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Micelles from self-assembled double-hydrophilic PHEMA-glycopolymers as multivalent scaffolds for lectin binding†

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We introduce a novel double-hydrophilic hydroxyethylmethacrylate (HEMA) based diblock glycopolymers which self-assembles into homogeneous spherical micellar structures in water. The micellar structure renders surface-oriented *N*-acetylglucosamine (GlcNAc) sugar moieties for strong multivalent glycan-mediated lectin binding. Structural analysis and lectin binding is performed by microscopy methods, dynamic light scattering (DLS) and two-focus fluorescence correlation spectroscopy (2fFCS), revealing a novel micellar type of multivalent sugar binding scaffold with high potential for biomedical applications.

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Introduction

In recent years, there has been an increasing attention on the synthesis of glycopolymers. Glycopolymers combine multivalent presentation of glycans with flexible distances of the glycan moieties as well as controllable synthesis.^{1–3} Moreover, polymers known to be biocompatible or biodegradable are promising for the synthesis of glyco-biomaterials.^{4–6} Together with controllable shape and behaviour in solution, glycopolymers are versatile artificial glycosylated scaffolds for the investigation of glycan-mediated interactions. Unlike polysaccharides, such polymers have a carbon backbone and pendant saccharide units. They comprise unique and tunable features in means of size, shape and phase behaviour together with

controlled glycan-ligand presentation. Due to the hydrophilicity of sugar moieties caused by their large number of hydroxyl groups, polymerisable neo-glycoconjugates have been used to synthesise amphiphilic block glycopolymers by conjugation to a hydrophobic polymer block for applications like emulsion stabilisation, drug delivery or for lectin binding studies.^{7–9} These amphiphilic diblock glycopolymers can self-assemble into various architectures such as micelles or vesicles. Their spherical and three dimensional structures may display much higher affinity towards binding lectins due to their larger surface area and therefore possible multivalency.^{10,11}

Lectins are generally known as carbohydrate binding proteins with considerable specificity for certain glycans.^{12,13} Plant lectins are often used in diagnostic assays and glycan analysis of glycoproteins.^{14–18} In human glycan-binding proteins have a huge impact in biomedical research.^{19–21} Lectins are known to play important roles in tumor progression and tumor angiogenesis depending solely on the cell type and its glycosylation pattern, the cell glyco-code.^{22–25} Furthermore, bacterial toxins are known to bind to their host cell *via* lectin domains as initial step of their pathogenicity.^{26,27} Lectins are therefore therapeutic and diagnostic targets which can be addressed by their specific carbohydrate ligands.

Glycan presentation in close proximity to each other enhances binding strength by orders of magnitude and is therefore the key element of effective glycan-functionalized materials in biomedical applications.^{28–30} Recently, glycovesicles have been shown to increase the affinity of a model lectin (Con A) about 40 times due to the multivalent presentation.³¹ Moreover, a drastic increase in binding ability was shown with sequence variation of hetero multivalent glycopolymers again in the case of ConA.³² Giving a good hint, that the general

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approach of using glycopolymers as scaffolds for multivalent presentation is certainly highly promising. ConA, however, is an example of a tetrameric lectin bearing both adjacent and opposite binding sites. But increased avidity towards multivalent scaffolds may not be found for all lectins just by increasing the number of presented ligands.³³ This highlights the urge of tailored materials to produce high-affinity ligands in glycoresearch, especially as there are various structures of lectins known.³⁴ Nevertheless, targeted drug release or tissue imaging may be realised by utilising glycan–lectin interactions as steering wheel, leading the containers to their destination.^{35–37} Such containers need to bear a more hydrophobic interior for interaction with the imaging dye or drug and a sugar coat for targeting and strong binding. Encouragingly it has been reported that amphiphilic mucin-mimicking glycopolymers are readily incorporated into cell membranes and undergo intracellular trafficking.³⁸ However, the formation of micelles and therefore the action as container of these structures is unlikely, because of the size relations between large hydrophilic glycopolymer and small hydrophobic endgroups.

