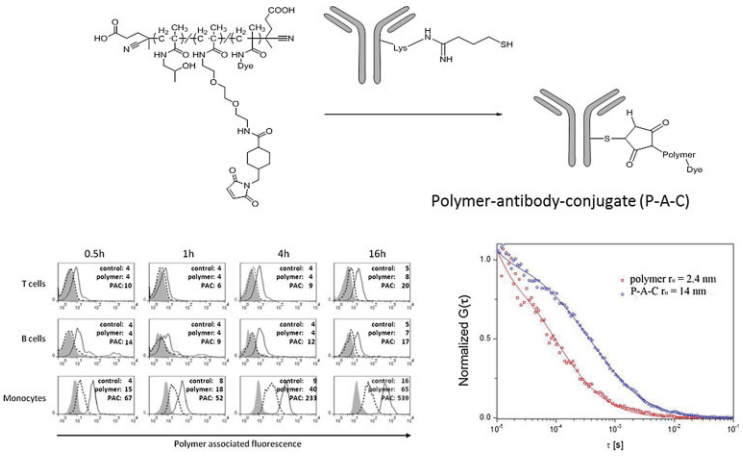
Synthesis of Maleimide-Functionalyzed HPMA-Copolymers and in vitro Characterization of the aRAGE- and Human Immunoglobulin (huIgG)–Polymer Conjugates[a]

Kristof Tappertzhofen, Verena V. Metz, Mario Hubo, Matthias Barz, Rolf Postina, Helmut Jonuleit, Rudolf Zentel*

Herein the synthesis of antibody–polymer conjugates, with a quite narrow dispersity based on the polymer HPMA, are reported. These conjugates are synthesized by coupling antibodies to maleimide-functionalized poly(*N*-(2-hydroxypropyl)-methacrylamide) (poly-HPMA) copolymers derived through reversible addition-fragmentation chain transfer (RAFT) polymerization of pentafluorophenyl methacrylate via the intermediate step of an activated ester polymer. We develop a protocol that allows the attachment of two different model antibodies, monoclonal anti-RAGE (receptor for advanced glycation end-products) antibody, and polyclonal human immunoglobulin (huIgG). Modification of the antibody and conjugation is monitored by SDS-PAGE electrophoresis. Preserved affinity is demonstrated by Western Blott and cell-uptake analysis, for example, to cells of the immune system.



K. Tappertzhofen, Dr. V. V. Metz, Dr. M. Barz, Prof. R. Zentel
Johannes Gutenberg-Universität Mainz, Institute of Organic Chemistry, Duesbergweg 10-14, 55099 Mainz, Germany
E-mail: zentel@uni-mainz.de
M. Hubo, Dr. H. Jonuleit
Department of Dermatology of the University Medical Center of the Johannes Gutenberg-University Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany

Dr. R. Postina
Johannes Gutenberg-Universität Mainz, Institute of Pharmacy and Biochemistry, Johann-Joachim Becherweg 30, 55128 Mainz, Germany

[a] **Supporting Information** for this article is available from the Wiley Online Library or from the author.

DOI: 10.1002/mabi.201200344

1. Introduction

Nanomedicines,[1] in particular polymer therapeutics[2] — that is, polymer–drug conjugates, polymer–protein conjugates, and polymeric micelles — have been extensively studied in recent decades.[3] In these systems a therapeutic agent is either encapsulated (hydrophobic drug) or covalently bound via a degradable spacer (hydrophilic drug) to a nanosized system and combined with a targeting moiety to direct the biodistribution to the desired target side.

Generally, the bodily distribution and unspecific cellular uptake of polymer therapeutics depends on their size, charge, and hydrophobic/hydrophilic balance. However, those factors can be controlled synthetically and "stealth-like" polymers can be achieved. Additional attachment of cell-specific targeting structures allows the cell-specific uptake of polymers. Various targeting moieties have already been introduced, such as folic acid,[4] peptides,[5] and sugars,[6] resulting in enhanced cellular uptake. In particular, monoclonal antibodies[7] are naturally specific for the binding motive, which can be a transmembrane protein expressed on the surface of a cell population.[8] It has to be kept in mind that, due to their size, polymer–antibody conjugates may not be an universal system for tumor imaging or anti-tumor therapy.[9] However, polymer–antibody conjugates do offer great potential in immunotherapy.[10] For this purpose, specific cells of the immune system, which are in systemic circulation, have to be addressed selectively. In addition recent advances in molecular biology, such as *phage display*, have enabled the production of human antibodies, thus leading to their clinical approval and application as targeting devices in therapy.[11]

