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Biomacromolecules, **Just Accepted Manuscript** • DOI: 10.1021/acs.biomac.5b01354 • Publication Date (Web): 21 Dec 2015

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ABSTRACT

Nano-carriers with carbohydrates on the surface represent a very interesting class of drug delivery vehicles since carbohydrates are involved in bio-molecular recognition events *in vivo*. We have synthesized biocompatible miktoarm star copolymers comprising glycopolypeptide and

poly(ϵ -caprolactone) chains, using ring opening polymerization and ‘click chemistry’. The amphiphilic copolymers were self-assembled in water into morphologies such as nanorods, polymersomes and micelles with carbohydrates displayed on the surface. We demonstrate that, the formation of nanostructure could be tuned by chain length of the blocks and was not affected by the type of sugar residue. These nanostructures were characterized in detail using a variety of techniques such as TEM, AFM, cryogenic electron microscopy, spectrally resolved fluorescence imaging and dye encapsulation techniques. We show that it is possible to sequester both hydrophobic as well as hydrophilic dyes within the nanostructures. Finally, we show that these non-cytotoxic *manno*-sylated rods and polymersomes were selectively and efficiently taken up by MDA-MB-231 breast cancer cells demonstrating their potential as nanocarriers for drug delivery.

INTRODUCTION

Synthetic glycopolypeptides, polypeptides with pendent carbohydrate units, have generated lot of interest in the area of biomedical applications owing to the prominent role played by carbohydrates in biology.¹⁻⁴ They are expected to be biocompatible as well as biodegradable and hence a desirable alternative to other synthetic glycopolymers.⁵⁻¹⁰ Glycopolypeptides model natural glycoproteins which are involved in regulation of cellular processes through polyvalent interaction with carbohydrate binding receptor proteins (lectins) that are observed on cell surfaces.¹¹⁻¹⁶ The overexpression of specific carbohydrate receptors on certain cancer cell surfaces such as the galactose binding asialoglycoprotein (ASGPR) receptor on liver cancer cells have stimulated the development of galactose containing macromolecules for drug delivery.¹⁷⁻²¹ Similarly, it is also observed that the mannose binding receptor MRC2 (mannose receptor C-type 2) is overexpressed on dendritic cells, macrophages and MCF-7/ MDA-MB-231 breast cancer

cells, a fact that suggests a possible targeting of drugs into these cells.²²⁻²⁵ As a result nanoparticles (viz. micelles, vesicles and nanorods etc.) with carbohydrates on the surface represent a very interesting class of nano-carriers in targeted drug delivery.²⁶⁻³³ However, engineering safe and efficient nanocarriers for drug delivery systems requires a higher level of understanding of the interaction between nanomaterials and biological components (proteins, plasma membranes, endocytic vesicles and organelles). A small alteration in physicochemical characteristics of nanomaterials can drastically influence the mechanisms of cellular uptake and ultimately intracellular fate.³⁴ Recent observations in biological systems suggest that particle shape has been considered to play an important role in cellular interactions with nanoparticles and shown to be important for certain aspects of drug delivery.³⁵⁻³⁷ For example, the rod-like particles get an appreciable advantage when it comes to internalization rates.³⁸ Discher et al. have demonstrated that filomicelles (long cylindrical micelles) had longer circulation times than spherical micelles because they are extended under flow conditions and hence not taken up by the cells.³⁹ However, the limited synthesis and information regarding the effects of particle shape on cellular responses hinder the use of nanomaterials for therapeutic applications. With this background, it would be interesting to investigate the effect of shape of carbohydrate-decorated nanostructures on cell uptake since they undergo receptor-mediated endocytosis. So far there is only one report in the area of glycopolymers that studied the cellular uptake of spherical and rod-like assemblies comprising acrylate copolymer blends.⁴⁰ Therefore, synthesis of glycopolypeptide-based amphiphilic polymer architectures and their self-assembly in aqueous solution in order to control the shape and morphology of the nanostructure is an important theme in this endeavor.^{10, 41-43}

We have recently reported controlled generation of nanostructures (micelles, nanorods and polymersomes) in solution from amphiphilic glycopolyptide-based block copolymers.^{41, 43} Research groups of Lecommandoux, Heise and Deming have reported spherical morphologies by tuning block lengths of linear and graft copolymers with glycopolyptide as one of the blocks wherein the hydrophobic block is also made up of a polypeptide backbone.⁴⁴⁻⁴⁶ We hypothesized that incorporating a non-peptidic biocompatible hydrophobic backbone instead would widen the scope of the resultant polymer properties. Poly(ϵ -caprolactone) (PCL), a well-known biocompatible and biodegradable FDA-approved polymer^{47, 48} was chosen as the hydrophobic block for conjugation with glycopolyptide to obtain amphiphilic block copolymers. Examples on polymer assemblies as drug delivery vehicles incorporating PCL as the hydrophobic block abound in the literature.⁴⁹⁻⁵⁷

We present here our results on synthesis and self-assembly study of biocompatible amphiphilic glycopolyptide-poly(ϵ -caprolactone) [nGP-(PCL_m)₂] star copolymers as potential candidates for targeted drug delivery vehicles. The self-assembly of these polymers will be influenced by the helical nature of hydrophilic glycopolyptides and hydrogen bonding interactions among them as well as by semi-crystalline nature of hydrophobic polycaprolactone block. Therefore, to understand their structure-property relationship, we have synthesized star copolymers with varied chain lengths of the both blocks, and compared the morphologies obtained by their solution assembly. We demonstrate controlled formation of morphologies of importance such as nanorods, and polymersomes along with spherical micelles from various nGP-(PCL_m)₂ conjugates (Scheme 1). We show that nanorods and polymersomes are able to encapsulate both hydrophobic and hydrophilic dyes as model drugs. Furthermore, the presented

synthetic methodology can be extended to formation of potential drug carriers with low cytotoxicity and the ability to target specific cancer cells.

Scheme 1: General chemical structures of $n\text{GP}-(\text{PCL}_m)_2$ star copolymers and nanostructures obtained by tuning the hydrophilic and hydrophobic block length.

