bulk density of magnetite, a polymersome with a diameter of 160 nm loaded with 4% iron oxide particles (mean diameter of 6 nm) contains 40 iron oxide particles on average. TEM imaging seemed to show a larger number of magnetic particles. However, since TEM allows only a limited view (spotlight) on a very small section of the sample, it is not statistically representative.

As shown in Fig. 1, an iron oxide content of 7.1% leads to a complete magnetic separation after 24 h. To further evaluate the magnetic polymersomes for magnetic targeting applications, experiments on the magnetophoretic mobilities of individual polymersomes with different iron oxide contents are in progress. Additionally, hyperthermia measurements to evaluate the therapeutic potential, also in combination with a controlled drug release, are planned.

Relaxometry measurements of magnetic polymersomes

Proton transverse relaxivities of magnetic polymersomes at 1.4 T with and without drug-loading are shown in Fig. 4A. The polymersomes loaded with 7.1% magnetic nanoparticles had a very high transverse relaxivity of 682 s⁻¹ mM⁻¹.

The polymersomes loaded with 7.1% magnetic nanoparticles and camptothecin had a reduced, but still significant, transverse relaxivity of 394 s⁻¹ mM⁻¹. In comparison, the transverse relaxivity of Feridex®, a frequently used MRI contrast agent, was only 111.5 s⁻¹ mM⁻¹.7 Hence, the camptothecin-loaded magnetic polymersomes have the capacity to generate significant T₂-weighted MRI contrast and potentially allow for direct monitoring of the biodistribution of the polymersomes. The r₁ relaxivities (Fig. 4B) were rather small (9.96 s⁻¹ mM⁻¹ for magnetic polymersomes and 3.60 s⁻¹ mM⁻¹ for camptothecin-filled magnetic polymersomes, respectively) in comparison to the r₂ relaxivities. Camptothecin-filled magnetic polymersomes, in particular, would thus not be suitable for generating T₁-weighted contrast for MRI.

Given the similarities in their physical properties (Table 1), it is unclear why the addition of camptothecin to the magnetic polymersomes results in lower transverse and longitudinal relaxivities. However, we observed less variability in the triplicate transverse relaxation rate measurements of the magnetic polymersomes with camptothecin, suggesting greater stability in the magnetic field of the relaxometer compared to the polymersomes with the drug. Hence, the less stable sample without camptothecin may have undergone more field-induced aggregation leading to a higher transverse relaxivity. The reduced longitudinal relaxivity after the addition of camptothecin suggests that the access of water molecules to the magnetic nanoparticles may be inhibited, but further studies are required to examine these observations.

Antitumor activity and cell-uptake studies

The antitumor activity of drug-loaded polymersomes was evaluated by the MTT method using prostate cancer cells (PC-3). Cytotoxicity of encapsulated camptothecin was comparable to the free drug formulation (Fig. 5A). Due to the low water solubility of camptothecin (1.3 μg mL⁻¹), drug dose is limited in the free drug formulation.35 Encapsulation of camptothecin in the
hydrophobic membrane of the polymersomes enabled a 10-fold higher drug dose and therefore a further reduction from 40% to 20% viability of prostate cancer PC-3 cells was reached. Non-toxicity of the empty carrier system containing magnetic nanoparticles was confirmed (Fig. 5B). Camptothecin loaded polymersomes showed a sustained release over several days as previously reported by our group.

Cell binding and uptake experiments including flow cytometry, fluorescence spectroscopy and confocal microscopy were performed to confirm successful targeting of bombesin-functionalized polymersomes. Bombesin (BN) is a 14 amino acid peptide with high affinity for gastrin releasing peptide receptors. Bombesin is over-expressed in several human tumours, particularly ovarian cancer, prostate cancer and breast cancer. Bound to the surface of polymersomes, bombesin might act as a targeting moiety for drug delivery.

