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Towards Targeted Drug Delivery by Covalent Ligand-Modified Polymeric Nanocontainers

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Summary: Here we present a novel strategy for specific cellular targeting of polymeric nanocontainers by using self-assembly of block copolymers consisting of either Polydimethoxysiloxane-b-Polymethyloxazoline-b-Polydimethoxysiloxane (PDMS-b-PMOXA-b-PDMS) or functionalized PDMS-b-PMOXA-b-PDMS. Covalent functionalization of the above copolymer was accomplished using either the fluorescent dye sulforhodamine B or a poly-guanosin ligand, the latter by using the Huisgen 1,3-dipolar cycloaddition. The success of the covalent modification of the block copolymer has been determined by studying functionalized sulforhodamine B by NMR and fluorescence correlation spectroscopy. The covalent click chemistry approach leads to efficiently functionalized polymeric nanocontainers which enables specific uptake by activated macrophages overexpressing the scavenger receptor A1.

Keywords: block copolymers; drug delivery systems; fluorescence; functionalization of polymers; nanoparticles

Introduction

Major problems in developing new successful therapies are related to the fact that i) most therapies do not have a targeting mechanism leading to the need for overdosing in order to reach its targeted tissue at a concentration corresponding to the therapeutic window, and a concomitant high risk of adverse affects, and ii) the active molecule is often degraded before it reaches its targeted tissue. Another reason for developing concepts for targeting and protecting the active molecule relates to the enormous interest in developing efficient therapies based on interfering RNA which will ultimately be dependent on the above mentioned strategies.[1] Previously, we have established methods to produce functional nanocontainers based on amphiphilic block copolymers with ideal properties for drug delivery applications.[2–5] A method to determine receptor-ligand interactions between proteins and polymer nanocontainers by using fluorescence correlation spectroscopy (FCS) has been established.[6] Here we present a novel strategy for specific cellular targeting by using polymeric nanocontainers comprising Polydimethoxysiloxane-b-Polymethyloxazoline-b-Polydimethoxysiloxane (hereafter referred to as ABA) and fluorescently labeled or ligand-functionalized ABA. First, the ABA polymer was functionalized with the fluorescent dye sulforhodamine B (SRB) (Figure 1). Second, click chemistry was utilized (Figure 1) to react alkyne terminated ABA polymer with an azide functionalized fluorophore. Contrary to direct endgroup functionalization an alternative modification approach was used where previously prepared vesicles made from ABA polymer was covalently modified with a 23 nucleotide long polyguanosin (poly$(G)_{23}$) moiety (Figure 1) by using click chemistry for specific targeting of the scavenger receptor A1 overexpressed by activated THP-1 macrophages. The success of the covalent

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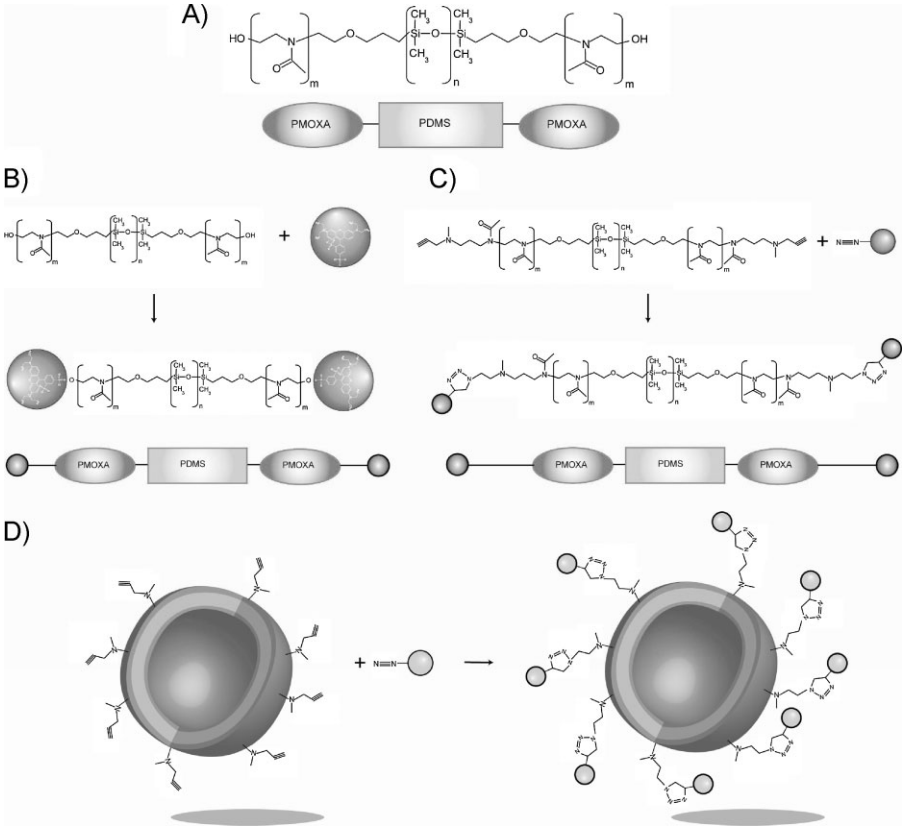