EXPERIMENTAL SECTION

Materials and Methods

HAuCl_4 , triphosgene and propargyl amine were obtained from Aldrich. All other chemicals used were obtained from Merck, India. FT-IR spectra were recorded on Perkin Elmer FT-IR spectrum GX instrument by making KBr pellets that were prepared by mixing 3 mg of sample with 97 mg of KBr. ^1H NMR spectra were recorded on Bruker Spectrometers (200 MHz, 400 MHz or 500 MHz). ^{13}C NMR and DEPT spectra were recorded on Bruker Spectrometer (50 MHz, 100 MHz or 125 MHz) and signals relative to deuterated solvent are reported. Gel permeation

chromatography/light scattering (GPC/LS) was performed on a VISKOTEK TDA 305-040 TRIPLE DETECTOR ARRAY refractive index (RI), viscometer (VISC), low angle light scattering (LALS), right angle light scattering (RALS) GPC/SEC MODULE. Separations were achieved by three columns (T6000M, GENERAL MIXED ORG 300X7.8 MM) and one guard column (TGAURD, ORG GUARD COL 10X4.6 MM), 0.05 M LiBr in DMF as the eluent at 60 °C. GPC/LS samples were prepared at concentrations of 5 mg/mL. System was calibrated by PMMA standards. A constant flow rate of 1 mL/min was maintained. GPC of all the polycaprolactone polymers was performed in the Agilent chromatogram, Model No: PL-GPC 220, using chloroform as running solvent at a constant flow rate of 1 mL/min. Transmission Electron Microscopy (TEM) measurements were performed at 200 kV on an FEI Technai F20 and F30 instrument. The cryo-TEM of samples was performed in a dedicated TEM of model Titan 80-300 Krios from FEI Company (Hillsboro, OR) by operating it at the accelerating voltage of 300 keV (Details are provided in supporting information). Atomic Force Microscopy (AFM) analysis was performed on Asylum MFP-3D instrument. Fluorescence experiments were carried out on fluorimeter QM40 from Photon Technology International, Inc.

Synthesis of glycopolyptide-polycaprolactone $[nAcGP-(PCL_m)_2]$ copolymers by “click reaction”

To a solution of alkyne end-functionalized acetyl protected glycopolyptide in THF/methanol/water/DMSO (2:1:0.2:1) was added azide functionalized polycaprolactone (0.9 eq) and resultant reaction mixture was degassed by three freeze-pump-thaw cycles. Premixed $CuSO_4 \cdot 5H_2O$ (0.2 eq), Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (0.2 eq) solution and sodium ascorbate (0.4 eq) were added and the reaction was allowed to proceed for 24 h at room temperature under argon atmosphere.⁵⁸ The progress of the reaction was monitored by the

disappearance of the azide stretch at 2115 cm^{-1} in FT-IR. When >95% of the azide had disappeared, the solvent was removed under reduced pressure. Crude product was dissolved in ethyl acetate and washed multiple times with 1N HCl followed by 5wt% disodium ethylenediamino tetraacetate (EDTA-Na salt) solution to remove the copper salt. Solvent was evaporated completely under reduced pressure and the residue was dried under vacuum overnight at $45\text{ }^{\circ}\text{C}$ to afford polymers as off-white solids.

Deprotection of acetyl protecting group of [nAcGP-(PCL_m)₂] co-polymers

Hydrazine monohydrate (25 equiv) was added to the solutions of acetyl protected nAcGP-(PCL_m)₂ block co-polypeptides in THF and the reactions were stirred for 7-8 hrs at room temperature (Scheme S2). Reactions were quenched by addition of acetone and then solvent was removed almost completely under reduced pressure. The solid residues were dissolved in a mixture of DMSO-deionized water (1:1), filtered through $0.45\text{ }\mu\text{m}$ filter paper and transferred to dialysis tubing (12kDa MWCO). The samples were dialyzed against deionized water for 3 days, with water changes once every two hours for the first day, and then thrice per day. Dialyzed polymer was lyophilized to yield glycopolypeptide-polycaprolactone conjugates as white fluffy solids (~80% yield).

Assembly of deprotected nGP-(PCL_m)₂ co-polymers in aqueous solution

Fully deprotected polymers [nGP-(PCL_m)₂] were dissolved in dimethyl sulfoxide and diluted by water to dimethyl sulfoxide: water = 1:1 so that final polymer concentration was 0.5 mg/mL. The homogeneous solution was filtered with $0.45\text{ }\mu\text{m}$ filter paper and dialyzed thoroughly against deionized water for 2 days changing water every 8 hours.

Dye encapsulation into nanostructures

Solution of the copolymer in DMSO (0.5 mg/mL) was added to DMSO solution of calcein (4 mg/mL) in water and diluted to 1 mL by gradual addition of DMSO-water mixture so that final solvent composition was 1:1 v/v. The solution was kept for 4 h under continuous stirring. The mixture was placed into a dialysis membrane (12kDa molecular weight cut-off) and then dialyzed against water for 24 h while changing deionized water at 4 h interval. Then it was dialyzed against phosphate buffer (pH 8.0) containing 50 mM phosphate, 50 mM NaCl, 0.2 mM di-sodium salt of EDTA. Within 16 hrs all the unencapsulated Calcein came out of from inside the dialysis membrane. After the removal of free Calcein the mixture was dialyzed against deionized water for 24 h with the water being changed 3 times.

Solution of copolymer in DMSO (0.5 mg/mL) was diluted to 1 mL by gradual addition of DMSO and water mixture to get final solvent composition 1:1 v/v. The solution was kept for 4 h under continuous stirring. It was then transferred to the dialysis tube (12kDa MWCO) and dialyzed against water for 24 h while changing deionized water at 4 h interval. Then, 10 μ L of the dye Nile Red or Rhodamine B octadecyl ester (RBOE) (1 mg/mL in DMSO) was added to the assemblies and kept for 1 h for encapsulation. This solution was filtered through 0.45 μ m filter paper before analysis.

Spectrally resolved fluorescence imaging of nanorods

A home-built spectrally resolved fluorescence imaging setup based on an inverted optical microscope (Nikon TE2000U) was used to obtain fluorescence image as well as spatially resolved emission spectra. Details of the experimental setup and analysis are provided elsewhere

⁴³. In brief, a 532 nm DPSS laser (30 mW) was used to excite the sample through objective lens

(Nikon 60X, 1.49NA oil immersion). Typically, $\sim 50 \mu\text{W}$ excitation power (adjusted using neutral density filters and measured just before objective) was used to illuminate $\sim 40 \times 40 \mu\text{m}^2$ area of the sample. The fluorescence emission was collected using the same objective, and passed through a dichroic mirror and emission filters (Semrock) and eventually imaged through a cooled interline CCD camera (DVC 1412AM) at exposure time of 50ms. To obtain spectral image, the fluorescence emerging from the sample was dispersed using a combination of an adjustable slit and a transmission grating (70 g/mm) placed in front of the CCD. All spectral images were obtained at identical excitation powers ($\sim 3 \text{ W/cm}^2$) and 300 ms integration time at 295K. Background corrected spectral images were used to extract spectral profiles and pixel to nm calibration performed using several laser lines. The spectral profiles were further corrected for the CCD response. 20 μl of Nile red loaded nanorods (in water) was drop cast on pre cleaned glass coverslips 201 (Fisher Scientific 25 $\times$ 25 mm) and solvent was evaporated at room temperature in dark. Control experiments were performed to optimize concentration such that the well separated spots of rods can be observed.