So far, less research has been directed towards the synthesis of double-hydrophilic glycopolymers, which are capable of micelle formation, owing to the synthetic difficulty. Most micellar or vesicular approaches combined glycans or glycopolymers with small aliphatic molecules to form multivalent glycovesicles.^{39–41} However, very good binding of lectins to these have been shown. Recently, we fabricated multivalent glycopolymer brushes on silicon wafers by surface-initiated atom transfer radical polymerisation (SI-ATRP) with additional biocatalytic elongations of glycans directly on the silicon wafer substrate.⁴² This system does not only show high multivalency but also enables the simultaneous binding of adjacent as well as opposing binding sites. Neighbouring binding sites may interact with two glycans on the same brush, whereas opposite sites bind two brushes by crosslinking. Both effects are maintained by flexibility of the brushes on the one hand and high grafting density on the other.

In this work, ATRP was applied for the synthesis of well-defined self-assembling glycopolymer micelles. The micelles should maintain the same good binding abilities as shown for surface grafted brushes due to low distance between two glycans in all dimensions. Interestingly, the micelles were formed although the diblock copolymer consists of two rather

hydrophilic blocks. The self-assembly of double-hydrophilic block copolymers by slight differences in overall hydrophilicity of the blocks was recently reported.⁴³ However, no biological application has been proven so far. Our structures were analysed by various methods such as TEM and DLS. The critical micelle concentration (CMC) was determined by incorporation of small hydrophobic molecules. Additionally, we investigated the polarity and container properties of the self-assembled aggregates by incorporation of the polarity-sensitive fluorescent dye Nile Red. Confocal laser scanning microscopy and fluorescence spectroscopy were carried out with the dyed micelles. Finally, lectin binding to the micelles and determination of the binding strength was carried out by 2fFCS.

Experimental section

Synthesis of diblock glycopolymer

The diblock glycopolymer consists of one block of poly(2-hydroxyethyl methacrylate) (PHEMA) and a block of poly(2-*O*-(*N*-acetyl- β -D-glucosamine)ethyl methacrylate) (PGlcNAcEMA) (Fig. 1). For the preparation of diblock glycopolymer PHEMA-*b*-PGlcNAcEMA (**4**), firstly PHEMA (**2**) was synthesised by ATRP starting from HEMA (**1**). A representative polymerisation reaction is as followed: 15.0 mL (123.50 mmol) HEMA was placed into the reaction flask and stirred in 15.0 mL of methyl ethyl ketone (MEK)/1-propanol = 7/3 (v/v). The reaction mixture was degassed with nitrogen and preheated to 50 °C for 20 min. Then, 354.32 mg (2.47 mmol) CuBr, 0.77 g (4.94 mmol) bpy and 362.5 μ L (2.47 mmol) ethyl α -bromoisobutyrate were added and stirred under a nitrogen stream until homogeneous dark brown solution is formed. The reactor was degassed with nitrogen for further 20 min. The polymerisation proceeded at 50 °C for 24 h. The second block based on a polymerisable *N*-acetyl-glucosamine (GlcNAc) derivative (GlcNAcEMA, **3**) was then added utilising ATRP again, now with the PHEMA-block as macroinitiator. 1.08 g (3.25 mmol) of the glycomonomer was stirred in 10 mL methanol/water = 1/1 (v/v) until the complete dissolution and deoxygenated by nitrogen purging. 5.15 mg (0.052 mmol) CuBr, 2.90 mg (0.013 mmol) CuBr₂, 20.30 mg (0.13 mmol) 2,2'-bipyridine and 291.64 mg (0.065 mmol) macroinitiator were added and stirred under a nitrogen stream for 20 min. The reaction was performed at

Fig. 1 General scheme for the synthesis of PHEMA-*b*-PGlcNAcEMA.

room temperature for 15 h. The purified polymer was freeze-dried and a white polymer was obtained.

The synthesised polymers were characterised by $^1\text{H-NMR}$, GPC and MALDI-ToF MS (see ESI†). The conversion of HEMA to PHEMA was determined to be 90% resulting in a theoretical degree of polymerisation (DP) of 45. For the PGLcNAcEMA-block a theoretical DP of approx. 10 was calculated. According to MALDI-TOF MS analysis the diblock glycopolymer shows an average DP of approx. 39 for the HEMA unit and a DP of 4 for the GlcNAcEMA unit. An average molecular weight M_n of approx. 6546 g mol^{-1} and a low polydispersity M_w/M_n of 1.1, indicating a uniform size of the polymers, was determined for PHEMA-*b*-PGLcNAcEMA.