Figure 1.
Fluorescent labeling of the amphiphilic block copolymer. (A) Generic molecular structure of the ABA polymer. (B) Schematic drawing showing the end group functionalization with sulforhodamine B chloride, and (C) showing the funtionalization using click chemistry. (D) Cartoon depicting functionalization of already prepared ABA vesicles by reaction of the alkyne moieties with the azide-functionalized fluorophore. The fluorophores and the poly$(G)_{23}$ ligand are depicted as red and yellow spheres, respectively.

modification of the SRB labeled ABA polymer has been confirmed by ^{1}H-NMR and FCS. Administration of poly$(G)_{23}$ functionalized ABA nanocontainers to activated macrophages leads to specific uptake, most probably via the scavenger receptor A1, which is overexpressed by this human monocytic cell line. In contrast to our previously published modular approach, covalent click chemistry modification enables a highly controlled and robust linkage between copolymer and ligand and reduces potential adverse immunogenicity due to exogenous protein necessary in the streptavidin-biotin method.[2]

Synthesis of PDMS-b-PMOXA-b-PDMS and Functionalized PDMS-b-PMOXA-b-PDMS

Synthesis of the amphiphilic coblock polymer PDMS-b-PMOXA-b-PDMS (hereafter referred to as ABA) has been published earlier by our group in great detail.[7] Sulforhodamine B acid chloride (SRB) was covalently coupled to the ABA polymer (SRB-ABA) using similar procedures described elsewhere (Figure 1 and Figure 2).[8] Under argon atmosphere dried hydroxyl endfunctionalized ABA polymer was dissolved in previously dried

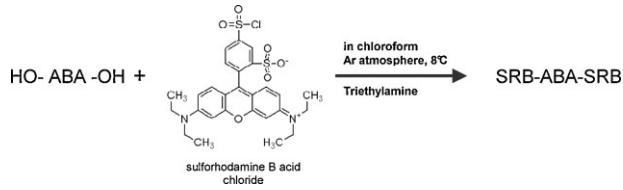

Figure 2.
Schematic diagram of the synthesis of fluorescently labelled ABA by covalent modification of the hydroxyl endfunctionalized ABA with sulforhodamine B.

chloroform and cooled down to 8 °C. Then triethylamine was added to deprotonate the ABA hydroxyl ends. A concentrated solution of SRB acid chloride in chloroform was added dropwise and the mixture was allowed to stir for 24h at 8 °C. After reaction the chloroform was evaporated and the dark red solid was dissolved in ethanol. The non-reacted free dye and triethylamine was removed by ultra filtration (membrane cutoff 5 kDa) with ethanol and water as solvent. As soon as the filtrate was nearly colorless, the solvent was evaporated and the SRB-ABA conjugate was dried under vacuum.

Alkyne-modification of the ABA polymer was performed by utilizing N-methylpropargylamine (Figure 2). The ABA was a symmetric poly-(2-methyloxazoline)-block-poly-(dimethylsiloxane)-block-poly-(2-methyloxazoline) ($PMOXA_{15}PDMS_{110}PMOXA_{15}$) polymer. The first step of the synthesis procedure involved acid-catalyzed polycondensation of dimethoxydimethyl silane in the presence of water and end-capper, resulting in butylhydroxy-terminated bifunctional PDMS. Liquid PDMS was purified by vacuum stripping at 80 °C and precipitation in a 1:1(by weight) water/methanol mixture. Purified PDMS was reacted with triflic acid anhydride in hexane at 10 °C for 3 h, resulting in triflate-PDMS bifunctional macroinitiator. The reaction mixture was then filtered under argon through a G4 filter. Hexane was evaporated under vacuum and dry ethyl acetate was added as reaction solvent. Addition of dry 2-methyl-2-oxazoline resulted in symmetric ring-opening cationic polymerization of PMOXA blocks on the macroinitiator. The reaction was terminated by addition of N-methylpropargylamine.