In vitro cytotoxicity assay

MDA-MB-231 cells were seeded in a flat bottomed 96-well plate at a density of 1×10^4 cells/well in DMEM containing 10% FBS. The plate was incubated at 37 °C with 5% CO₂ for 24 h. Polymersomes and nanorods were prepared and added to make a final concentration of 10, 20, 40, 60, 80, 100, 200, 300 $\mu\text{g/mL}$, respectively in serum free DMEM. Cells were further incubated for 4 h at 37 °C with 5% CO₂, and then the medium was replaced with DMEM containing 10% FBS. After another 40 h at 37 °C with 5% CO₂ incubation, the media was removed and 110 μL solution of DMEM containing 10% FBS with filter sterilized MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.45 mg/mL) was added into

each well and further incubated at 37 °C with 5% CO₂ for 4 h. At the end of incubation media was aspirated from the wells and 100 µL DMSO was added to dissolve insoluble Formosan crystals formed. The absorbance was measured at 550 nm using a microtitre plate reader. The relative % cell viability was calculated from the following equation: Relative percent cell viability = ($A_{\text{test}}/A_{\text{control}}$) × 100% (A_{test} is the absorbance of the sample treated cells and A_{control} is the absorbance of the untreated cells. Each absorbance was taken to be the mean of triplicate measurements). The cell viability was represented as a percentage relative to untreated cells as a control.

Cellular uptake study using Epifluorescence Microscopy

MDA-MB-231 cells were seeded in 24 well plate at a density of 50,000 cells/well in DMEM containing 10% FBS. The plate was incubated at 37 °C with 5% CO₂ for 24 h. After incubation media was replaced with 100 µg/mL of RBOE encapsulated *manno*-sylated polymersomes and nanorods respectively and incubated for 2 h at 37 °C with 5% CO₂. The concentration of encapsulated RBOE was measured by UV-Vis measurement and according to that fluorescent intensity data was normalized. To examine the carbohydrate specific endocytosis of polymersomes and nanorods, the cells were treated with 0.5 mM mannose/glucose/galactose for 1.5 h. Then RBOE encapsulated *manno*-sylated polymersomes and nanorods at concentration of 100 µg/mL were loaded onto cells and incubated for 2 h. After incubation finished, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. Nucleus was stained with DAPI. Images were acquired using Epifluorescence Microscope by Carl Zeiss. Mean fluorescent intensity difference were calculated by using imaging software ZEN PRO 2012 (From Carl ZEISS), the contour tool in graphics has been selected to draw the cell boundary which gives mean intensity value for each channel used to acquire particular image. All images for RBOE are

acquired by keeping 500 ms exposure time throughout the assay and at least 50 cells from different areas were chosen to calculate fluorescent intensity distribution.

Energy dependent cellular uptake study: MDA-MB-231 cells were seeded in 24 well plates at a density of 50,000 cells/well in DMEM containing 10% FBS. The plates were incubated at 37 °C with 5% CO₂ for 24 h. After incubation, the media was replaced with 100 µg/mL of RBOE encapsulated polymersomes and incubated with 5% CO₂ for 2 h either at 37 °C or at 4 °C. After the duration of incubation, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. Nucleus was stained with DAPI. Images were acquired using Epifluorescence Microscope. Fluorescent intensity distribution was calculated by following previously stated method.

Comparison of cellular uptake of *manno*-sylated and *galacto*-sylated polymersomes: MDA-MB-231 cells were seeded in 24 well plates at a density of 50,000 cells/well in DMEM containing 10% FBS. The plates were incubated at 37 °C with 5% CO₂ for 24 h. After incubation, the media was replaced with 100 µg/mL of RBOE encapsulated *manno*-sylated polymersomes or the RBOE encapsulated *galacto*-sylated polymersomes (control). The concentration of encapsulated RBOE in both samples was measured by UV-Vis measurement and according to that fluorescent intensity data was normalized. Both plates were incubated for 2 h at 37 °C with 5% CO₂. After the duration of incubation, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. Finally, Nucleus was stained with DAPI. Images were acquired using Epifluorescence Microscope. Fluorescent intensity distribution was calculated by following previously stated method.

RESULTS AND DISCUSSION

Synthesis and characterization of $n\text{GP}-(\text{PCL}_m)_2$ copolymers

Establishing structure-property relationship for these new copolymers, particularly in the context of self-assembly is important in view of potential future applications in drug delivery. Therefore, $n\text{GP}-(\text{PCL}_m)_2$ copolymers were synthesized in a modular fashion so that molecular parameters of each block could be tuned independently and their effect on morphologies could be studied.

a) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Na-ascorbate, THPTA, THF/Methanol/ H_2O /DMSO, b) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ /THF

Scheme 2: Synthesis of $n\text{GP}-(\text{PCL}_m)_2$ miktoarm star copolymers

A series of miktoarm star copolymers were synthesized by coupling linear GP chains with branched PCL chains. Hydrophilic weight fraction (W_{philic}) is an important parameter used to control the morphology in aqueous solution.^{59, 60} Two chain lengths of 25 and 50 were targeted for each arm of the branched PCL so that W_{philic} would be maintained the same by connecting it to GP of appropriate chain length. Branched PCL was designed with an azide at the junction of the two arms. For the GP block, three different chain lengths were targeted each with a DP of 10, 20 and 40. When GP with 10 and 20 units is attached to branch PCL with 25 and 50 repeat units, respectively, two miktoarm star copolymers with same W_{philic} but one having twice the chain

lengths as the other will be obtained. Thus, this design allows us to study the effect of chain length within the same polymeric architecture.

Table 1: Molecular parameters of the synthesized acetyl protected nAcGP-(PCL_m)₂ star copolymers. n = average chain length of *manno*-GP, m = average chain length of each arm of branched PCL.