Protein treatment

Lectins GS-II were purchased from Vector Laboratories (Burlingame, CA, US) as FITC conjugates. As residual unbound dye was interfering with the measurements, it was purified *via* size exclusion chromatography (SEC) utilising Sephadex® G-25 with LB (with 10 mM HEPES-NaOH, pH = 7.5, 150 mM NaCl, 0.1 mM CaCl_2) to give a protein concentration of $20 \mu\text{g ml}^{-1}$.

CMC determination

For CMC determination 5 mg benzoylacetone (Sigma-Aldrich, Steinheim, Germany) was dissolved in 500 mL bidest water. Different concentrations of PHEMA-*b*-PGLcNAcEMA were dissolved in 100 μL of this solution and the absorption spectra were recorded utilising a microtiterplate reader. The CMC is determined as the concentration where the absorption band at 315 nm reaches its minimum.

Viscosity determination

The dynamic viscosity is an important parameter in the Stokes-Einstein equation, in order to obtain the hydrodynamic radius from the diffusion coefficient. The dynamic viscosity was calculated by the equation $\eta = \nu \cdot \rho$, whereas the density was determined with a DMA-5000 density meter (Anton-Paar, Germany). The kinematic viscosity was measured with a Lauda PVS viscosity system. For this purpose a Micro-Ostwald capillary (No. 1065402, capillary constant $0.0106 \text{ mm}^2 \text{ s}^{-2}$) was used. Thus, a dynamic viscosity of $0.9195 \pm 0.0009 \text{ mPa s}$ was obtained for aqueous buffer solution (LB) used in DLS and 2fFCS measurements. The temperature was set to 25°C .

Dynamic light scattering (DLS)

DLS measurements were performed on a standard ALV setup equipped with HeNe laser (JDS Uniphase, 633 nm, 35 mW), digital hardware correlator (ALV 5000), two avalanche photo diodes (Perkin Elmer, SPCM-CD2969), goniometer (ALV, CGS-8F) and light scattering electronics (ALV, LSE-5003). Highly diluted samples were prepared to prevent multiple scattering. Before measurements, the samples were filtered several times through regenerated cellulose filters (Sartorius) with a pore size of $0.2 \mu\text{m}$. Measurements were recorded in pseudo-cross correlation mode. The scattering angle was varied between 30° and 142° in steps of 4° for PHEMA-*b*-PGLcNAcEMA

and between 34° and 124° in steps of 3° for GS-II. Measurement times were 90 s and 240 s, respectively. The temperature was set to 25°C . Data were evaluated in the following way: intensity autocorrelation functions were transformed to electric field autocorrelation functions by use of the Siegert relation. A second order cumulant analysis was applied to obtain the decay rate Γ_2 . The cumulant fit range was limited between the lag time of 10 μs and the lag time where the electric field autocorrelation function amplitude had decayed to 30 percent of the experimental maximum value. Γ_2 was plotted against the square of the scattering vector q , the mean diffusion coefficient $\bar{D}$ was obtained from the equation

$$\Gamma_2 = \bar{D} q^2 \quad (1)$$

The hydrodynamic radius R_h was calculated by use of the Stokes-Einstein equation.

Two-focus fluorescence correlation spectroscopy (2fFCS)

2fFCS measurements were performed on a time-resolved confocal fluorescence microscopy system (PicoQuant, Micro-Time200 with dual-focus option). For excitation, two perpendicular polarised pulsed laser beams (wavelengths of 470 nm or 637 nm were used) are combined with a broadband polarising beamsplitter cube (Ealing Catalog) and then coupled into a polarisation maintaining single mode fiber. The laser pulses are generated by two identical laser diode heads (Picoquant, LDH-P-C-470 or LDH-P-C-635B) with an overall repetition rate of 40 MHz and thus a temporal distance of 25 ns between successive pulses. The synchronisation of the laser pulses (so-called pulsed interleaved excitation⁴⁴) with alternating orthogonal polarisation is achieved by laser driver electronics (PicoQuant, PDL 828 “Sepia II”). At the fiber output the light is collimated by a lens and afterwards reflected by a dichroic mirror (AHF-Analysentechnik, z470/532/637rpc) towards the water-immersion objective (Olympus, UPLSAPO 60xW, 1.2 N.A.). Prior to the dichroic mirror, the total laser power was adjusted to approx. $25 \mu\text{W}$. In front of the objective a Nomarski prism (Olympus, U-DICTHC) is located, which deflects the incoming laser pulses in two different directions according to their corresponding polarisation. After focusing the light through the objective of the inverted microscope (Olympus, IX71), two overlapping but laterally shifted foci are generated in the sample. The particle diffusion is only dominated by the time a particle needs to diffuse from the first focus to the second focus, so it only depends on the distance between the focal volumes but not their exact size and shape. Because this interfocal distance remains constant over time for a given wavelength, it enables us to measure absolute values of the diffusion coefficient.⁴⁵ The fluorescence light is collected by the same objective, passed through the prism and the dichroic mirror. It is then focused onto a confocal pinhole of $75 \mu\text{m}$ diameter to block out-of-focus light and achieve a good signal-to-noise ratio. After additional collimation, the light passes through laser clean up filters (AHF-Analysentechnik, HQ505/30m for 470 nm, HQ687/70m

