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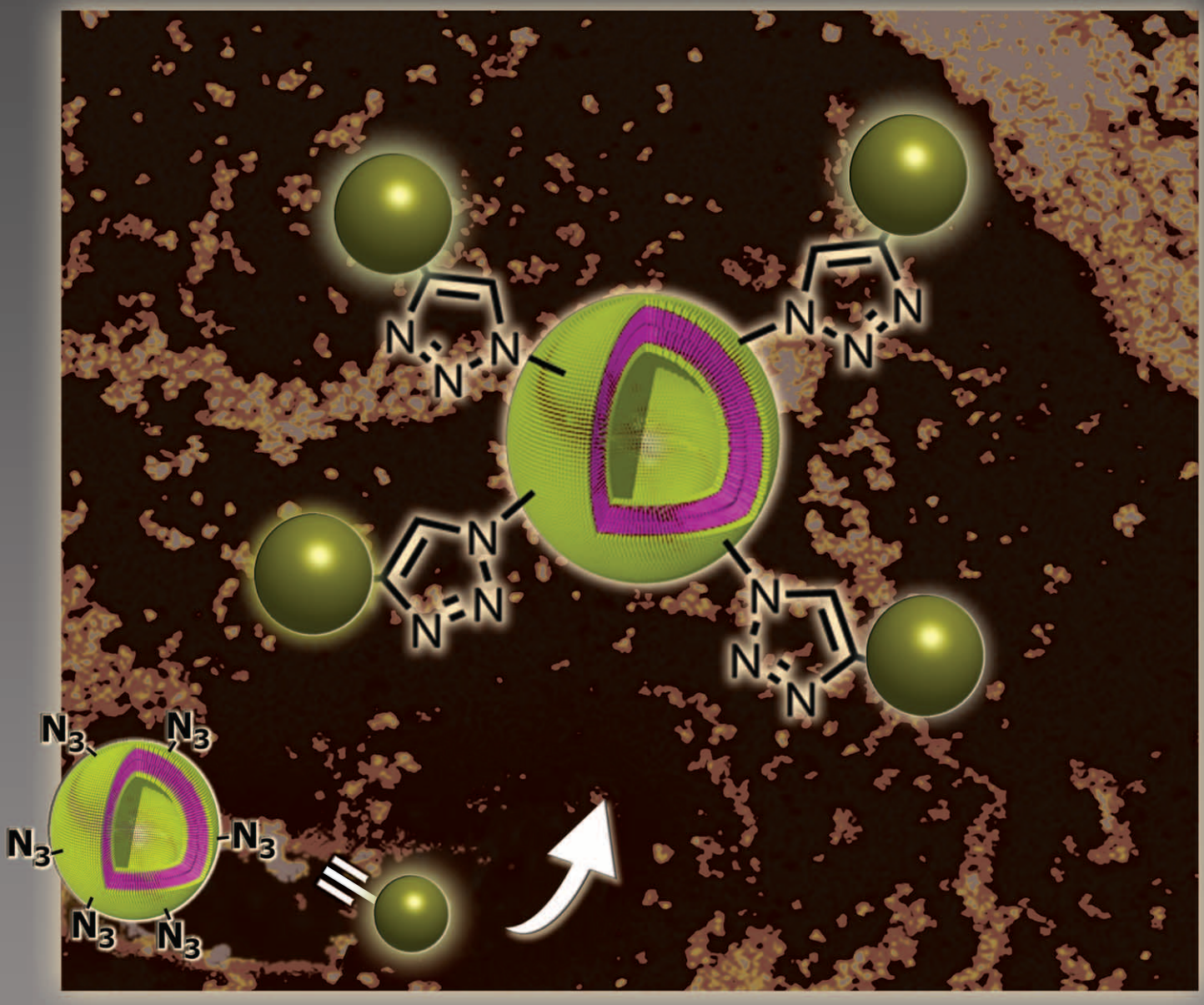

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"Clickable" polymersomes†

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Polymersomes, composed of amphiphilic polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA), with the periphery being covered with azide groups, were used for further functionalization using "click" chemistry.

Block copolymers represent an interesting class of materials for nanotechnology and drug delivery systems, due to their ability to assemble on a mesoscopic length scale into multiple, highly regular morphologies.[1] In solution, amphiphilic block copolymers form a variety of different structures, ranging from micelles and micellar rods to vesicular morphologies. Vesicles composed of block copolymers, often referred to as polymersomes, are spherical shell structures embracing exceptional stability compared to their low molecular weight counterparts.[2] Conjoined with the possibility of readily tailoring the chemical, physical and biological properties of block copolymers, these polymersomes are ideal candidates for utilization as nanoreactors[3] or drug delivery vehicles.[4]

Here we report the synthesis of functional polymersomes, which have the capacity to be conjugated at their periphery to (bio)active moieties, such as targeting ligands or enzymes. Thus, first the desired, stable aggregates are formed of which the exterior is covered with reactive groups which, subsequently, can be used for further functionalization. In this way, large functional moieties can be introduced on the outside of polymersomes without disrupting the aggregate morphology.