Entry no.	Protected nAcGP-(PCL _m) ₂ polymer	M _n ^a (×10 ³ g/mol) of GP	DP ^b of GP	M _n ^a (×10 ³ g/mol) of PCL	DP ^b of PCL (branched)	Deprotected nGP-(PCL _m) ₂ polymer
1	10AcGP-(PCL ₂₅) ₂	5.6	10	12.4	26×2	10GP-(PCL ₂₅) ₂
2	20AcGP-(PCL ₂₅) ₂	11.6	21	12.4	26×2	20GP-(PCL ₂₅) ₂
3	20AcGP-(PCL ₅₀) ₂	11.6	21	23.0	50×2	20GP-(PCL ₅₀) ₂
4	40AcGP-(PCL ₅₀) ₂	22.1	40	23.0	50×2	40GP-(PCL ₅₀) ₂

^aNumber average molecular weight calculated from GPC. ^bDP was calculated from ¹H NMR.

Branched PCL blocks were synthesized by ring opening polymerisation (ROP) of ε-caprolactone from an azide functionalized dihydroxy initiator. GPs containing pendent acetyl protected-glycosyl unit and an alkyne at the chain end were synthesized by following our previously developed methodology that involves propargyl amine initiated ROP of the glycosyl-*N*-carboxyanhydride (NCA) monomer (Table S1).¹⁹ Glycopolypeptides with different sugar residues *viz.* mannose and galactose were selected to investigate their influence on morphology. The NCA monomer was synthesized from *l*-lysine as has been described before.¹⁹ All the copolymer building blocks, alkyne-GP and azide-PCL, had low PDI (Figure S1 and S2) and chain length of each polymer was determined from ¹H NMR spectra using end group analysis

(Table 1). Alkyne terminated GPs were then coupled with azide functionalized branched PCLs by using Cu-catalyzed azide alkyne cycloaddition (CuAAC) reaction - the most well-known member of the ‘click chemistry’ as shown in Scheme 2. We have performed a SEC analysis of the nAcGP-(PCL_m)₂ block copolymer to analyze efficiency of the click reaction. The GPC of the “clicked” product (Figure S3) displayed a new peak at higher molecular weight which was distinctly shifted from the parent glycopolypeptide and azide-(PCL₂₅)₂ suggesting the formation of the coupling product. In addition, the GPC curve shows that more than 90% of the starting materials was consumed and a very small shoulder of starting glycopolypeptide was observed. The trace amount of glycopolypeptide remained since we have used 0.9 equivalent of azide-(PCL₂₅)₂ with respect to glycopolypeptide during the click reaction. Dialysis (using 12 kDa dialysis membranes) of the deprotected coupling product is expected to completely remove the excess of glycopolypeptide (molecular weight of free glycopolypeptide is 3.5 or 7 kDa). Moreover, the complete absence of the resonances characteristic of both azide and alkyne containing starting materials in NMR (Figure S29-32) and IR analysis (Figure S4) suggest near quantitative click reaction. The acetyl groups on the GP block were deprotected using hydrazine hydrate under very mild conditions without affecting the polycaprolactone chains to afford the desired, nGP-(PCL_m)₂ star copolymers with free sugar units (Table 2). Helical nature of the GP block in the final copolymers was confirmed by circular dichroism measurements in aqueous solution, which displayed two minima at 208 and 222 nm, characteristic of the α -helix motif (Figure S5). Structural parameters for all copolymers are given in (Table 2).

Table 2: Synthesized acetyl deprotected nGP-(PCL_m)₂ block copolymers and nanostructures observed in TEM analysis of the self-assembled polymers at 0.5 mg/mL in DI water. n = average chain length of *manno*-GP, m = average chain length of each arm of branched PCL.

Entry no.	Deprotected polymer	% helicity ^a of GP block	Hydrophilic weight fraction (W_{philic})	Morphology obtained
1	10GP-(PCL ₂₅) ₂	23	36%	Nanorod
2	20GP-(PCL ₂₅) ₂	33	53%	Vesicle
3	20GP-(PCL ₅₀) ₂	32	37%	Sheet, tapes
4	40GP-(PCL ₅₀) ₂	52	54%	Micelle

Self-assembly of nGP-(PCL_m)₂ copolymer in aqueous medium

The synthesized nGP-(PCL_m)₂ amphiphilic copolymers were dissolved in DMSO and equal volume of water was added so that polymer concentration reached 0.05 wt%. This clear solution was dialyzed thoroughly against deionized water to obtain a translucent solution in pure water. A drop of this solution was placed on the carbon coated copper grid and negatively stained with uranyl acetate (0.2 wt%) solution. When the solution of 10GP-(PCL₂₅)₂ was imaged using transmission electron microscopy (TEM), nanorods with an average diameter of ~16 nm were observed (Figure 1a). These nanostructures that appear to be hollow are more than hundred nanometers in length and are stable in aqueous solution for about two weeks. Using Cryo-TEM we were able to image the nanorods in their native solution state (Figure 1b and Figure S6) and the extracted average diameter from these measurements was found to be ~18 nm.

Figure 1: (a) TEM images of 0.05wt% aqueous solution of 10GP-(PCL₂₅)₂ in water with uranyl acetate as a negative stain, (b) cryo-TEM image shows single rod as well as aggregates of rods.

When the star copolymer with twice the GP chain length, 20GP-(PCL₂₅)₂, was analyzed using TEM well-dispersed polymersomes with a characteristic dark rim were observed (Figure 2a). These had an average diameter of 55±10 nm. Under cryogenic transmission electron microscopy, 20GP-(PCL₂₅)₂ showed vesicular morphology and the measured average diameter corroborates well with that from TEM measurement (Figure 2b). Further evidence for vesicular morphology was obtained from atomic force microscopy (AFM) analysis on silicon wafer (Figure S7). It revealed a thickness of 6-8 nm for the spherical structure that gives a height/diameter ratio of ~0.08, close to that expected for a hollow vesicular nanostructure.⁶¹

Figure 2: (a) TEM image and (b) cryo-TEM image of 20GP-(PCL₂₅)₂ star copolymer in aqueous solution (0.5 wt%). Inset of (b) indicating polymersomes morphology.