To allow for bombesin coupling, carboxylation of Pluronic® L121 was performed prior to polymersome preparation. [Lys3]-bombesin (pGlu-Gln-Lys-Leu-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2), where the third amino acid arginine is exchanged with lysine, was chosen for surface functionalization of the polymersomes. Conjugation to the polymersome surface with the free amino group of the lysine was carried out via carbodiimide chemistry. A control experiment without coupling agents was performed to confirm the covalent binding after purification with dialysis. The coupling efficiency was >80% determined by HPLC amino acid analysis after acidic hydrolysis.

In addition to the targeting ligand the fluorescent label Alexa Fluor® 647 was covalently attached to the polymersomes. Cell uptake of polymersomes with [Lys3]-bombesin at the surface was observed already after two hours incubation (Fig. 6). Even

![Fig. 5](image-url) (A) Cytotoxicity of drug loaded polymersomes compared to free camptothecin tested on prostate cancer PC-3 cells after 72 h incubation. (B) Empty polymersomes (L121) and magnetic polymersomes (L121-MNP) without drug loading in the highest applied polymer concentration (300 mg L\(^{-1}\)) are non-toxic to PC-3 cells.

![Fig. 6](image-url) Uptake of fluorescent magnetic polymersomes in human prostate cancer cells: cells were incubated with magnetic polymersomes for 2 h at 37 °C. Fluorescence confocal microscopy: nucleus is stained with DAPI (blue) and cell membrane is labelled with a fluorescein conjugate of wheat germ agglutinin (green), Alexa Fluor® 647-labelled magnetic polymersomes are shown in red. Flow cytometry: histogram of Alexa Fluor® 647 of PC-3 cells: control cells (grey), cells incubated with Alexa Fluor® 647 polymersomes without (L121-MNP) and with [Lys3]-bombesin (L121-MNP-BN) respectively (pink), block experiment with bombesin pre-incubation of PC-3 cells (blue). Uptake was calculated using the following equation: % uptake = (fluorescence intensity value in the cell lysates)/(total fluorescence intensity value of the nanoparticles added into the cell culture).
though unspecific uptake of non-targeted polymersomes also occurred, uptake of targeted polymersomes was significantly higher. This was confirmed by FACS analysis where we obtained a mean fluorescence intensity of 4415 for bombesin-polymersomes compared to 2279 for the blank polymersomes. A blocking experiment with an excess of free bombesin confirmed the receptor-specificity of the observed binding for the targeted polymersomes. Confocal microscopy showed that the nanocarriers are inside the cells and not just bound to the surface (Fig. 6). This fact was also confirmed by a fluorescence uptake study where the surface-bound polymersomes were removed by treating the cells with glycine–HCl pH 2.2 and analysing the remaining fluorescence after cell lysis. Uptake after 2 hours incubation was two times higher for the bombesin-functionalized polymersomes compared to the non-functionalized polymersomes.

Bombesin and its analogues have already been shown to be promising targeting peptides for cancer diagnosis and selective radiotherapy. In particular, [Lys3]-bombesin has been reported to bind very well to prostate cancer cells (PC-3) with a 50% inhibitory concentration value for DOTA-[Lys3]-bombesin of 2.2 ± 0.5 nM. Moreover, in vivo PET imaging studies in mice with 64Cu-DOTA-[Lys3]-bombesin confirmed its ability to detect GRPR-positive prostate cancer.

The combination of a powerful targeting peptide with a biocompatible carrier system with prolonged blood circulation time, as reported for PEG-polymersomes, as well as the ability to transport therapeutic drugs and simultaneously track the resulting drug carrier via MR imaging is going to be a powerful tool in the fight against cancer. The magnetic properties might even help to guide the carrier magnetically to the diseased tissue and further decrease the interaction with healthy cells, potentially resulting in low toxicity.

In vivo studies to investigate the fate of our carrier system in tumor animals are planned. The magnetic properties of our drug-containing polymersomes will help to track their biodistribution and determine their pharmaco-kinetics and target uptake.