The general requirements for polymers suitability are water solubility, and the absence of toxicity and immunogenicity. In this context, the usage of systems based on polyethylenglycol (PEG), poly[*N*-(2-hydroxypropyl) methacrylamide] (poly-HPMA), poly(glutamic acid) (PG), and dextrane have been explored. PEG is by far the most frequently used and its structure[13] and functionality[14] are constantly being diversified. Besides PEG, poly-HPMA-based drug conjugates are probably the most carefully investigated polymers, since HPMA–drug polymers have entered clinical trials.[15] The fixation of antibodies to polymers is usually performed using standard protocols for protein fixation, which include the reaction of activated carboxylic acids with the amino groups of proteins. These protocols were primarily designed for surface modifications of beads, which display enough functional units/antibodies, although the binding process may be of rather limited selectivity. Also poly(HPMA) based polymers made by free-radical polymerization have been functionalized with various antibodies and an increased uptake has been reported.[12] However, there is little information on the quantitative yields of the linking reaction and conserved target affinity and specificity. For the functionalization of polymers, or polymer aggregates, efficient binding strategies with high chemical yields are required allowing a selective binding of new structures.

Advances in controlled radical polymerization have enabled the synthesis of defined poly-HPMA copolymers by atom transfer radical polymerization (ATRP)[16] or reversible addition-fragmentation chain transfer (RAFT) polymerization;[17] in addition, selectively end-group functionalized polymers have become available.[18] Consequently the use of living radical polymerization to design polymer–protein bioconjugates has gained much interest.[19] Another approach towards HPMA-based copolymers is the RAFT-polymerization of pentafluorophenyl methacrylates leading to well-defined reactive polymeric precursors,[20] which can be converted into HPMA copolymers by subsequent post-polymerization modification reactions with 1-amino-2-propanol (HPA). This approach enables the controlled synthesis of multifunctional copolymers.[21–25]

To extend the synthetic approach to multifunctional polymer–antibody conjugates we have synthesized copolymers bearing maleimide groups for selective coupling to antibodies. In this work we report a methodology for efficient synthesis of poly-HPMA–antibody conjugates. The synthesis of conjugate and biological activity has been evaluated for two different model antibodies, the monoclonal anti-RAGE (receptor for advanced glycation end-products) antibody and the polyclonal human immunoglobulin. We report conjugate purity and binding efficiency investigated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) electrophoresis, Western blots, and cellular uptake studies (fluorescence-activated cell sorting, FACS, analysis).

2. Experimental Section

2.1. Materials

All chemicals were reagent grade and obtained from Sigma-Aldrich. Chemicals were used without further purification unless otherwise indicated. Oregon green (OG) cadaverine 488 and tetramethylrhodamine cadavarine 554 were obtained from Invitrogen. The pentafluoro-phenol was obtained from Fluorochem (UK). Anti-RAGE, clone mAbA11 was purchased from Millipore. Dioxane and tetrahydrofuran used in the synthesis was freshly distilled from sodium. 2,2-azobis(isobutyronitrile) (AIBN) was recrystallized from diethyl ether and stored at −7 °C. Dialysis was performed using Spectra/Por® 3 membranes (MWCO 3500 g/mol) obtained from Carl Roth GmbH+ Co. KG (Germany). Amicon® Ultra centrifugal filters 0.5 mL (MWCO 100 000 g mol^{-1}) were purchased from Millipore.

2.2. Characterization

^{1}H and ^{19}F nuclear magnetic resonance (NMR) spectra were obtained at 300 or 400 MHz using a Fourier-transform (FT)