Self-assembly of nGP-(PCL_m)₂ star copolymers comprising both GP and PCL blocks with twice the chain lengths as that in the above polymers was then studied. Polymer 20GP-(PCL₅₀)₂ displayed sheets and tape kind of morphology (Figure S8a and S8b) although its W_{philic} is same as that of 10GP-(PCL₂₅)₂, which forms nanorods. On the other hand, star copolymer 40GP-(PCL₅₀)₂ exhibited well-dispersed micelles with average diameter of 40 nm in TEM analysis (Figure S8c). This polymer has same W_{philic} as that of 20GP-(PCL₂₅)₂ that displays vesicles. The above result suggests that, hydrophilic to hydrophobic ratio is not the only parameter that controls the assembly of these copolymers; there are possibly other parameters that actually influence the assembly process. Now, a look at the structural parameters of four copolymer structures in Table 2 reveals that the helicity of GP block increases from 23% for 10GP-(PCL₂₅)₂ to ~32% for 20GP-(PCL₅₀)₂ and 20GP-(PCL₂₅)₂ and then to 52% for 40GP-(PCL₅₀)₂. Based on our previous study, the structure of the copolymer 10GP-(PCL₂₅)₂ (with both GP and PCL chains of shorter length) is expected to be roughly helix-coil where the helical GP

segment appears to be quite flexible and dynamic in solution.⁴³ We think that the flexible helical conformation of the GP segment would certainly provide efficient packing at the surface of nanorods accompanied by shift in the conformational equilibrium towards the helical structures during the assembly process resulting in a morphology bearing lower curvature. Similar nanorod formation had been observed for self-assembly of short GP helix-dendron conjugate as reported by our group earlier.³⁹ Even though helicity increases with increasing chain length of GP block, assemblies with higher curvature such as vesicles and micelles are obtained for 20GP-(PCL₂₅)₂ and 40GP-(PCL₅₀)₂, respectively (Figure S8c). Similar trend was observed in our earlier report wherein GP-dendron conjugate with longer GP block formed micelles.⁴¹ The role of PCL chains in the formation of these assemblies is also expected to be significant. Although, PCL is crystalline even at low molecular weights, the extent of crystallinity depends on the nature of copolymer and the perfection of crystalline domain generally increases with chain length.⁶² Thus, there appears to be an intricate interplay of three factors *viz.* W_{philic} , helicity of GP block and crystallinity of PCL chains that governs the formation of the observed morphologies for these star copolymers.

The effect of sugar residue on the assembly into nanostructures was studied using star copolymers containing galactose *viz.* *galacto*-20GP-(PCL₂₅)₂. The star copolymers formed polymersomes similar to the mannose-containing analogue suggesting that varying the sugar isomers does not influence the self-assembly (Figure S9). This observation is similar to that in our earlier report on dendron-conjugated GPs.⁴¹

Among all the nGP-(PCL_m)₂ conjugates studied, 10GP-(PCL₂₅)₂ and 20GP-(PCL₂₅)₂ generate interesting morphologies in the form of hollow nanorods and polymersomes, and hence we were interested to probe if these nanostructures were able to encapsulate both hydrophilic as

well as hydrophobic dyes. Such studies are important for understanding the utility of these materials for drug delivery applications. Calcein, a hydrophilic dye, was added during the self-assembly process and hence was expected to be encapsulated in the hydrophilic interior of the assemblies. UV-vis spectra of calcein-encapsulated polymersomes show a characteristic peak (λ_{max}) at 493 nm (Figure S10a). The encapsulation efficiency (EE) was determined using the equation $EE = (W_1/W_2) \times 100$, where, W_1 is the amount of calcein encapsulated and W_2 is the amount of calcein taken in the feed. The value of W_1 was calculated from the absorbance at 493 nm (extinction coefficient for calcein in water is $76000 \text{ M}^{-1} \text{ cm}^{-1}$), from which the encapsulation efficiency of calcein was estimated to be $\sim 7.1\%$.

The assemblies obtained from 10GP-(PCL₂₅)₂ are also capable of encapsulating a dye-labeled hydrophilic macromolecule, FITC-Dextran (mol. wt. = 3.5 kD) (Figure S10a), which clearly indicates that the rod-like structures are hollow. To verify the encapsulation of hydrophobic dyes within the bilayer membrane of rod structure, spectrally resolved fluorescence microscopy was performed on Nile red-loaded nanorods, drop-cast on a glass substrate. Figure 3 (a-c) shows the fluorescence intensity image obtained using 532 nm excitation of an area of the sample where several individual bright spots can be visualized over dark background. It was observed that majority ($\sim 70\%$) of these bright emission spots are diffraction-limited (≤ 200 nm) while others resemble long streaks of few microns. It should be noted that the rods with size less than ~ 200 nm appear as dots while the longer rods or agglomerates of several smaller rods appear as one dimensional streak which are brighter due to large amount of dye therein. Irrespective of the shape (dots or long streaks) that appears in the fluorescence image, all of these bright spots show characteristic Nile red spectra with emission maxima (λ_{max}) at ~ 625 nm. These spectra of Nile red within the bright spots are different from the free Nile red on the glass substrate ($\lambda_{\text{max}} \sim 605$

nm) as indicated by green arrow in the spectral image. Red shifted emission maxima ($\lambda_{\text{max}} \sim 625$ nm) of Nile red shows that it exists in non-polar microenvironment, consistent with the hydrophobic shell of the rods. Similar behavior was also observed in aqueous solution of Nile red encapsulated nanorods and polymersomes (Figure S11).

Figure 3: (a) Wide-field fluorescence intensity image of Nile red encapsulated 10GP-(PCL₂₅)₂ for an area of $\sim 22 \times 22 \mu\text{m}^2$ obtained using 532 nm excitation, a small area within the image is zoomed to indicate rod like structure, (b) Fluorescence spectrally resolved image from a region showing the spectral positions of Nile red in bright spots and in the background, (c) spectra from various such regions are plotted for integration time of 0.3s.

To test the stability of polymersomes, aqueous solution of calcein encapsulated 20GP-(PCL₂₅)₂ was then dialyzed against phosphate buffer solution for 2 days. Absorption spectra of the PBS outside the dialysis tube indicated leakage of small amount of dye and almost no leakage after the second dialysis cycle (Figure S10c). Dynamic light scattering (DLS) analysis of the polymersome solution before and after dialysis with PBS showed negligible change in size

from 55 nm to 40 nm (Figure S12). These two experiments together suggest that the vesicles are quite stable in buffer solutions with little leakage of cargo. We have then performed DLS experiment to see the aggregation behavior of the nanorods. The DLS data suggest that the rods are not in the aggregated state in aqueous solution (Figure S13a). To see that the nanorods are quite stable in biological medium also, DLS of the rods in DMEM media kept in sterile conditions was followed for 4 days. No precipitation was observed and only a very small fraction of aggregated nanorods was observed in DLS measurement (Figure S13b-c).