Owing to the reduced reactivity on the periphery of polymersomes, a highly efficient coupling procedure is required. In this respect, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes is very suitable.[5] Beside of the eminent efficiency, this type of "click" reaction is highly selective, thereby tolerating the presence of other functional groups. This is an important advantage when compared to other coupling methods, such as the recently described bioconjugation to tosylated shell polymeric nanoparticles.[6] Moreover, this type of chemistry is applicable under aqueous conditions, which is a prerequisite since the polymersomes are formed in an aqueous environment. "Click" chemistry recently received much attention in for instance polymer[7,8] and dendrimer[9] synthesis, as conjugation methodology for preparing biohybrid polymers,[10] and in the functionalization of both the core and the corona of micelles and shell-crosslinked nanoparticles.[11]

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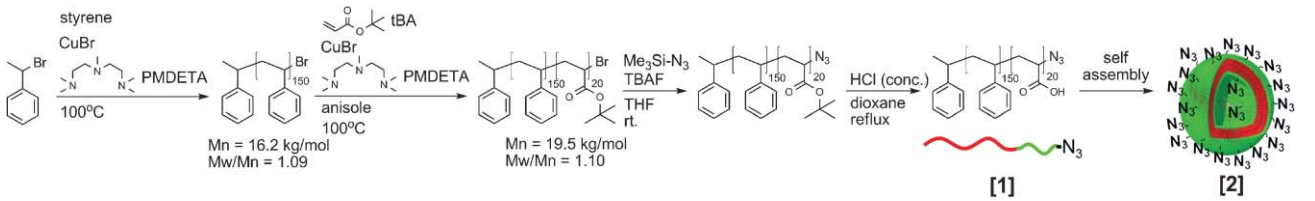
† Electronic supplementary information (ESI) available: Experimental procedures and details. SEC traces of the synthesized polymers. Fluorescence experiments conducted to estimate degree of functionalization in vesicles. See DOI: 10.1039/b704568a

Amphiphilic polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) block copolymers were prepared by atom transfer radical polymerization (ATRP).[12] Styrene and *tert*-butyl acrylate (tBA) were polymerized consecutively in good control (Scheme 1). The bromide end groups were substituted for azides using azidotrimethylsilane ($Me_3Si–N_3$) and tetrabutylammonium fluoride (TBAF),[7,13] prior to acidic hydrolysis of the tBA block. The resulting terminal azide functional PS-*b*-PAA block copolymer [**1**] was allowed to self-assemble into vesicular aggregates *via* slow addition of water to a block copolymer solution in dioxane. The organic solvent was subsequently removed by extensive dialysis against water.[14]

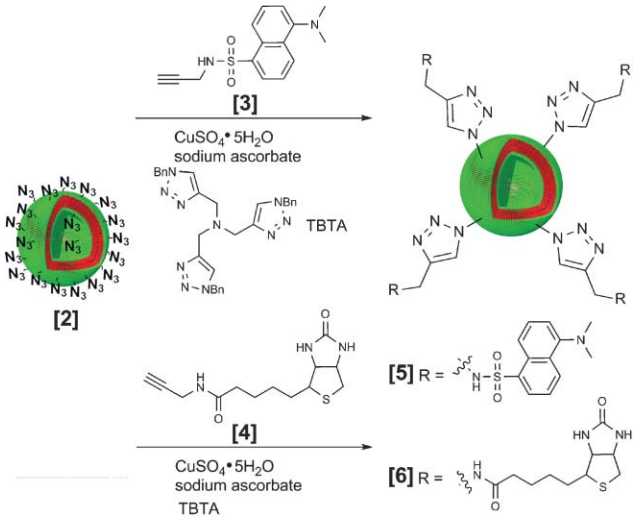
In order to test the exploitation of azide-functionalized polymersomes as scaffolds for further conjugation, these vesicles were subjected to an aqueous solution of acetylene-functionalized dansyl probe [**3**] in combination with a copper catalyst using *tris*-(benzyltriazolylmethyl)amine (TBTA) as a ligand (Scheme 2). After 24 h of reaction, unreacted probe and catalyst were removed by extensive dialysis against a 0.55 mM solution of ethylenediamine-tetraacetic acid tetrasodium salt tetrahydrate (EDTA). Using transmission electron microscopy (TEM), it was determined that no change in aggregate morphology occurred due to the "click" functionalization. Furthermore, as can be seen in Fig. 1 c and d, confocal laser-scanning microscopy (CLSM) images display significant fluorescence of the polymersomes in contrast to the background, implying attachment of the fluorescent dansyl probe [**3**]. Additionally, the dansyl-functionalized vesicles [**5**] were dissolved in chloroform and measured with size exclusion chromatography (SEC) to ascertain covalent attachment of the probe to the exterior of the polymersomes. As depicted in Fig. 2, after conjugation of the dansyl moiety, the block copolymers exhibited absorption at 345 nm, whereas the bare block copolymer was not detectable at this wavelength. From these SEC traces it can be deduced that the periphery of the polymersomes was successfully functionalized in a covalent fashion.