Experimental section

Materials

Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic® L-121), (S)-(+) camptothecin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-(N-morpholino)ethanesulfonic acid (MES), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) hydrochloride, N-hydroxysuccinimide ( NHS), ethylenediamine and succinic anhydride were purchased from Sigma-Aldrich (Germany or St. Louis, MO, USA). Dialysis membranes (MWCO: 1 and 12 kDa) were obtained from Spectrum® Laboratories Inc., Rancho Dominguez, USA. Gibco® RPMI Medium 1640, fetal bovine serum (FBS), penicillin streptomycin (Pen Strep), wheat germ agglutinin (WGA) fluorescein conjugate, ProLong® Gold anti-fade reagent with DAPI and Alexa Fluor® 647 NHS ester were from Invitrogen (Carlsbad, CA, USA). The human prostate cancer cell line PC-3 was obtained from American Type Culture Collection (ATCC, Rockville, MD). Ferrofluid was purchased from Webraft GmbH (Germany). [Lys3]-bombesin was obtained from Kinexus Bioinformatics Corporation (Vancouver, Canada). FluoraldehydeTM (OPA) reagent solution, dimethyl sulfoxide (DMSO), triethylamine, tetrahydrofuran (THF) and acetonitrile (HPLC grade) were from Pierce Thermo Fisher Scientific (Rockford, USA).

Preparation of loaded polymersomes

The microfluidic mixing device consists of a syringe pump (Postnova, Germany), a HPLC pump (Smartline, Knauer, Germany) and a stainless steel capillary micromixer (model CPMM-V1.2-R300-12-ss-wt, Institut für Mikrotechnik Mainz, Germany). Polymer solutions were prepared with Pluronic® L121 (PEO–PPO–PEO, as purchased or carbosylated by succinic anhydride as described below) in THF. Self-assembly was performed at room temperature with 10 g L−1 starting polymer concentration, with a total flow rate of 10 mL min−1 and a mixing ratio of 1:10 (polymer solution–water). The final polymer concentration after evaporation of the non-selective solvent was 3 g L−1. In situ loading of the polymersomes was performed by adding camptothecin and/or magnetic nanoparticles to the starting polymer solution. Magnetic nanoparticles (ferrofluid) were dialysed against THF before use.

Dynamic light scattering and zeta potential measurements

Dynamic light scattering (DLS) was measured with an ALV-CGS-3 MD goniometer system, 632 nm HeNe laser (22 mW), APD, dual ALV-7004 Multiple Tau Digital Real Time Correlator (ALV, Germany) after filtration through 0.45 μm filters (LCR or PVDF, Millipore, Germany). Zeta potential was measured in water with a Zetasizer Nano ZS (Malvern Instruments Ltd UK).

Transmission electron microscopy (TEM)

TEM measurements were carried out with a FEI Tecnai 12 or Philips Electron Microscope EM420 on carbon coated copper grids at 120 kV acceleration voltage and the images were taken with a CCD camera. Cryogenic TEM imaging was performed under liquid N2 cryo conditions on holey carbon-coated copper grids after freezing in liquid propane.

Drug encapsulation efficiency

Camptothecin entrapment was determined by HPLC. One milligram of drug-loaded polymersomes was dissolved in 1 mL DMSO, diluted with an appropriate amount of PBS pH 6 and analysed using HPLC (Waters Millenium system with Novapak C18 waters column and spectrophotometric detection at 368 nm). The mobile phase consisted of 23% (v/v) acetonitrile and 77% triethylamine acetate buffer (1% (v/v) triethylamine in water, acidified to pH 5.5 with glacial acetic acid). The flow rate was set to 1 mL min−1. The entrapment efficiency of hydrophobic magnetic nanoparticles was determined by thermogravimetric analysis with a TGA Q500 instrument (TA Instruments).
Cell viability MTT assay