The availability of saccharides units at the surface of nanostructure was investigated by using turbidimetric assay (Figure S14). The protein ConA is known to specifically bind to mannosyl residues. In nanorods/polymersomes, multiple copies of the manno-sylated glycopolypeptides are displayed onto the outer surface and they are expected to interact “polyvalently” with the carbohydrate binding site of the ConA. Upon addition of nanorods/polymersomes solution to the ConA, the turbidity first increases with increasing concentrations and finally reaches a plateau. At this point, the turbidity does not increase with increasing concentration of nanorods/polymersomes and can be considered as an optimum value for the binding to ConA. This implies that the mannose moieties present at the surface of the nanorods/polymersomes are available to interact with cell-surface receptors.

Cell viability and cellular uptake study

Cellular entry experiments of polymersomes and nanorods were conducted to determine whether they were able to enter mammalian cells. Since cell viability of the polymersomes and nanorods are a precondition, we first performed cytotoxicity test (MTT assay) with *manno*-20GP-(PCL₂₅)₂ and *manno*-10GP-(PCL₂₅)₂ that self-assemble into polymersomes and nanorods, respectively.

The experiments were performed by incubation of the polymer assemblies with MDA-MB-231 cells for 40 h. Results of MTT assay show that both 10GP-(PCL₂₅)₂ and 20GP-(PCL₂₅)₂ were non-toxic up to 300 µg/mL since more than 85% of the cells remained viable at this concentration (Figure 4).

Figure 4: MTT assay for the polymer 10GP-(PCL₂₅)₂ and 20GP-(PCL₂₅)₂ that assembled into nanorod (red) and polymersomes (blue) respectively.

The uptake of RBOE encapsulated polymersomes and nanorods was investigated on MDA-MB-231 cells by epifluorescence microscopy (Figure 5). The results revealed the rapid cellular endocytosis within 2h for *manno*-sylated RBOE encapsulated polymersomes and nanorods at 100 µg/mL. The quantification of fluorescence intensity present inside the cells reveals that there is no effect of shape of the morphology on cellular uptake (~7% difference in normalized fluorescence intensity; Figure 5), the observation being similar to that in a recent report.⁴⁰

Figure 5: Cellular uptake of the RBOE encapsulated polymersomes and nanorods to the MDA-MB-231 cell lines: fluorescence microscopy image of cells treated with RBOE encapsulated polymersomes (a) and corresponding fluorescence intensity profile (b); fluorescence microscopy image of cells treated with RBOE encapsulated polymersomes (c) and corresponding fluorescence intensity profile (d). (Red color showing internalized RBOE and nucleus was stained with DAPI)

In order to study the receptors responsible for the uptake of *manno*-sylated polymersomes and nanorods, we have carried out competition assays using additional free mannose at the concentrations of 0.5 mM. Figure 6 displays the difference in uptake of *manno*-sylated polymersomes without and with the treatment of different sugar molecules. Although the added mannose did not block all of the internalization, the competition of free mannose decreased the

internalization by 37% compared with that without free mannose treatment. Free D-glucose led to decrease by 8% and free D-galactose showed a decrease by 10% in the uptake for 100 $\mu\text{g/mL}$ *manno*-sylated polymersomes. The same trend was also observed for *manno*-sylated nanorods uptake (Figure S17). These results suggest that there may be mannose receptors pathways for up taking these nano-assemblies.

Figure 6: Results of the cellular uptake analysis of the *manno*-sylated polymersomes with or without the competition of different free sugar molecules.

We next proceeded to study of the cellular internalization mechanism this would be critical in evaluating the therapeutic potential of these nano-assemblies. Endocytosis is an active process involving the energy obtained from hydrolysis of ATP.⁶³ However, in mammalian cells the ATP synthesis and utilization is maximum near 37°C and is known to be significantly less at 4°C. Therefore, the low temperatures can inhibit receptor mediated endocytosis significantly.⁶⁴ To determine whether the nano-assemblies were up taken via receptor mediated endocytosis process, *manno*-sylated polymersomes (RBOE encapsulated) were incubated with the MDA-MB-231 cells at different temperatures: 4°C and 37°C for 2 h. The quantification of fluorescence

intensity (Figure S18-19) present in the cells reveal that for uptake at 4°C, the fluorescence from RBOE in the polymersomes was only 58% in comparison to what was observed for uptake at 37°C (Figure 7). This reveals that the mechanism for cellular internalization of *manno*-sylated polymersomes by MDA-MB-231 cells is predominantly receptor mediated endocytosis.

Figure 7: The *manno*-sylated polymersomes uptake is an energy dependent process: fluorescence microscopy images of MDA-MB-231 cells were treated with RBOE encapsulated *manno*-sylated polymersomes at 37 °C (a), same at 4 °C (b) and fluorescence intensity analysis of the cellular uptake (c). (Red color showing internalized RBOE and nucleus was stained with DAPI)

The MDA-MB-231 cells are known to have overexpressed mannose specific MRC2 receptors. Hence receptor mediated endocytosis should show specificity of uptake with nano-assemblies bearing mannose groups in comparison to other carbohydrates. In order to verify this, the uptake

efficiency of *manno*-sylated polymersomes was compared with *galacto*-sylated polymersomes. MDA-MB-231 was incubated with RBOE encapsulated *manno*-sylated and *galacto*-sylated polymersomes at 37°C for 2 h respectively. The quantification of fluorescence intensity (Figure S20-21) present in the cells reveal uptake of *galacto*-sylated polymersomes, the fluorescence from RBOE in the polymersomes was only 54% in comparison to what was observed for uptake of *manno*-sylated ones (Figure 8). This result suggests the preference of the MDA-MB-231 cells for *manno*-sylated polymersomes.