637 nm). Both beam contributions are guided by a non-polarising beam splitter cube onto the active area of two single photon avalanche diodes (Micro Photon Devices, PDM series). A dedicated time-correlated single photon counting (TCSPC)⁴⁶ electronics (PicoQuant, PicoHarp 300) is used to refer every detected photon to its arrival time from the start of the experiment and with respect to the last laser pulse. It is known for every recorded photon which laser pulse has excited it and thus in which focus the photon was generated. Autocorrelation functions (ACFs) for each focus and cross correlation functions (CCFs) between the two foci are calculated from the photon arrival time traces with a specific algorithm based on a multiple-tau approach,⁴⁷ but only for photons from different detectors to avoid after pulsing artifacts.⁴⁸ A custom-made Matlab routine was used for computation.

For subsequent data fitting it has to be taken into account that the fluorescence intensity depends on the detection efficiency and the shape of the molecular detection function (MDF) in both foci. In 2fFCS the MDF is described by a two-parameter model based on a modified three-dimensional elliptic Gaussian distribution:⁴⁵

$$U(\vec{r}) = \frac{\kappa(z)}{w^2(z)} \exp\left[-\frac{2}{w^2(z)}(x^2 + y^2)\right] \quad (2)$$

with the beam waist function

$$w(z) = w_0 \left[1 + \left(\frac{\lambda_{\text{ex}} z}{\pi w_0^2 n}\right)^2\right]^{1/2} \quad (3)$$

and amplitude function

$$\kappa(z) = 1 - \exp\left(-\frac{2a^2}{R^2(z)}\right), \quad (4)$$

where $R(z)$ is given by

$$R(z) = R_0 \left[1 + \left(\frac{\lambda_{\text{em}} z}{\pi R_0^2 n}\right)^2\right]^{1/2}. \quad (5)$$

In the above equations $\{x, y, z\}$ are Cartesian coordinates with z along the optical axis, λ_{ex} refers to the excitation wavelength, λ_{em} to the center emission wavelength, n is the refractive index of the immersion medium (water), a denotes the radius of the confocal pinhole divided by magnification, and w_0 and R_0 are two initially unknown model parameters that are determined by the fit. Taking together the parameterisation of the MDF for each focus and the solution of the diffusion equation (Green function), the following function is used to fit the ACFs and CCFs:

$$g(\tau, \delta) = g_\infty(\delta) + \frac{\varepsilon_1 \varepsilon_2 c}{4} \sqrt{\frac{\pi}{D\tau}} \int_{-\infty}^{+\infty} dz_1 \int_{-\infty}^{+\infty} dz_2 \frac{\kappa(z_1)\kappa(z_2)}{8D\tau + w^2(z_1) + w^2(z_2)} \times \exp\left[-\frac{(z_2 - z_1)^2}{4D\tau} - \frac{2\delta^2}{8D\tau + w^2(z_1) + w^2(z_2)}\right], \quad (6)$$

where τ is the correlation or lag time, δ the interfocal distance, D the diffusion coefficient, c the concentration of particles and

ε_1 and ε_2 denote the overall detection efficiencies from both focal volumes. This model curve can be applied to the measured ACFs ($\delta = 0$, $\varepsilon_1 \varepsilon_2$ replaced by either ε_1^2 or ε_2^2) and CCFs simultaneously in a global fit with a linear least-square fitting approach including, among others, δ and D as fit parameters. By measuring fluorescent solutions with known diffusion coefficient δ can be calculated.⁴⁹ In this work, interfocal distances of $\delta = 259$ nm (for $\lambda_{\text{ex}} = 470$ nm) and $\delta = 287$ nm (for $\lambda_{\text{ex}} = 637$ nm) were determined. The model curve can be extended by additional terms to account for two fluorescent components or triplet blinking.