spectrometer from Bruker and analyzed using MestReNova 6.0.2. IR spectra were recorded on a Bruker FT/IR-4100 using an attenuated total reflectance (ATR) unit. The polymers were dried at 40 °C overnight under vacuum and afterward characterized by gel permeation chromatography (GPC). GPC of hydrophobic pentafluorophenyl methacrylate was performed in tetrahydrofurane (THF) as solvent and with following parts: pump PU 1580, auto sampler AS 1555, UV detector UV 1575, refractive index (RI) detector RI 1530 from Jasco and mini DAWN Tristar light scattering detector from Wyatt. Columns were used from MZ-Analysentechnik, 300 × 8.0 mm: MZ-Gel SD plus 10^6 Å 5 μm, MZ-Gel SDplus 10^4 Å 5 μm and MZ-Gel SDplus 10^2 Å 5 μm. The elution diagram was evaluated with PSS WinGPC from Polymer Standard Service Mainz. The flow rate was set to 1 mL min^{-1} at a temperature of 25 °C. Calibration was done using polystyrene standards. GPC of hydrophilic HPMA-copolymers was performed in hexafluoroisopropanol (HFIP) containing 3 g L^{-1} potassium trifluoroacetate as solvent and with following parts: column packed with modified silica (PFG columns particle size: 7 μm, porosity: 100 and 1000 Å) and an RI detector (G1362A RID). The elution diagram was evaluated with PSS WinGPC from Polymer Standard Service, Mainz. Calibration was done using PMMA standards (Polymer Standard Service, Mainz). The flow rate was set to 0.8 mL min^{-1} at a temperature of 40 °C. The amount of fluorescent dye was determined using 3 mg per 20 mL (free dye) and 3 mg per 1 mL (polymer) solutions in methanol. Optical absorption was measured at a wavelength of $\lambda = 554$ nm (tetramethylrhodamine) or $\lambda = 498$ nm (Oregon green) through a 1 cm quartz cell using a Jasco V-630 photo spectrometer. GPC of the polymer–antibody conjugates was performed in buffered aqueous solution (0.05 M sodium phosphate/0.15 M sodium chloride; pH 7) with the following parts: Jasco pump (pU-2086 Plus series), Jasco UV/vis detector (UV-2077 Plus), and Jasco RI detector (Jasco RI 2031 Plus series). The flow rate was set to 0.4 mL min^{-1} using a Superose™6 10/300 GL column. Calibration was done using protein standards. The elution diagram was evaluated with PSS WinGPC from Polymer Standard Service, Mainz.

2.3. Synthesis of 4-Cyano-4-((thiobenzoyl)sulfanyl)-pentanoic Acid (Acid CTA)

The 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid was synthesized according to the literature.[26] 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 7.92–7.90 (d, 2H), 7.60–7.55 (t, 1H), 7.42–7.37 (t, 2H), 2.81–2.40 (m, 4H), 1.94 (s, 3H).

2.4. Synthesis of Pentafluorophenyl Methacrylate (PFPMA)

Pentafluorophenyl methacrylate (PFPMA) was prepared according to the literature.[27] 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 6.45 (s, 1H), 5.91 (s, 1H), 2.03 (s, 3H). 376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm]= −152.70 (d, 2F), −158.12 (t, 1F), −162.41 (d, 2F).

2.5. General Synthesis of Polymers

RAFT synthesis of PFPMA were performed in a Schlenk tube. The reaction vessel was loaded with 9.3 mg of 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid (acid-CTA), 0.55 mg 2,2-azobis(isobutyronitrile (AIBN) (molar ratio of CTA/AIBN 10:1), and 2 g of PFPMA in 4 mL of dry dioxane. After four freeze–thaw–vacuum cycles the tube was immersed in an oil bath at 70 °C for 12 h. The polymer was reprecipitated from tetrahydrofurane in hexane three times, isolated by centrifugation and dried over night at 30 °C in the vacuum oven. 950 mg (48%) of a slightly red powder were obtained.

2.6. End-Group Removal of Poly-Pentafluorophenyl Methacrylate (PR)

A Schlenk tube was loaded with 500 mg of the polymer (0.017 mmol) and 118 mg (0.4 mmol, 25 eq.) of 4,4-azobis(4-cyanovaleric acid) (ACVA), and 4 mL of dioxane were added. The tube was immersed in an oil bath at 85 °C for 4 h. Then, the polymer was precipitated three times in hexane, isolated by centrifugation and dried for 12 h at 30 °C under vacuum. 460 mg (92%) of a colorless powder were obtained. IR: $\nu = 3050$ (C−H), 1778 (CO). 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 2.75–2.00 (br s,3H), 1.53–1.36 (m, 3H). 376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm]= (−150)-(−152) (m, 2F), −157 (br s, 1F), −162 (br s, 2F).

2.7. Synthesis of *N*-(tert-Butyloxycarbonyl)-aminoethyloxyethyloxyethylamine

N-(tert-Butyloxycarbonyl)aminoethyloxyethyloxyethylamine was synthesized according to the literature.[28]