Fluorescent labeling of the alkyne-functionalized ABA polymer was carried out in DMF by modifying SRB acid chloride with the bifunctional linker 11-Azido-3,6,9-trioxaundecan-1-amine. After 24 hours of stirring at room temperature the reaction mixture was purified with HPLC and characterized by Maldi-TOF MS (data not shown).

Preparation of Ligand Modified Polymeric Nanocontainers

Additionally to the covalently SRB-ABA we prepared $Poly(G)_{23}$ modified polymeric nanocontainers by first preparing fluorescent nanocontainers and then adding an azide functionalized $Poly(G)_{23}$ using click chemistry for specifically targeting the scavenger receptor A1 from activated macrophages. The polymeric nanocontainers were prepared by mixing three different ABA polymers: ABA 1 ($PMOXA_{12}PDMS_{60}PMOXA_{12}$), ABA 2 (alkyne-functionalized ABA), and SRB-ABA, respectively, at a final polymer concentration of 0.4% (wt/vol) using weight ratios between ABA 1, ABA2, and SRB-ABA of 16 to 3.7 to 1, respectively. The solvent was evaporated under high vacuum and the resulting polymer film was rehydrated in bidistilled water, extruded through a polycarbonate filter (0.2 μm pore size) and purified over a Sepharose 2B size exclusion column. A solution (1.5 ml) was prepared by mixing the ABA nanocontainer solution

with bidistilled water, the Poly$(G)_{23}$ ligand, sodium ascorbate solution, $CuSO_4$ solution, and a Tris[(1 - benzyl - 1H - 1,2,3 - triazol - 4 - yl)methyl] amine (TBTA) solution at a volume ratio of: 25/120/2/1/1/1 using concentrations mentioned elsewhere.[9] As a negative control ABA nanocontainers without added Poly$(G)_{23}$ reaction mix were used.

Characterization of the SRB Labeled ABA Polymer and SRB Labeled Nanocontainers

All calculations were related to the integral of the protons of the butyl-linker methylene group at 0.56 ppm (Figure 4). The peaks between 0.0 and 0.2 ppm correspond to the PDMS methyl groups, the peak between 2.0 and 2.3 ppm to the methyl groups of the PMOXA. Hence, we obtain an average block copolymer composition of $PMOXA_{11}PDMS_{91}PMOXA_{11}$ which corresponds to a molecular weight of 8756 g/mol. To quantify the conjugation of SRB acid chloride to the polymer, the integral of a peak corresponding to the aromatic region (Figure 4) was compared to the reference integral at 0.56 ppm. The estimated degree of SRB conjugation to the polymer is 2.6%. Additional ^{1}H-NMR diffusion-ordered spectroscopy (DOSY) experiments were carried out to further corroborate the SRB conjugation. The diffusion coefficients determined from the peaks at 6.71 ppm and 0.17 ppm are $2.41^{*}10^{-10}$ m^2/s and $2.36^{*}10^{-10}$ m^2/s, respectively, indicating that the SRB moiety diffuses with the ABA polymer. This diffusion coefficient is one order of magnitude smaller then the one of water, which is around $2.3^{*}10^{-9}$ m^2/s. This is a fairly good, but not an absolute proof for the conjugation of the dye to the polymer. The average block copolymer composition, $PMOXA_{10}PDMS_{87}PMOXA_{10}$, was also estimated for the SRB-conjugated ABA using the above method indicating a molecular weight of 8276 g/mol.

Additionally to chemical characterization by NMR, the SRB-ABA polymer was analyzed by fluorescence correlation spectroscopy (FCS). FCS is a powerful spectroscopic method which enables the simultaneous determination of the absolute concentration and the diffusion properties of the fluorescently labeled molecule. Since FCS is able to discriminate between a given fluorescent species with different sizes with a resolution of about 6-8 in molecular weight difference, it is possible to distinguish the free SRB (559 g/mol) from the