In this work, measurements took place in sealed sample cells to prevent solvent evaporation and to make use of home-made temperature regulation.⁵⁰ The sample temperature was set to 25 °C with an absolute accuracy of ± 0.1 °C. LiChrosolv (Merck, No. 115333), clean water for chromatography, was used for sample preparation. The measurement time was chosen in such a way to detect around 4×10^6 photons per measuring point. The collected photons were divided into 2×10^6 photon packages, thus two packages per point and overall 20 at least (10 packages for inhibition measurements at least). The resulting ACFs and CCFs (two of each for every package) were averaged and fitted by a two-component model in the case of GS-II and a one-component model including triplet blinking in the case of BSA.

Fluorescence spectroscopy

A 0.8 mM Nile Red stock solution was prepared in ethanol and diluted 1000-fold in the diblock glycopolymer system. Fluorescence spectra were obtained at room temperature using a Jasco FP-6500 Spectrofluorometer under the following conditions: excitation wavelength at 595 nm and fluorescent emission range between 610 and 750 nm, 3 nm slit width for excitation and emission, resolution 0.1 nm and a scan speed of 60 nm min⁻¹.

Microscopy

Confocal laser scanning fluorescence microscopy is performed on a Leica TCS SP8 to visualize fluorescent micelles. For this purpose, we used the same Nile Red concentration in the micellar diblock glycopolymer solution as with fluorescence spectroscopy measurements.

TEM images were obtained using a Zeiss LibraTM 120 Microscope. The accelerating voltage of the electron beam was set at 120 kV. A drop of the sample was trickled on a carbon-coated copper grid. Then, the copper grid was air-dried under ambient conditions.

In situ TEM experiments were performed at a Zeiss Libra 200FE apparatus equipped with in-column energy filter and Köhler illumination at 200 kV. An about 100 eV energy window at the most probable loss of the water plasmon was utilised for image generation. The sample was prepared by pipetting a 0.5 μ L droplet between two Si3N4-membranes, which were in a Hummingbird scientific *in situ* sample holder. To reduce contamination artifacts and improve the wettability of the otherwise hydrophobic membranes, an air-plasma was applied for

10 s. The water-plasmon in the EELS-spectrum was repeatedly captured during imaging to prove the presence of a liquid film in the sample. Furthermore, occasional diffusion of particles into or out of the focus plane was taken as proof for the presence of the solvent. Magnification was from 16 000 to 25 000 times.

MALDI-ToF MS analysis

The PHEMA-*b*-PGlcNAcEMA sample was dissolved in bidest water to a concentration of 5 mg ml⁻¹ and the DHB matrix to a concentration of 20 mg ml⁻¹ in TA30; sample and matrix solution were mixed in a ratio of 1 : 1. The cationisation solution of 0.1 mol l⁻¹ sodium trifluoroacetate in TA 30 was prepared as a thin-layer on the target plate (ground steel target, Bruker) and 1 µl of the sample/matrix solution added on top of it. Mass spectra were acquired using a 1 kHz Laser Bruker UTX MALDI-ToF mass spectrometer (Bruker, Bremen, Germany) with pulsed ion extraction. The spectra were recorded in the reflector mode with a repetition rate of 1000 Hz signals from positive ions; 3000 spectra were added to a sum spectrum. While recording the sample stage moved in a random fashion on a sample spot allowing 500 laser shots per position. The calibration was carried out externally using a mixture of proteins (ProteinCalibrationStandard I, #206375 from Bruker Daltonics, Bremen, Germany) prepared on a near neighbour spot. The spectra were baseline subtracted (Top hat algorithm) and mass peak annotation was obtained with the centroid detection algorithm (width 0.5 at 80% height).

The characterisation of the copolymer molecular weight distributions, determination of polydispersity and mixture deconvolution were performed with the Polytools™ software (Bruker Daltonics, Bremen) and the Polymerix™ software tool from Sierra Analytics, Modesto, CA 95356 USA.

Results and discussion

The diblock glycopolymer was dissolved in water at a molar concentration of 3.2×10^{-4} mol L⁻¹ for electron microscopy analysis, Fig. 2a–c presents the micrographic images of PHEMA-*b*-PGlcNAcEMA. Uniformly distributed dark spherical structures are obtained by TEM measurements (Fig. 2a and b)

with sizes around 21 nm in radius. In addition, about 5% of the particles occur as large aggregates, which can be also observed, as shown in the *in situ* TEM micrograph (c). However, the smaller micellar structures are the dominating species and monodisperse in size distribution.