Figure 8: The *manno*-sylated polymersomes were preferentially taken up by MDA-MB-231 cells with respect to *galacto*-sylated polymersomes: fluorescence microscopy images of MDA-MB-231 cells were treated with RBOE encapsulated *manno*-sylated polymersomes (a); MDA-MB-231 cells were treated with RBOE encapsulated *galacto*-sylated polymersomes (b) and

fluorescence intensity analysis of the cellular uptake (c). (Red color showing internalized RBOE and nucleus was stained with DAPI)

CONCLUSIONS

We have prepared a set of dial-in morphologies from biocompatible star copolymers based on mannose containing glycopolypeptides. The hydrophobic block in these copolymers comprises biodegradable non-peptidic backbone viz. poly(ϵ -caprolactone) unlike polypeptide blocks used in the literature reports on amphiphilic GP copolymers. A series of amphiphilic star copolymers with varied chain length of each block was synthesized in a modular fashion using ‘click chemistry’. Well-defined hollow rod-like, vesicular and micellar nanostructures were observed selectively for each polymer and their formation was attributed to the dynamics between hydrophilic-hydrophobic ratio, helicity of GP block and crystallinity of PCL. Assembly of control polymers with different sugar residue, galactose afforded same morphologies as mannose substituted polymers. Two self-assembled nanostructures, tubular nanorods and polymersomes, were studied in detail using electron microscopy, fluorescence microscopy and dye encapsulation, and shown to be non-cytotoxic using MTT assay thus demonstrating their potential for drug delivery applications. Preliminary studies showed that these *manno*-sylated polymersomes and nanorods were efficiently taken up by MDA-MB-231 breast cancer cells. These polymersomes and nanorods also display the carbohydrate-specific uptake towards MDA-MB-231 cells and do not show noticeable effect of shape of morphology. Therefore, these glycopolypeptide-based nanostructures could be useful for receptor-mediated targeted delivery by virtue of carbohydrate display on the surface.

SUPPLEMENTARY INFORMATION

Experimental details of the synthesis, sample preparation, characterization, and NMR and optical spectroscopic data are provided.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. Dharmendar Kumar Sharma and Prof. Arindam Chowdhury for TIRF measurements, and Dr. P Rajmohan for NMR support. DP and SD thanks CSIR and NGP thanks UGC for research fellowship. AVA acknowledges financial support from CSIR-NCL (project code MLP028826). SSG acknowledges CSIR Network Project CSC0302 and CSC0134 for financial support.

REFERENCES

1. Yamazaki, N.; Kojima, S.; Bovin, N.V.; Andre, S.; Gabius, S.; Gabius, H.-J. *Adv. Drug Delivery Rev.* **2000**, *43*, 225–244.
2. Murrey, H. E.; Hsieh-Wilson, L.C. *Chem. Rev.* **2008**, *108*, 1708–1731.
3. Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364.
4. Becker, D. J.; Lowe, J. B. *Glycobiology* **2003**, *13*, 41–53.
5. Aoi, K.; Tsutsumiuchi, K.; Okada, M. *Macromolecules* **1994**, *27*, 875–877.
6. Bonduelle, C.; Lecommandoux, S. *Biomacromolecules* **2013**, *14*, 2973–2983.
7. Pati, D.; Shaikh, A. Y.; Hotha, S.; Sen Gupta, S. *Polym. Chem.* **2011**, *2*, 805–811.
8. Kramer, J. R.; Deming, T. J. *J. Am. Chem. Soc.* **2010**, *132*, 15068–15071.
9. Krannig, K. S.; Doriti, A.; Schlaad, H. *Macromolecules* **2014**, *47*, 2536–2539.

10. Krannig, K.-S.; Schlaad, H.; *J. Am. Chem. Soc.* **2012**, *134*, 18542–18545.
11. Yamaguchi, H. *Trends Glycosci. Glycotechnol.* **2002**, *14*, 139–151.
12. Wells, L.; Vosseller, K.; Hart, G. W. *Science* **2001**, *291*, 2376–2378.
13. Kalovidouris, S. A.; Gama, C. I.; Lee, L. W.; Hsieh-Wilson, L. C. *J. Am. Chem. Soc.* **2005**, *127*, 1340–1341.
14. Sandi, C.; Rose, S. P. R.; Mileusnic, R.; Lancashire, C. *Neuroscience* **1995**, *69*, 1087–1093.
15. Mammen, M.; Chio, S. K. Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.
16. Bonduelle, C.; Huang, J.; Mena-Barragán, T.; Mellet, C. O.; Decroocq, C.; Etamé, E.; Heise, A.; Compain, P.; Lecommandoux, S. *Chem. Commun.*, **2014**, *50*, 3350–3352.
17. Cho, C. S.; Seo, S. J.; Park, I. K.; Kim, S. H.; Kim, T. H.; Hoshiba, T.; Harada, I.; Akaike, T. *Biomaterials* **2006**, *27*, 576–585.
18. Manning, D. D.; Hu, X.; Beck, P.; Kiessling, L. L. *J. Am. Chem. Soc.* **1997**, *119*, 3161–3162.
19. Pati, D.; Shaikh, A.; Das, S.; Nareddy, P. K.; Swamy, M. J.; Hotha, S.; Sen Gupta, S. *Biomacromolecules* **2012**, *13*, 1287–1295.
20. D'Souza, A. A.; Devarajan, P. V. *J. Controlled Release* **2015**, *203*, 126–139.
21. Parry, A. L.; Clemson, N. A.; Ellis, J.; Bernhard, S. S. R.; Davis, B. G.; Cameron, N. R. *J. Am. Chem. Soc.* **2013**, *135*, 9362–9365.

22. Gary-Bobo, M.; Mir, Y.; Rouxel, C.; Brevet, D.; Basile, I.; Maynadier, M.; Vaillant, O.; Mongin, O.; Blanchard-Desce, M.; Morère, A.; Garcia, M.; Durand, J. O.; Raehm, L. *Angew. Chem. Int. Ed.* **2011**, *50*, 11425–11429.
23. Brevet, D.; Gary-Bobo, M.; Raehm, L.; Richeter, S.; Hocine, O.; Amro, K.; Looock, B.; Couleaud, P.; Frochot, C.; Morère, A.; Maillard, Garcia, M.; Durand, J. O. *Chem. Commun.* **2009**, 1475–1477.
24. Sheikh, H.; Yarwood, H.; Ashworth, A.; Isacke, C. M. *J. Cell Sci.* **2000**, *113*, 1021–1032.
25. Wienke, D.; Davies, G. C.; Johnson, D. a.; Sturge, J.; Lambros, M. B. K.; Savage, K.; Elsheikh, S. E.; Green, a. R.; Ellis, I. O.; Robertson, D.; Reis-Filho, J. S.; Isacke, C. M. *Cancer Res.* **2007**, *67*, 10230–10240.
26. Wu, D. Q.; Lu, B.; Chang, C.; Chen, S. C.; Wang, T.; Zhang, Y. Y.; Cheng, S. X.; Jiang, X. J.; Zhang, X. Z.; Zhuo, R. X. *Biomaterials* **2009**, *30*, 1363–1371.
27. Suriano, F.; Pratt, R.; Tan, J. P. K.; Wiradharma, N.; Nelson, A.; Yang, Y.-Y.; Dubois, P.; Hedrick, J. L. *Biomaterials* **2010**, *31*, 2637–2645.
28. Otsukaa, H.; Nagasakib, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2003**, *55*, 403–419.
29. Wang, X.; Sun, H.; Meng, F.; Cheng, R.; Deng, C.; Zhong, Z. *Biomacromolecules* **2013**, *14*, 2873–2882.
30. Schatz, C.; Louguet, S.; Meins, J.-F.; Lecommandoux, S. *Angew. Chem., Int. Ed.* **2009**, *48*, 2572–2575.
31. You, L.; Schlaad, H. *J. Am. Chem. Soc.* **2006**, *128*, 13336–13337.