2.8. Synthesis of 4-(*N*-Maleimidomethyl)cyclohexane Carboxylic Acid

The synthesis of 4-(*N*-maleimidomethyl)cyclohexane carboxylic acid was adapted from the literature.[29] Briefly 10 g (0.064 mol) of 4-(*N*-aminomethyl)cyclohexane carboxylic acid were dissolved in 100 mL acetic acid and 4.3 g (0.064 mol) maleic anhydride, dissolved in 50 mL acetic acid, was added dropwise and the resulting suspension was stirred for 5 h. The precipitate was filtered and recrystallized from methanol. Yield: 11 g (0.043 mol; 67%). The resulting colorless solid was suspended in 900 mL of toluene and heated under reflux for 15 h with a water separator. The solution was filtered and the solvent evaporated under reduced pressure. 400 mL of water were added to the residue and the pH value was adjusted to 2 with 1 M HCl. The product was extracted three times with ethyl acetate and the solvent was evaporated under reduced pressure. The crude product was recrystallized from toluene yielding 5 g (0.021 mol, 49%) of the desired product. 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm]= 6.70 (s, 2H), 3.38–3.36 (d, 2H), 2.30–2.20 (tt, 1H), 2.06–2.00 (m, 2H), 1.77–1.72 (m, 2H), 1.69–1.60 (m, 1H), 1.45–1.31 (m, 2H); 1.07–0.94 (m, 2H) FD-MS: [m/z]= 237.3 ([M + H], calc. 237.1).

2.9. Synthesis of 1-Pentafluorophenyl-4-(*N*-maleimidomethyl)cyclohexane Carboxylate

Pentafluorophenyl trifluoroacetate was synthesized according to the literature.[30] 0.9 g (3.8 mmol) 4-(*N*-maleimidomethyl)cyclo-

hexane carboxylic acid was dissolved in 30 mL of dry THF, 2.1 g (7.5 mmol) pentafluorophenyl trifluoroacetate and 0.77 g (7.5 mmol) NEt_3 were added through a syringe in a argon atmosphere. The resulting solution was stirred for 3 h at room temperature, the solvent evaporated under reduced pressure and 150 mL of water were added. The product was extracted three times with dichloromethane, dried over magnesium sulfate and the solvent was removed under reduced pressure. The product was purified via silica gel flash chromatography using petrol ether/ethyl acetate 5:1 as the eluent yielding 1.09 g (2.7 mmol, 71%) of the desired product. $R_f = 0.18$ (petrol ether/ethyl acetate 10:1); 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm]= 6.72 (s, 2H), 3.42–3.40 (d, 2H), 2.66–2.57 (tt, 1H), 2.21-2.16 (m, 2H), 1.84-1.79 (m, 2H), 1.76-1.68 (m, 1H), 1.62-1.48 (m, 2H), 1.17–1.02 (m, 2H); 376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm]= −153.10 (d, 2F), −158.18 (t, 1F); −162.39 (t, 2F).

2.10. Post-Polymerization Modification

In a typical reaction 100 mg (0.4 mmol) of the polymer with removed thioester end-groups PR were dissolved in 1.5 mL dioxane/DMSO 2:1, then 4 mg of one dye and 40 μg of NEt_3 were added and stirred for 4 h at 50 °C. Afterwards 10 mg (0.04 mmol) of *N*-(tert-butyloxycarbonyl)-aminoethyloxyethyloxyethylamin, 60 mg 1-amino-2-propanol, and 64 μg NEt_3 were added and stirred for 3 d at 35 °C. Complete conversion of the polymeric precursor was confirmed by ^{19}F-NMR spectroscopy. The polymer was precipitated in diethyl ether, dialyzed against pure water and freeze dried. Afterwards the polymer was stirred for 3 d in 10% TFA/water mixture for removal of the Boc-protecting group. Remaining water/TFA was removed by codistillation with toluene. The polymer was dialyzed against pure water and freeze-dried, yielding 48 mg of amine-functionalized polymer with Oregon green as dye and 55 mg of amine-functionalized polymer with tetra-methylrhodamine as dye.

2.11. Synthesis of Maleimide-Functionalized Polymer (P1/P2)

48 mg of the polymer with Oregon green as dye and 55 mg of the polymer with tetra-methylrhodamine as dye were dissolved in dioxane/DMSO 1:3 and 13 mg/16 mg of pentafluorophenyl-4-(*N*-maleimidomethyl)cyclohexane carboxylate and 43 μL/60 μL DIPEA were added respectively. The solution was stirred for 12 h at room temperature. The polymers were precipitated in ethyl ether, dialyzed against pure water and freeze dried yielding 40 mg of the polymer P1 (dye: Oregon green) as an orange powder or 52 mg of the polymer P2 (dye: tetramethylrhodamin) as a red powder. The polymers were further purified by preparative SEC chromatography using Sephadex Hi Trap™ desalting columns. IR: ν = 3200–3600 (–OH), 2970–2930 (C−H), 1707 (maleimide C−N), 1638 (amide CO). 300 MHz ^{1}H-NMR (DMSO-d_6): [δ/ppm]= 7.36 (br, s, 1H), 7.01 (s, 0.10H), 4.71 (br, s, 1H), 3.67 (br, s, 1H), 2.90 (br, s, 2H), 2.33–0.67 (m, 8H).