Actually, the synthesised block copolymers are composed of two hydrophilic blocks: one water soluble glycooligomer consisting of PHEMA conjugated to a very hydrophilic sugar and a water-soluble pure PHEMA block. It has been reported that PHEMA homopolymers with a DP of 39, which corresponds to our PHEMA block (see ESI†), are considered as water-soluble, but possess a cloud point of at least 32 °C. As all experiments were carried out at room temperature, the PHEMA block can be considered fully soluble.⁵¹ Despite the existence of two hydrophilic blocks, these block glycopolymers form spherical particle structures in water. We assume that these structures have a micellar structure with the more hydrophilic glycan moieties at the outside. Other studies so far found a hydrophilic PHEMA corona or at least outer core in blockcopolymers micelles at elevated temperatures.⁵¹

We determined the critical micelle concentration (CMC) by analysing the absorption spectra of benzoylacetone (BZA) at different concentrations of blockcopolymer (see ESI, Fig. S10†). BZA is known to exhibit two absorption bands; one at about 250 nm, the other at about 315 nm due to the existence of ketonic- and enolic forms, respectively. In hydrophobic environments the enolic form is more pronounced, whereas in aqueous solution the ketonic form and therefore the band at 250 nm is more present. For this, BZA is utilised as marker molecule for CMC determinations, as the environment inside a micelle changes the hydrophilicity in comparison to the surrounding media.^{52–54} Interestingly, our measurements showed that at high concentrations of the block copolymer the band at 315 nm was more pronounced indeed, whereas at very low concentrations the band at 250 nm was dominating. In this way we were not only able to determine a CMC of approx. 0.4 g L⁻¹ (0.06 mmol L⁻¹), but also to get a hint that the interior of the structure is more hydrophobic than the surrounding media indeed.

These results indicate that the glycans are localised on the outside and the PHEMA block forms the interior. However, the band at 315 nm, even at high concentrations of 4, is not as pro-

Fig. 2 Micrographs of PHEMA-*b*-PGlcNAcEMA. (a) and (b) are obtained by TEM. (c) By *in situ* TEM. The scale bars represent 100 nm.

nounced as with classical surfactant systems. This may be caused by the water-soluble and still hydrophilic properties of PHEMA, which gives an excellent opportunity of incorporation of small hydrophobic molecules and usage of our system as molecular container.

To probe the abilities of our system to act as a transporter for, e.g., imaging purposes, we tried to incorporate the fluorescent dye Nile Red. It was shown that the fluorescence of Nile Red is environment-sensitive and especially enhanced in a hydrophobic environment.^{55–57} We found a dominant increase in the fluorescence intensity as well as a spectral blue shift of the emission wavelength in our diblock glycopolymer solution compared to the fluorescence in pure water. Both is caused by exposition of Nile Red to a more hydrophobic environment provided by the interior of the micelles and only occurs above the CMC. As shown in Fig. 3a the emission wavelength decreases by 10 nm. Nile Red was used previously as highly polarity-sensitive fluorescent probe to determine hydrophobicity of protein surfaces or polymer films.^{56–58} Therefore we expect to gain more insight about the assembly and polarity of the aggregates. Polarity is often related to mixtures of water and organic solvents.⁵⁷ Taking that into account a decrease of 10 nm in emission maximum equals the polarity of a binary mixture containing 50% methanol in water.⁵⁸ This corresponds to a surprisingly high change in relative permittivity, considering the low concentration of polymer in solution. Hence, this change may only be explained by incorporation of the dye molecules into a more hydrophobic nano-environment, with high local concentration of residues with lower relative permittivity, as expected for the micellar structure we suggest. Furthermore the blue shift of emission wavelength also confirms that the interior of the micelles is not as hydrophobic as observed for different polymers and nonpolar organic solvents, where the spectral shift is more pronounced.^{56–58}