In order to determine the degree of functionalization of the polymersomes, a reference compound was made by coupling PS-*b*-PAA and dansyl probe [**3**] in solution. Comparison of the fluorescence of this model compound with the block copolymers functionalized in the polymersome led to the estimation that 23 percent of the azide moieties present within the polymersomes were coupled. Since the azide functionalities in the interior of the polymersomes were not available for conjugation, this implies that 40 to 50 percent of the azides on the periphery were functionalized.

In order to improve the degree of functionalization, four variables were altered, namely temperature, the copper stabilizing ligand, the copper concentration and the pH value of the reaction mixture. As an alternative ligand bathophenantrolinedisulfonic



Scheme 1 Preparation of PS-*b*-PAA polymersomes [**2**] with peripheral azide moieties.



Scheme 2 "Click" modification of the exterior of polymersomes with both acetylene bearing fluorescent dansyl probe [**3**] and biotin [**4**].

acid sodium salt (BPhT) was chosen, which is known to be an excellent and very water soluble catalyst.[15] The degrees of functionalization were obtained by comparison of the fluorescence with that of the model compound as well. As can be seen in Table 1, no significant improvement in the degree of functionalization was obtained, implying the optimum degree of functionalization was attained. A plausible explanation can be that not all azide moieties were sufficiently available for reaction, owing to the dense packing of the polymer chains in the vesicular aggregates.

Additionally, by applying the same methodology, acetylene bearing biotin [**4**] was attached to the polymersomes. Biotin is a high affinity binding ligand for the enzyme streptavidin ($K_d \cdot 10^{-14}$). Consequently, in a next step streptavidin labeled with colloidal gold particles of 6 nm was complexed to the biotinylated vesicles [**6**]. After a 24 h treatment of the vesicles with an aqueous streptavidin solution, residual streptavidin was removed by dialysis against water.

As illustrated in Fig. 1 a and b, no change in aggregate morphology was observed due to the complexation of gold labeled streptavidin. Furthermore, this polymersome–streptavidin complex was visualized by CLSM (Fig. 1 e and f).

To demonstrate the direct conjugation of proteins to the exterior of polymersomes, enhanced green fluorescent protein (EGFP), which was prepared by expression in *Escherichia coli*, was accommodated with acetylene functionality by reaction of pentynoic acid *N*-succinimidyl ester [**7**] with one or more of the 20 lysine residues exposed on the outside of the protein, as schematically depicted in Scheme 3.

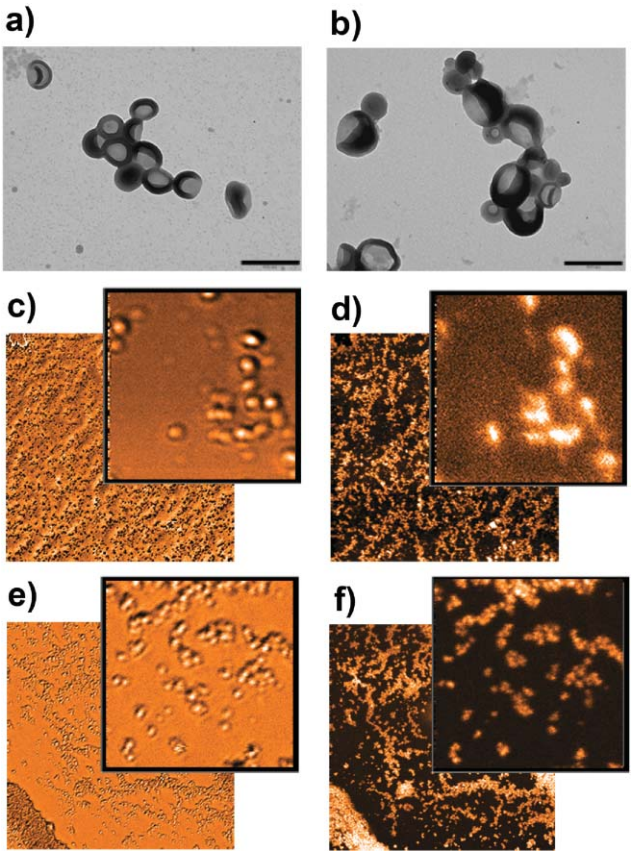


Fig. 1 Transmission electron micrographs of PS-*b*-PAA vesicles [**6**] before (a) and after (b) attachment of gold labeled streptavidin (black scale bar represents 500 nm). Confocal laser-scanning microscopy images (transmission and fluorescence excited at 411 nm, respectively) of dansyl functionalized polymersomes [**5**] (c and d) and biotin-streptavidin-gold functionalized vesicles (e and f).