2.12. Deactivation of Maleimide Group

7 mg of polymer P1 or P2 (0.004 mmol maleimide groups) was dissolved in 1 mL of DMSO and 4 mg of cysteine chloride (0.03 mmol) was added; the mixture was stirred for 2 h at room temperature, dialyzed in pure water, and freeze-dried, yielding 7 mg of polymer P1b and P2b with deactivated maleimide group. The polymers were further purified by preparative SEC chromatography using Sephadex Hi Trap™ desalting columns.

2.13. Preparation of 2-IT Modified/TCEP-Reduced Antibody

In a typical procedure the pH value of a solution of 100 μg (6.7×10^{-10} mol) of the native antibody in 400 μL PBS buffer solution containing 0.001 M EDTA was adjusted to 8, then 0.0037 mg (2.7×10^{-8} mol, 40 eq.) 2-iminothiolane were added via a stock solution and stirred for 1 h at room temperature. The solution was dialyzed against 0.001 M EDTA PBS buffer solution at 4 °C for 12 h and aliquots were taken for SDS-PAGE analysis. Subsequently 0.0048 mg (1.6×10^{-8} mol, 25 eq.) tris-(carboxyethyl)phosphine (TCEP) were added via a stock solution, stirred for 1 h at room temperature and dialyzed against 0.001 M EDTA PBS buffer at 4 °C. The amount of thiol groups per antibody was determined using Ellman's assay.

2.14. Synthesis of Polymer–Antibody Conjugates (P1K/P2K)

In a typical procedure 2 mg (9.9×10^{-8} mol chains, 1.1×10^{-6} mol maleimide groups) of the polymer P1/P2 were dissolved in 20 μL of DMSO and added to 100 μg (6.7×10^{-10}) mol antibody, 5.3×10^{-9} mol SH-groups) of the modified antibody in 400 μL PBS buffer solution and stirred for 1 d at room temperature. Excess of polymer was removed by centrifugal filtration using Amicon® centrifugal filter devices with a molecular weight cut off of 100 000 g mol^{-1}. The purification procedure was repeated seven times for 15 min until no fluorescent signal of the polymer could be detected in the filtrate. The conjugate was dissolved in PBS buffer for further experiments. Aliquots for SDS-PAGE analysis were taken before and after removal of free polymer.

2.15. Fluorescence Correlation Spectroscopy

The experiments were performed on a semi commercial setup based on an inverted microscope IX70 (Olympus, Japan) combined with the FluoView300 confocal laser scanning unit (Olympus, Japan) and an FCS upgrade kit (PicoQuant, Germany). The latter is fiber-coupled to the FluoView300 and has two detection channels separated by a dichroic mirror and possessing separate emission filters and single photon avalanche diode (τ-SPAD) detectors. A TimeHarp 200 time-correlated single-photon counting card in combination with the software package SymPhoTime (both PicoQuant, Germany) was used for data acquisition and analysis. An Olympus UPLSAPO 60XW, 60×/NA 1.2 water immersion objective was used in all studies. The fluorescent species were excited by an argon-ion laser at $\lambda = 488$ nm and their emission was detected after filtering with a LP505 long pass filter. An eight-well, polystyrene-chambered cover glass (Laboratory-Tek, Nalge Nunc International) was used as sample cell. For each sample a series of 10 measurements with a total duration 5 min were performed.

The confocal observation volume was calibrated using a reference dye with a known diffusion coefficient, i.e. Alexa Fluor 488.

2.16. Cell Culture

SK-N-MC and RAGE expressing HEK Flp-In cells[31] were maintained in DMEM supplemented with fetal calf serum (10%), glutamine (0.002 M), sodium pyruvate (0.001 M), penicillin and streptomycin (100 U mL^{-1}). Medium and cell culture supplements were from PAA/Austria and PromoCell/Germany. Cells were cultured in an incubator at 37 °C in a humid atmosphere of 5% CO_2 and split twice a week.

2.17. Western Blot

Cell-lysates were separated by 10% SDS-PAGE and blotted to nitrocellulose membranes (GE Healthcare). Membranes were probed with anti-RAGE antibody, detection with a second antibody labeled with horse radish peroxidase and ECL substrate or anti-RAGE-polymer-conjugate P1K (2.5 μg mL^{-1}), detection by fluorescent dye (tetramethylrhodamin) of the polymer using the Stella 8300 system (Raytest, Straubenhardt, Germany) and the YFP filter (ex: 500 nm +/− 10 nm).