32. Chen, G.; Amajjahe, S.; Stenzel, M. H. *Chem. Commun.* **2009**, 1198–1200.
33. Ladmiral, V.; Semsarilar, M.; Canton, I.; Armes, S. P. *J. Am. Chem. Soc.* **2013**, *135*, 13574–13581.
34. Nel, A. E.; Mädler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. *Nat. Mater.* **2009**, *8*, 543–557.
35. Herd, H.; Daum, N.; Jones, A. T.; Huwer, H.; Ghandehari, H.; Lehr, C. M. *ACS Nano* **2013**, *7*, 1961–1973.
36. Shi, J.; Choi, J. L.; Chou, B.; Johnson, R. N.; Schellinger, J. G.; Pun, S. H. *ACS Nano* **2013**, 10612–10620.
37. Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. *Nano Lett.* **2006**, *6*, 662–668.
38. Gratton, S. E.; Ropp, P.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 11613–11618.
39. Geng, Y.; Dalhaimer, P.; Cai, S.; Tsai, R.; Tewari, M.; Minko, T.; Discher, D. E. *Nat. Nanotechnol.* **2007**, *2*, 249–255.
40. Dag, A.; Lu, H.; Stenzel, M. *Polym. Chem.* **2015**, *6*, 7812–7820.
41. Pati, D.; Kalva, N.; Das, S.; Kumaraswamy, G.; Sen Gupta, S.; Ambade, A. V. *J. Am. Chem. Soc.* **2012**, *134*, 7796–7802.
42. Dhaware, V.; Shaikh, A. Y.; Kar, M.; Hotha, S.; Sen Gupta, S. *Langmuir* **2013**, *29*, 5659–5667.
43. Das, S.; Sharma, D. K.; Chakrabarty, S.; Chowdhury, A.; Sen Gupta, S. *Langmuir* **2015**, *31*, 3402–3412.

44. Kramer, J. R.; Rodriguez, A. R.; Choe, U.-J.; Kamei, D. T.; Deming, T. J. *Soft Matter* **2013**, *9*, 3389–3395.
45. Huang, J.; Bonduelle, C.; Thévenot, J.; Lecommandoux, S.; Heise, A. *J. Am. Chem. Soc.* **2012**, *134*, 119–122.
46. Bonduelle, C.; Huang, J.; Ibarboure, E.; Heise, A.; Lecommandoux, S. *Chem. Commun.* **2012**, *48*, 8353–8355.
47. Ghoroghchian, P. P.; Li, G.; Levine, D. H.; Davis, K. P.; Bates, F. S.; Hammer, D. A.; Therien, M. J. *Macromolecules* **2006**, *39*, 1673–1675.
48. Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O.C.; Margalit, R.; Langer, R. *Nat. Nanotechnol.* **2007**, *2*, 751–760.
49. Oerlemans, C.; Bult, W.; Bos, M.; Storm, G.; Nijssen J. F. W.; Hennink, W. E. *Pharm. Res.* **2010**, *27*, 2569–2589.
50. Ahmed, F.; Discher, D. E. *J. Controlled Release* **2004**, *96*, 37–53.
51. Nasongkla, N.; Shuai, X.; Ai, H.; Weinberg, B. D.; Pink, J.; Boothman, D. A.; Gao, J. *Angew. Chem., Int. Ed.* **2004**, *43*, 6323–6327.
52. Allen, C.; Han, J.; Yu, Y.; Maysinger, D.; Eisenberg, A. *J. Controlled Release* **2000**, *63*, 275–286.
53. Coombes, A. G. A.; Rizzi, S. C.; Williamson, M.; Barralet, J. E.; Downes, S.; Wallace, W. A. *Biomaterials* **2004**, *25*, 315–325.
54. Park, E. K.; Lee, S. B.; Lee, Y. M. *Biomaterials* **2005**, *26*, 1053–1061.

- 1
2
3 55. Nakayama, M.; Okano, T.; Miyazaki, T.; Kohori, F.; Sakai K.; Yokoyama, M. *J. Controlled*
4 *Release* **2006**, *115*, 46-56.
5
6
7
8
9 56. Hong, G.; Yuan, R.; Liang, B.; Shen, J.; Yang X.; Shuai, X. *Biomed. Microdevices* **2008**, *10*,
10 693-700.
11
12
13
14 57. Yang, X.; Grailer, J. J.; Pilla, S.; Steeber D. A.; Gong, S. *Bioconjugate Chem.* **2010**, *21*, 496-
15 504.
16
17
18
19
20 58. Hong, V.; Presolski, S. I.; Ma, C.; Finn, M. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 9879–9883.
21
22
23 59. Zhang, L.; Eisenberg, A. *Science* **1995**, *268*, 1728–1731.
24
25
26 60. Discher, D. E.; Eisenberg, A. *Science* **2002**, *297*, 967–973.
27
28
29
30 61. Kazakov, S.; Kaholek, M.; Kudasheva, D.; Teraoka, I.; Cowman M. K.; Levon, K. *Langmuir*
31 **2003**, *19*, 8086–8093.
32
33
34
35 62. Peponi, L.; Navarro-Baena, I.; Baez, J. E.; Kenny, J. M.; Marcos-Fernández, A. *Polymer*,
36 **2012**, *53*, 4561–4568.
37
38
39
40
41 63. Silverstein, S.C.; Steinman, R.M.; Cohn, Z.A. *Annu. Rev. Biochem.* **1977**, *46*, 669–722.
42
43
44 64. Chithrani, B. D.; Chan, W.C. *Nano Lett.* **2007**, *7*, 1542–1550.
45
46
47
48
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Tunable Nanocarrier Morphologies from Glycopolypeptide-based Amphiphilic Star Copolymers and Their Cellular Internalization
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