Acetylene bearing EGFP [**8**] was coupled to azide functionalized vesicles [**2**] utilizing similar "click" conditions in 0.1 M PBS buffer of pH 7.2. Subsequent to the removal of unreacted EGFP by extensive dialysis, successful functionalization of the polymersomes was determined by visualization of the fluorescent behavior of the vesicles by CLSM, which stemmed from the attachment of EGFP (Fig. 3). Due to the high dilution of the reaction mixture, no crosslinking was observed. As a control experiment, equal conditions were applied with omitting the addition of $CuSO_4 \cdot 5H_2O$. In this case, the vesicles displayed no fluorescence, leading to the conclusion that all unreacted EGFP can be removed by dialysis and, hence, the fluorescence was caused by covalently coupled EGFP.

In conclusion, we have demonstrated a facile methodology for the functionalization of polymeric vesicular aggregates. The

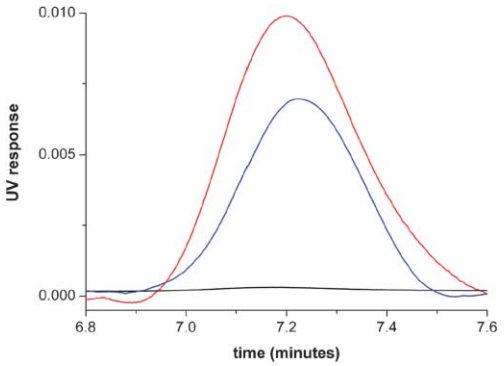
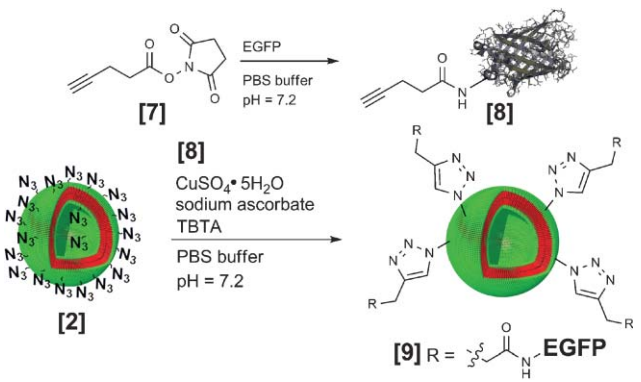


Fig. 2 SEC traces of PS-*b*-PAA-N_3 [**1**] measured at 254 nm (blue) and 345 nm (black) and dissolved dansyl functionalized vesicles [**5**] measured at 345 nm (red) in chloroform.

Table 1 Reaction conditions used and the degrees of functionalization obtained for the "click" functionalization of polymersome [**2**] and dansyl probe [**3**]

Temperature/°C	Ligand	[Cu(II)]/mM	pH	Degree of functionalization
30	TBTA	5.0	7.4	26.0%
ambient	TBTA	5.0	6.0	24.7%
30	BPhT	5.0	6.0	25.6%
ambient	BPhT	5.0	7.4	24.7%
30	TBTA	0.5	6.0	25.3%
ambient	TBTA	0.5	7.4	24.2%
30	BPhT	0.5	7.4	25.4%
ambient	BPhT	0.5	6.0	23.9%



Scheme 3 Functionalization of EGFP with acetylene moiety and subsequent "click" conjugation to azide functionalized polymersomes.

amphiphilic block copolymers were prepared readily by consecutive ATRP polymerizations. The azide functionality was introduced conveniently and further modification of the azide functionalized vesicles only required the application of the desired acetylene functionalized moiety and a copper-catalyst.

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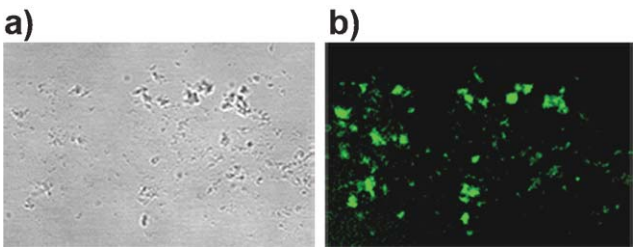

Fig. 3 Confocal laser-scanning microscopy images (transmission (a) and fluorescence excited at 488 nm (b)).

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