PC-3 cells, human prostate cancer cell line, were cultured in RPMI 1640 containing 10% (v/v) FBS and 1% (w/v) penicillin–streptomycin at 37 °C in a humidified 5% CO2–95% air atmosphere. In vitro cytotoxicity was evaluated using the MTT assay, based on mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into blue formazan crystals by viable cells.48 PC-3 cells were seeded at a density of 5000 cells per well in 96-well plates and were allowed to adhere overnight. The cells were washed with PBS and incubated for 72 h in a fresh medium containing various concentrations of free camptothecin and polymersome formulations. The supernatant was carefully removed and cells were washed twice with PBS. 100 μL of media and 20 μL of a 5 mg mL−1 MTI solution were added and incubated for 3 more hours. The supernatant in each well was aspirated and 150 μL of DMSO added to solubilize the cells and MTT crystals. After 1 h of shaking on an Eppendorf Thermomixer at 37 °C and 400 rpm to dissolve all crystals, absorbance at 540 nm was measured in a multiwell scanning spectrophotometer.

Relaxometry measurement of magnetic polymersomes

Proton relaxation rates of a series of sample concentrations were measured at 25 °C using a Bruker Minispec mq60 NMR Analyzer (Bruker, Ettlingen, Germany) operating at a magnetic field of 1.4 T. Proton transverse relaxation rates (R2) were determined from fitting a monoexponential decay curve to signal data generated by a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with an echo spacing of 1 ms and a repetition time of 5 s. Proton longitudinal relaxation rates (R1) were determined from fitting a monoexponential recovery curve to signal data generated by an inversion recovery (IR) pulse sequence using 10 logarithmically spaced inversion times between 10 and 10 000 ms. Three measurements of R1 and R2 were made for each sample and the results averaged.

Iron concentrations were verified via inductively coupled plasma atomic emission spectrometry following acid digestion. Longitudinal and transverse relaxivities, r1 and r2, were determined from a linear fit of relaxation rates versus iron concentration expressed in mM. All samples showed a linear variation of R1 and R2 versus concentration.

Carboxylation of Pluronic® L-121

Esterification of Pluronic® L-121 was performed with a 20 fold excess of succinic anhydride in THF with stirring at 80 °C for 72 h. After purification by dialysis in THF and vaporization, IR analysis showed the typical carbonyl peak between 1670 and 1820 cm−1 and confirmed the successful conversion of hydroxyl into carboxyl end-groups.

Alexa Fluor® 647 labelling and [Lys3]-bombesin conjugation

Polymersomes (2 g L−1) self-assembled from carboxylated Pluronic® L-121 were activated with EDC hydrochloride (5 mM) and NHS (5 mM) in 0.1 M MES buffer pH 5.5 for 1 h at 20 °C. The reaction mixture was adjusted to pH 7 with 0.2 M borate buffer and [Lys3]-bombesin (0.3 mM) was added and shaken (700 rpm, Eppendorf Thermomixer) overnight. The remaining active ester groups were reacted with ethylenediamine (1.5 mM) for 6 h. Purification of [Lys3]-bombesin conjugated polymersomes was performed by dialysis (MWCO: 12 kDa). Purified [Lys3]-bombesin polymersomes were fluorescent labelled with Alexa Fluor® 647 NHS ester according to manufacturer’s protocol. Control samples without targeting ligand were prepared identically except instead of [Lys3]-bombesin an equal volume of water was added. The fluorescence intensity of Alexa Fluor® 647 polymersome samples differs less than 10% as confirmed by fluorescence spectrometric analysis.

Quantification of peptide attached to the polymersome surface

Determination of conjugated [Lys3]-bombesin was performed by HPLC quantitative amino acid analysis according to Bartolomeo and Maisano with slight modifications.26 Briefly, polymersome samples were treated with 5 N HCl at 110 °C overnight to hydrolyse the peptide. After cooling, neutralisation and filtration through 0.45 μm filters (LCR, Millipore), pre-column derivatisation with Fluoraldehyde reagent solution was carried out as follows: 50 μL borate buffer and 50 μL sample were mixed for 30 s, 50 μL OP solution was added and mixed for 90 s, 600 μL sodium acetate buffer (50 mM, pH 7) was added and mixed for 30 s. Sample analysis using a HPLC Waters Millenium system with Novapak C18 waters column and spectrophotometric detection at 338 nm started immediately after derivatisation. The mobile phase consisted of (A) 50 mM sodium acetate buffer pH 7 and (B) acetonitrile (gradient starting after 7 min A with 10% acetonitrile and ending at 80% acetonitrile after another 5 minutes). The injection volume was 10 μL and the flow rate was set to 1 mL min−1. Calibration was performed with glutamic acid and recovery rate for the hydrolysis step was determined with the tripeptide glutathione.