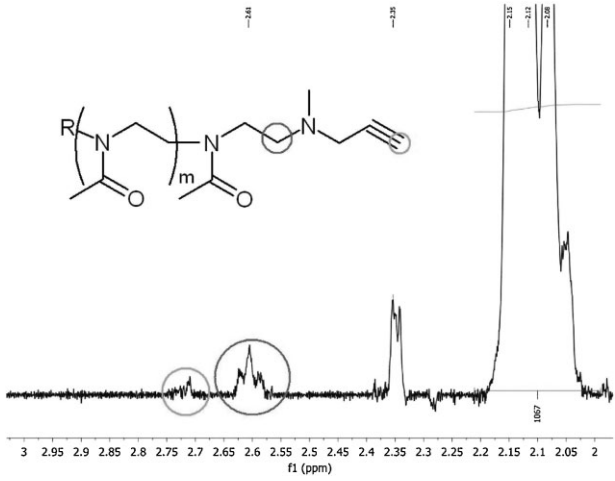

Figure 3.
^{1}H-NMR spectra of alkyne endfunctionalized ABA in $CDCl_3$. The two triplets between 2.6 and 2.8 ppm origin from the protons of the conjugated alkyne linker N-methylpropargylamine.

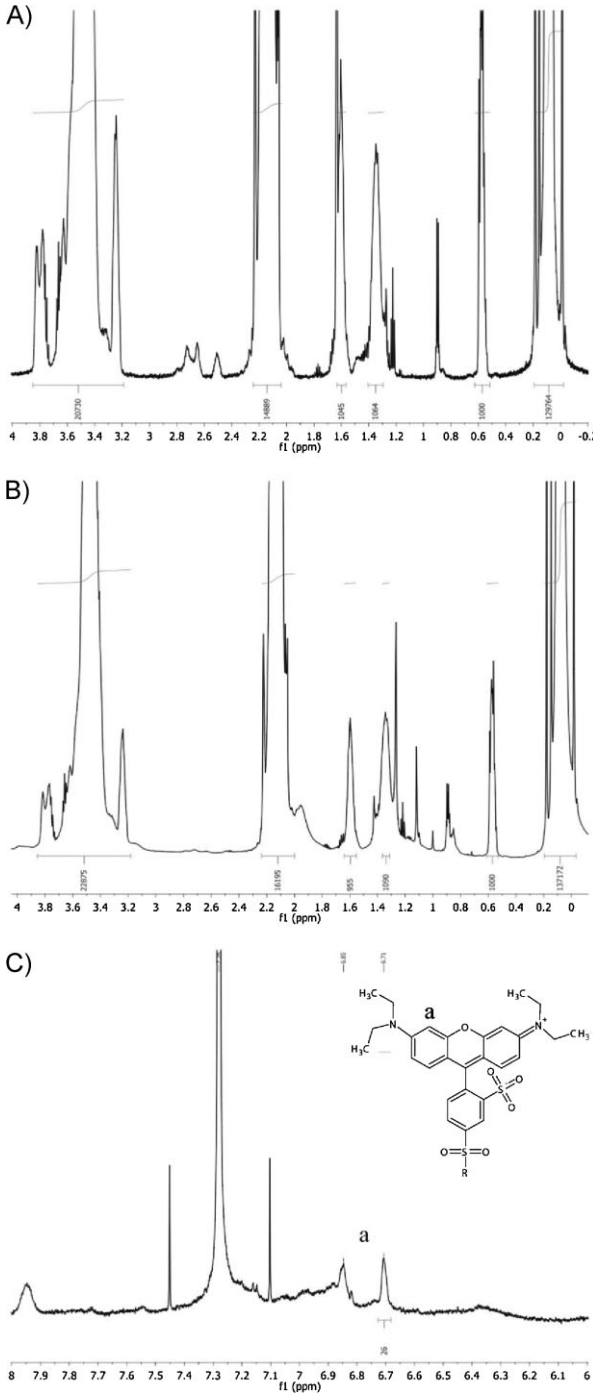

Figure 4.
[1]H-NMR of ABA and SRB-ABA polymers. (A) Integrals of peaks corresponding to the ABA polymer. (B) Integrals of peaks corresponding to the SRBABA polymer. (C) Close-up of the SRB-ABA spectrum between 6 and 8 ppm showing the doublet (a) at around 6.71 ppm corresponding to the proton at position (a) as indicated by the molecular structure of SRB. Spectra were acquired using $CDCl_3$ and a 600 MHz NMR spectrometer from Bruker.