2.18. Microscopy

For cell microscopy SK-N-MC cells or HEK/RAGE cells were plated in 35 mm dishes containing coverslips (diameter of 18 mm) and incubated until confluence. The next day cells were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min. After an additional washing step cells were treated with coupled RAGE antibody P1K in PBS/5% FCS (25 μg mL^{-1}) for 1 h at room temperature. Then cells were washed twice with PBS and mounted in Mowiol 4-88 before imaging. Images were acquired using a Zeiss Axiovert 100 microscope with a ×100/1.30 Fluar objective and a MicroMax CCD camera (Princeton Instruments, Trenton, NJ) driven by MetaView Imaging System software (Universal Imaging Corporation).

2.19. Silver Staining

Low amounts of proteins separated by SDS-PAGE (7.5%) were detected with the silver staining method, after Wray.[32]

2.20. Ellman Assay

The quantification of free thiol groups in solution was conducted using Ellman's reagent and measuring molar absorptivity after manufacturer's instructions (Thermo Scientific, USA).

2.21. Isolation of Human PBMC

Human PBMC were obtained from buffy coats of healthy volunteers by ficoll density gradient centrifugation.

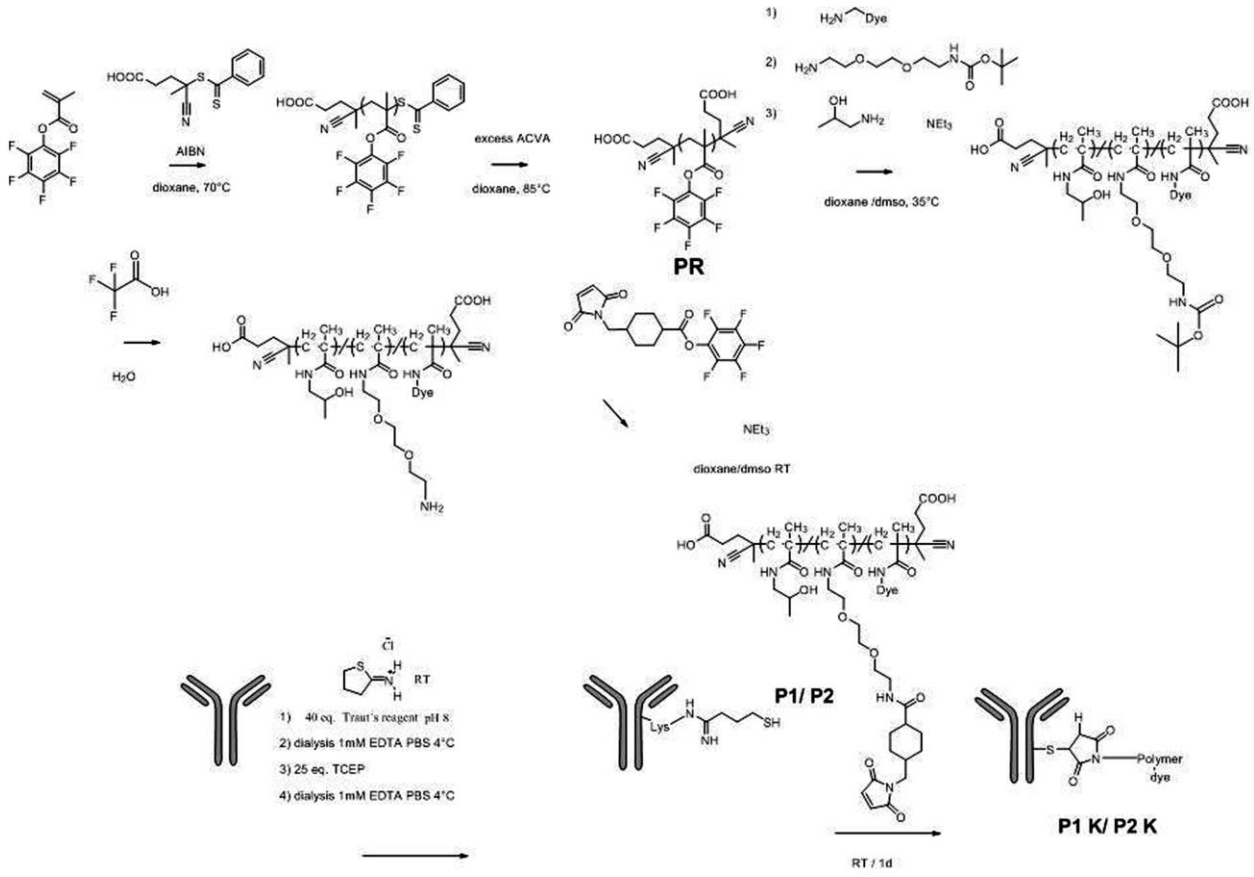
2.22. Flow Cytometry

T cells, monocytes or B cells, PBMC were stained with anti-CD3 (HUCT1, Pe-Cy5 conjugated, BD), anti-CD14 (M5E2, APC-conjugated, BD), and anti-CD19 monoclonal antibodies (HIB19, PE conjugated, BD) and sorted. Stained PBMC were then incubated with unconjugated or human IgG-conjugated HPMA-polymers (10 μg mL^{-1}) for different time points (0.5, 1, 4, and 16 h) at 37 °C and 5% CO_2. Harvested PBMC were washed twice and analyzed by flow cytometry (LSR II, BD). Data was evaluated using FlowJo software (Celeza) with a blue laser (488 nm) and a emission filter for 530 nm. To determine cell viability, PBMC were treated with 7-AAD (eBioscience) following the manufacturer's instructions.

3. Results and Discussion

To establish a synthetic protocol for the controlled linkage of different types of antibodies to HPMA-based polymers we have chosen the selective *Michael-like* addition of thiol groups to maleimides. Thus, we had to handle two tasks: first, we had to synthesize stable, well-defined HPMA-based copolymers bearing maleimide groups; and, second, we needed a route to create a small, but sufficient number of free thiol groups at the antibody for conjugation. For this purpose we developed the synthetic pathway shown in Scheme 1. The properties of the resulting polymers are compiled in Table 1.