Moreover, we could image an overview of the micelles in water using confocal laser scanning fluorescence microscopy. Again we detect some large aggregates (Fig. 3b), but also visualise nanosized entities with red fluorescence (Fig. 3c). We

conclude that the polymers although combining rather hydrophilic blocks are not only able to self-assemble into micellar structures in aqueous environment, but also may serve as nano-containers for small hydrophobic molecules. The phenomena of controlled self-assembly of double-hydrophilic block copolymers in water has been mostly overlooked so far, thus proven and studied for poly(ethyleneoxide)-poly(2-methyl-2-oxazoline) diblock copolymers.⁴³

However, the usage of PHEMA as less hydrophilic block in our case gives the opportunity to use these copolymers in biomedical applications due to the biocompatibility of PHEMA. Furthermore, the glycopolymer block bears a high biological coding capacity for tissue or cell targeting. Hence, we wanted to elucidate, if the glycans are accessible for lectin binding. If binding is detected, this will confirm our idea of less hydrophilic PHEMA forming the inside of the micelles and PGEMA forming the hydrophilic corona. That may lead to the possibility of glycan-targeted dye or drug transport.

More detailed analysis of the structures especially with respect to biological interaction was carried out *via* DLS and 2fFCS measurements. As the micelles are presenting GlcNAc we choose lectin II from *Griffonia simplicifolia* (GS-II) as GlcNAc specific lectin to specify the binding in a biological context. GS-II is known as a homodimer with four carbohydrate recognition domains (CRD) and has a M_w of 120 kDa.^{59,60}

DLS was used as complementary technique to get more precise information about the particle sizes of diblock glycopolymer and lectin. GS-II was dissolved in aqueous buffer solution (LB: 10 mM HEPES-NaOH, pH = 7.5, 150 mM NaCl, 0.1 mM CaCl_2) to keep the lectin structure stabilised and provide Ca^{2+} -ions for binding. A molar concentration of $9 \mu\text{mol L}^{-1}$ was used. PHEMA-*b*-PGlcNAcEMA was dissolved in the same buffer solution with a molar concentration of 0.5 mmol L^{-1} , thus above the CMC. Both solutions were filtered through a $0.2 \mu\text{m}$ filter. Fig. 4a presents the decay rate obtained from the second order cumulant fit plotted against the square of the scattering vector q for PHEMA-*b*-PGlcNAcEMA (squares) and GS-II (circles). The diffusion

Fig. 3 (a) Emission spectra of Nile Red in pure water (black line) and in micellar solution from PHEMA-*b*-PGlcNAcEMA (red line). The dashed vertical lines indicate the position of the respective emission maximum; (b) and (c) images obtained by confocal laser scanning microscope. The scale bars represent $5 \mu\text{m}$.

Fig. 4 (a) 2nd order decay rate with q^2 for GS-II (circles) and PHEMA-*b*-PGlcNAcEMA (squares). Linear regressions are indicated by solid lines. (b) Normalised cross correlation functions with lag time for GS-II (circles) and GS-II upon addition of PHEMA-*b*-PGlcNAcEMA (triangles). Two-component fits are indicated by solid curves. The dashed lines display the particular inflection points.

coefficients were calculated from the slope of linear regressions shown as solid lines in Fig. 4a. The good linearisation of PHEMA-*b*-PGlcNAcEMA data indicates a relatively narrow size distribution of micellar structures in solution.⁶¹ A hydrodynamic radius of 16.7 ± 0.1 nm was calculated, which verifies the observations made by microscopy. For GS-II a hydrodynamic radius of 7.5 ± 0.1 nm was obtained.

Fluorescence correlation spectroscopy (FCS) is well-suited to monitor binding processes by measuring changes in diffusion characteristics.^{32,62} However, standard FCS measurements are affected by different properties of the optical setup, like refractive index mismatch, and the photophysics of the used fluorophores, so that it is rather difficult to obtain absolute values of the diffusion coefficient. These problems can be neglected using 2fFCS as an improved version of the standard confocal FCS setup.^{45,49} In this work, the protein binding was analysed using FITC labeled GS-II at a molar concentration of 17 nmol L^{-1} . Unlabeled block glycopolymer was used at a molar concentration of 0.25 mmol L^{-1} . Complete diagrams with auto and cross correlation functions and their fits, for GS-II and PHEMA-*b*-PGlcNAcEMA, respectively, are presented in the ESI (Fig. S11 and S12[†]). Cross correlation functions (CCFs) were fitted using a two-component fit model of diffusion (Fig. 4b, solid curves), which accounts for the presence of residual FITC (not chemically attached to GS-II). Indeed, the GS-II stock solution was purified by SEC (see ESI, Fig. S9[†]), but a certain fraction of 30% of free dye still remained.