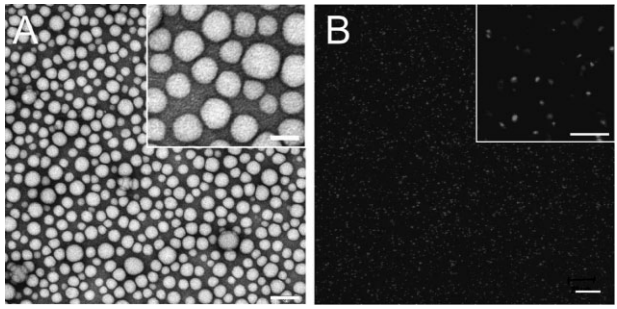

Figure 5.
Transmission electron and fluorescence micrographs of polymer nanocontainers. (A) Transmission electron micrographs of nanocontainers prepared from ABA. Scale bar in large image is 200nm and in inset image 100nm. (B) Fluorescence micrographs showing polymeric nanocontainers prepared by mixing ABA with SRB labeled ABA. Scale bar in large image is 20 μm and 5 μm in the inset image.

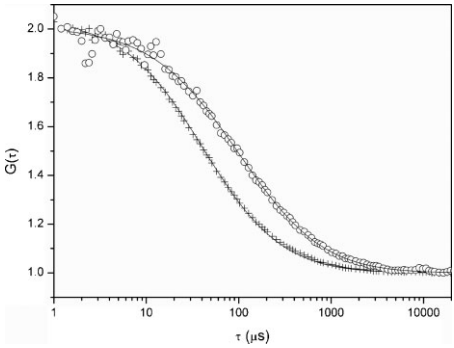

Figure 6.
Normalized autocorrelation curves of free SRB (crosses) and SRB labeled ABA polymer (open circles) using FCS. Scattered symbols correspond to experimental data and lines to fitted data.

SRB-ABA polymer (8276 g/mol). The diffusion properties of labeled polymer were measured in ethanol as a solvent since the use of the standard ABA solvent chloroform lead to a significant aggregation already of the free SRB dye (data not shown). The diffusion time for free SRB in ethanol was determined to 58.5 μs which is as expected. In contrast, the diffusion time of SRB-ABA was with 150 μs significantly higher than the free SRB dye suggesting the successful covalent modification (Figure 6). The molecular weight ratio of the SRB-ABA to SRB is approximately 15 which indicates that the expected diffusion time of the SRB-ABA should be around 146 μs, in perfect agreement with our measured value.

To verify whether it is possible to modify our alkyne-functionalized ABA nanocontainers once they are formed we analyzed the diffusion properties of above nanocontainers after the reaction with azide-functionalized SRB. After the reaction SRB-functionalized nanocontainers were lyophilized, dissolved in ethanol and filtered

Table 1.
Parameters obtained by FCS of SRB and SRB-functionalized ABA polymers.

Sample	Count rate (kHz)	N[a]	τ_1 (μs)[b]	F_1 (%)[c]	τ_2 (μs)[b]	F_2 (%)[c]
SRB	36.5	23.5	51	100	–	–
ABA-SRB[d]	34.8	32.5	51	63.5	116	36.5
ABA-SRB[e]	25.6	28.5	51	0.8	124	99.2
SRB + ABA[f]	36.7	23.9	51	6.9	53	93.1

[a]Number of molecules.
[b]Diffusion time of molecular species.
[c]Fraction molecules with a certain diffusion time.
[d]SRB conjugated to ABA using click chemistry.
[e]SRB conjugated to ABA using the SRB acid chloride.
[f]SRB mixed with ABA.

through a 0.2 μm hydrophilic membrane. The obtained sample was measured by FCS using 514 nm excitation and the diffusion properties were compared with other SRB samples (Table 1). These data indicate that reaction of SRB-azide to alkyne-functionalized polymersomes worked properly. Furthermore mixing unfunctionalized ABA polymer with SRB does not lead to an increase in the diffusion time indicating that non-specific adhesion of SRB to hydrophobic parts of the polymer is negligible.