In vitro cell binding and cell uptake study

The cellular binding and uptake behaviour of [Lys3]-bombesin-conjugated and Alexa Fluor® 647-labeled polymersomes was analysed by flow cytometry and confocal laser scanning microscopy. Furthermore, an in vitro uptake assay using a fluorescence spectrometer was performed which enables to distinguish between surface-bound and internalized polymersomes. PC-3 cells were seeded in 12-well plates (1.5 × 107 cells per well) and were allowed to adhere overnight. Cells were washed twice with PBS and incubated with Alexa Fluor® 647-labeled polymersomes (0.3 g L−1 polymer concentration) ([Lys3]-bombesin-conjugated and non-functionalized, respectively) in PBS supplemented with 5% BSA at 37 °C, 5% CO2 for 2 h. Blocking experiments to confirm the receptor specificity of functionalized polymersomes were performed with a pre-incubation with excess of free [Lys3]-bombesin (15 μM).

For the flow cytometry measurements cells were washed 3 times with PBS and detached with 2.3 mM EDTA in PBS and analysed with a BDTM LSR II Flow Cytometer (BD Biosciences, USA). Data were analysed with Cylogic software (Cyfluo Ltd, Finland). For the CLSM study cells were seeded on polylsine
coated coverslips, after 3 times washing cells were fixed with 4% paraformaldehyde, stained with WGA fluorescein conjugate, according to manufacturer’s protocol, and mounted onto glass microscope slides with DAPI containing Prolong® Gold mounting medium. Images were acquired with an Olympus FV10i confocal microscope.

For the in vitro uptake assay (n = 6) after incubation and washing, cells were treated with glycine–HCl pH 2.2 to remove surface-bound polymersomes. Surface fraction was collected and fluorescence intensity (Ex 630 nm, Em 650 nm, BioTec Synergy Multi-Mode Microplate Reader (Winooski, CT)) was measured. Cells were lysed with 0.3 M NaOH and analysed as well. Data are reported as uptake (%) = (fluorescence intensity in the cell lysate)/(total fluorescence intensity of polymersomes added into the cell culture).

Conclusions
Magnetic polymersomes have a great potential for both diagnostic and therapeutic applications. Diagnostically, tracking drug carriers inside the body, for example by MRI to ensure therapeutic monitoring, would be a huge benefit in nanomedical development. Therapeutically, hybrid polymersomes filled with magnetic nanoparticles can also be valuable tools for hyperthermia treatment, magnetic drug release triggering and magnetic targeting.

The here-presented magnetic and drug loaded polymersomes show enhanced anti-cancer activity against prostate cancer cells compared to the free drug formulation. The feasibility of encapsulation of both hydrophobic and hydrophilic components allows the design of highly effective complex drug formulations, where synergistic effects between the single components can be exploited and efficacy can be further enhanced. The MR contrast properties enable the tracking of polymersomes inside the body. Biodistribution and target uptake studies as well as investigations on the therapeutic efficacy in vivo are in progress.

The continuous and reliably controllable straightforward preparation method with micromixer technology enables the production of well-defined nanocarriers that can be individually designed. The covalent attachment of targeting moieties, in combination with magnetic guidance of the drug carriers, will help to modulate their biodistribution, minimize unwanted side effects and maximize the therapeutic effects in the diseased areas. Magnetic polymersomes as theranostic nanomedicines are suitable systems for monitoring drug delivery, which will provide real-time feedback on target site localisation. Regarding personalized medicine a pre-screening with individual validation and optimization of the delivery system can be performed. Patients showing high levels of target site accumulation could then be treated safely with highly potent drugs. Thus, therapeutic response to the targeted therapy can be improved, whereas risk of treatment is minimized.

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Notes and references