RAFT polymerization of pentafluorophenyl methacrylate resulted in a narrowly distributed ($D = 1.3$) and reactive precursor polymer **PR**, which allows the introduction of functionalities in high yields. In addition, the homopolymer can be precisely characterized by GPC (THF). In order to avoid side reactions during aminolysis during postpolymerization modification the polymer end-groups were removed with an excess of 4,4-azobis(4-cyanovaleric acid) (see Figure S8 in the Supporting Information, SI). In the next step, subsequent post-polymerization modification was carried out to attach a fluorescent marker (quantified by fluorescence spectroscopy) as well as a maleimide group resulting in the HPMA-copolymers **P1/P2**. Thereby, a linker was used to decouple the maleimide groups from the polymer backbone, enhancing accessibility. The maleinimide groups have to be introduced in several steps, since they react rapidly with 1-amino-2-propanol under the post-polymerization modification conditions. Thus in the first step a short, monoprotected Jeffamine was introduced together with the dye followed by the 1-amino-2-propanol. Complete conversion of the polymeric precursor **PR** to HPMA-copolymers was ensured by ^{19}F-NMR spectroscopy (Figure S10 in the SI) and IR spectroscopy (Figure S11). After removal of the Boc-protecting group, the free amino group was coupled to the activated maleimide (1-pentafluorophenyl-4-(*N*-maleimidomethyl)cyclohexane carboxylate), which exhibits a comparable reactivity to commercial



Scheme 1. Synthesis of HPMA copolymers and their conjugation to antibodies.

available succinimidyl-4-(-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC). This yields the polymers **P1** and **P2**, which are identical except for the fluorescent dye (Scheme 1 and Table 1). **P1** and **P2** are water-soluble, slightly amphiphilic, and cannot be characterized with a usual GPC setup, due to their amphiphilic nature, which leads to interaction with the column material. To ensure that the quality of the polymer was not reduced during the post-polymerization modification we performed GPC measurements in HFIP as solvent. The GPC analytics displayed a dispersity of 1.3 (see Table 1). Thus, the well-defined nature of the polymers **P1** and **P2** remained untouched during

Table 1. HPMA-based copolymers (see Scheme 1), **P1** and **P2** are identical except for the fluorescent dye; **P1K** and **P2K** are derived from **P1** or **P2** by conjugation with the two different antibodies; **P1ref** and **P2ref** are reference polymers for **P1K** and **P2K** and are derived from **P1** and **P2** by reaction with cysteine.