Specific Uptake of Poly(G)$_{23}$ Modified Nanocontainers by THP-1 Cells

In order to verify the specific cellular uptake we used the human monocytic cell

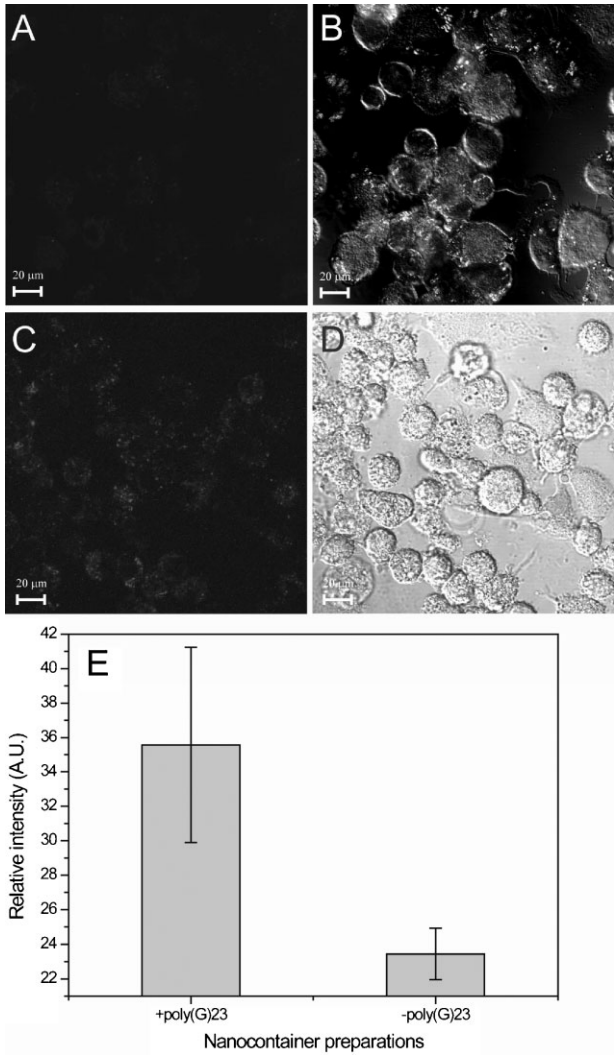

Figure 7.
Specific uptake of Poly(G)$_{23}$ modified nanocontainers by activated macrophages (THP-1 cells). (A) Confocal micrograph of THP-1 cells after 24 hours incubation of nanocontainers lacking the ligand, and (B) corresponding differential intereference (DIC) image. (C) Confocal micrograph of THP-1 cells after 24 hours incubation of poly(G)$_{23}$ modified nanocontainers, and (D) corresponding DIC image. (E) Graph indicating the relative intensity differences between positive and negative control.

line THP-1. Previous research in our group have shown the feasibility of specific uptake of Poly$(G)_{23}$ modified polymeric nanocontainers by THP-1 cells using the biotin-streptavidin interaction as a link for the ligand.[2,10,11] Although modular and with a high affinity, biotin-streptavidin interactions have the drawback that the number of binding sites of the nanocontainer has to be precisely determined in order to prevent excess free ligand significantly reducing uptake, and that the streptavidin protein might lead to adverse immunogenicity. Two days prior to imaging, THP-1 cells were activated with phorbol-12-myristate-13-acetate (PMA, 100 nM) to differentiate the monocytes into macrophages and approximately 500 000 cells/ml were seeded into each well of the tissue culture slide (Lab-tek, NUNC). Both Poly$(G)_{23}$ modified nanocontainers and control ABA nanocontainers lacking the ligand were added to the activated cells and incubated (37 °C, 5% CO_2) for 1, 2, 4, and 24 hours, respectively. Confocal fluorescence imaging using the 543nm laser line of a ZEISS LSM 510 microscope was used to determine the extent of specific cellular uptake (Figure 7). Significantly higher relative fluorescence intensity is found in images (Figure 6E) corresponding to THP-1 cells which had seen poly$(G)_{23}$ modified nanocontainers in contrast to THP-1 cells treated with nanocontainers lacking the poly$(G)_{23}$ ligand.

Conclusion

A first step in developing a robust and versatile ligand modified polymeric self-assembled delivery system is the successful covalent modification of our amphiphilic ABA coblock polymers. In this report we have shown that it is possible to directly endfunctionalize the ABA polymer by synthesizing a fluorescently labled ABA polymer using the dye SRB, but we have also shown that it is possible to functionalize the polymeric nanocontainers once they are formed by linking a poly$(G)_{23}$ ligand to the outer polymer membrane by a click chemistry approach. A combined analytic approach using both ^{1}H-NMR and FCS indicate successful conjugation of the SRB dye to the ABA polymer using two different coupling chemistries. To detemine whether this system is viable for targeting specific cells, poly$(G)_{23}$ modified polymeric nanocontainers were given to activated macrophages resulting in specific cellular uptake. Further studies are necessary to optimize the covalent modification of the ABA polymers, other promising receptor-ligand interactions have to be screened for optimal uptake and specificity, and ultimately release of biological active molecules have to be accomplished in order to develop a successful polymeric platform for targeted drug delivery.

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