Fig. 4b shows the normalised and averaged CCFs plotted against the lag time of freely diffusing GS-II (circles) and GS-II upon addition of PHEMA-*b*-PGlcNAcEMA (triangles). The dashed lines in Fig. 4b indicate that the inflection points of the CCFs are significantly different. This corresponds to a change in diffusion time meaning that, after addition of PHEMA-*b*-PGlcNAcEMA, GS-II was bound to the diblock glycopolymer. The hydrodynamic radii obtained from the fits are in good agreement with DLS and TEM analysis: 6.8 ± 0.1 nm for GS-II and 15.4 ± 0.2 nm for GS-II upon addition of PHEMA-*b*-PGlcNAcEMA. The slightly larger radius provided by TEM may be caused by flattening of the soft micelles on the target. We

conclude that little or no unbound GS-II remained in solution after addition of diblock glycopolymer, which hints to strong multivalent glycan-mediated protein adsorption hence.

To get more information about the binding properties of our glycopolymer we performed 2fFCS measurements under inhibiting conditions for GS-II binding by varying concentrations of the free sugar ligand GlcNAc (Fig. 5). Displacement experiments were carried out by adding GlcNAc after GS-II has been incubated together with the micelles and the binding events are assumed to be completed. Competitive inhibition in contrast describes the simultaneous addition of GS-II and GlcNAc to the micelles. Therefore, in the latter approach the lectin “decides” which presentation mode of GlcNAc is more favoured: dissolved free GlcNAc or multivalent micelle. From these experiments IC₅₀ values, describing the concentration of inhibitor, where 50% of maximum binding is left, are obtained. Meanwhile GS-II and PHEMA-*b*-PGlcNAcEMA concentrations remained unchanged. The inhibition experiments gave IC₅₀ values of 1.627 ± 0.3950 mM and 1.049 ± 0.2613 mM for displacement and competitive inhibitions, respectively.

Fig. 5 Inhibiting the binding of lectin GS-II to the micelles with different concentrations of GlcNAc, via displacement (●), that is the inhibition of binding by adding GlcNAc after incubation of micelles and GS-II as well as competition (□), which is the addition of GlcNAc and GS-II simultaneously to the micelles.

These values are surprisingly high in comparison with the inhibition of hemagglutination activity of GS-II by soluble GlcNAc, which is about 0.048 mM for complete inhibition.⁶³ This supports our assumption that the glycans are on the outside of the micelles and accessible with high multivalency of the glycopolymer which assures outstanding binding capabilities towards lectins. Because these binding capabilities could also be rest on unspecific binding, further experiments were done by substituting GS-II with bovine serum albumin (BSA) labeled with Alexa Fluor 647. BSA is not known to have any affinity to glycan moieties. The measurements showed that the diffusion coefficient of BSA did not change upon addition of PHEMA-*b*-PGlcNAcEMA (see ESI, Fig. S13 and S14†). This result supports that our synthesised diblock glycopolymer acts as highly specific ligand for GS-II.

Conclusions

Herein, PHEMA-*b*-PGlcNAcEMA diblock glycopolymer was synthesised and its structure in water was observed using microscopy measurements such as TEM and *in situ* TEM. Although the synthesised polymer is composed of two rather hydrophilic parts, the resulted double-hydrophilic diblock glycopolymers have shown to self-assemble into micellar particle structures in aqueous systems. The measured size of micellar particles was around 16 nm in radius by DLS and 2fFCS. TEM showed slightly larger values due to flattening on the target. The lectin binding ability of the micelles was observed by 2fFCS, showing excellent specific binding of lectin GS-II with very high IC₅₀ values for the inhibition of binding. Moreover, we proved the incorporation of small hydrophobic hardly water-soluble molecules leading to the conclusion that the micelles may serve as transporter. Comparison of the emission spectra of the polarity-sensitive fluorescent probe Nile Red in different reported systems showed a surprisingly high shift in relative permittivity. This proves the formation of micelles as this shift may be explained by a high local concentration of hydrophobic molecules as it is found in micelles. Although double-hydrophilic, the micelles are capable of solubilisation and incorporation of small hydrophobic molecules. Hence, our system may be elaborated to act as nano-container for drugs or dyes with specific targeting by multivalent and strong glycan-protein interactions.

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