	MI/chain[g] (NMR)	$\overline{M}_n/\overline{M}_w$	*D*	AB [wt%][d]	R_H [nm][f]
PR		29 700/38 300[a]	1.29		
P1/P2	11	20 200/26 050[b]	1.31[c]		2.4
P1K/P2K		308 000/469 000[e]	1.6[e]	aRAGE/huIgG 48	14
P1ref/P2ref		20 200/26 050[b]			

[a]As determined by GPC with THF as eluent; [b]the values for P1/2 were recalculated from the molecular structure; [c]as determined by GPC with HFIP as solvent to ensure consistency of dispersity after post-polymerisation modification; [d]as estimated by molecular weight of the conjugate obtained by GPC in buffered aqueous solution; [e]as measured by GPC in buffered aqueous solution; [f]as determined by FCS measurement in buffered aqueous solution; [g]number of maleimide groups per polymer chain.

the post-polymerization modification. The content of maleimide groups was determined by ^{1}H-NMR spectroscopy as 9 mol% (see Figure S4). This is in good agreement with the intended degree of functionalization of 10 mol% and corresponds to an average of 11 maleimide groups per polymer.

The *Michael-like* addition of maleimides to thiol groups is widely used for the conjugation of biological active molecules since it takes place rapidly at physiological conditions with no byproducts; thus providing mild reaction conditions.[33] It has already successfully been used to attach antibodies as well as fragment antigen binding (FAB) fragments.[34] The maleimide group is, however, known to be sensitive to hydrolysis under basic conditions.[35] To overcome this problem the polymers were always dissolved in dry DMSO and added directly to a solution of the reaction partners.

In addition, we synthesized the reference polymers **P1ref** and **P2ref** in which the maleimide groups (see Table 1) were quenched with cysteine. Those polymers were used to demonstrate the absence of unspecific interactions between the polymers and antibodies, or polymers and targets. The quenching is also necessary whenever the controls **P1ref** and **P2ref** are applied to cellular uptake studies, since unspecific covalent binding of the polymer free thiols on the cell surface needs to be prevented.[36]

Second, to prove applicability of the conjugation concept, two different antibodies were chosen: one monoclonal and the other polyclonal. The monoclonal antibody anti-RAGE was chosen, since the receptor for advanced glycation end-products (RAGE) is involved in many diseases[37] and thus may serve as an interesting therapeutical target. The second antibody used in this study is the polyclonal antibody human immunoglobulin, which allows the targeting of immune cells, for example, monocytes, B-cells, T-cells, and dendritic cells, a major task for successful immunotherapy.[10]

Natural antibodies do not possess free thiol groups. Thus, those groups have to be introduced before polymer conjugation. This can be done either by use of mild reduction of some of the naturally occurring disulfide bonds stabilizing the different subunits of the antibody or by using Traut's reagent[38] (2-iminothiolane)[39]. The 2-iminothiolane converts ε-amino groups of lysine residues to thiol groups by a nucleophilic ring-opening reaction. There are several reports that reduction is superior since epitope binding sites are less affected. The use of 1×10^{-4} M dithioerithrol (DTT) was reported to reduce the disulfide bridges only in the hinge region, thus introducing thiol groups for conjugation to proteins without affecting binding sites. During our experiments (see Figure S2 in the SI) we found, however, that under the use of 1×10^{-4} M DTT, as described in the literature,[40] binding to polymers was not efficient. Working with such a small dose of reducing agents and under mild conditions, only a few percent of antibodies were reduced leading to a large number of unmodified antibodies. However, at higher concentrations of reducing agent (1.0 M DTT), as described in the literature, [41] complete or partial decomposition of the antibody was observed (see Figure S1). This demonstrates that there is more work needed to obtain an efficient modification of the antibody by this strategy.

Thus, we decided to use Traut's reagent. The amount of modification can be adjusted by the ratio of Traut's reagent to lysine residues. We tried to keep the level of modification as low as possible to minimize alterations on the antibody itself. We found that the use of a twentyfold excess of Traut's reagent to antibody resulted in polymer conjugates with still unbound antibody left (see Figure S3 in the SI). Increasing the excess of Traut's reagent to antibody to fortyfold resulted in conjugates with no residual unbound antibody.

Generally during reaction with Traut's reagent an additional band with about the double mass of the antibody can be observed in the SDS-PAGE for both antibodies (lane 2, Figure 1 and 2). This occurs most likely due to partial oxidation of the introduced thiol groups leading to cross-linked antibodies with increased molecular weight. The disulfide groups responsible for aggregate formation can be reduced by adding TCEP. This treatment resulted in a complete disappearance of the bands at double molecular weight (lane 3, Figure 1 and 2). 25 eq. tris-(2-carboxyethyl)phosphine (TCEP) were sufficient to yield the monomeric thiol modified antibody. The structural integrity of the antibody remained untouched under those conditions. The amount of thiol groups per antibody was afterwards quantified using Ellman's reagent resulting in an average of 8 thiol groups (see Figure S5 in the SI).

In the next step, a 100-times excess of polymer compared to antibody was used to ensure complete conjugation

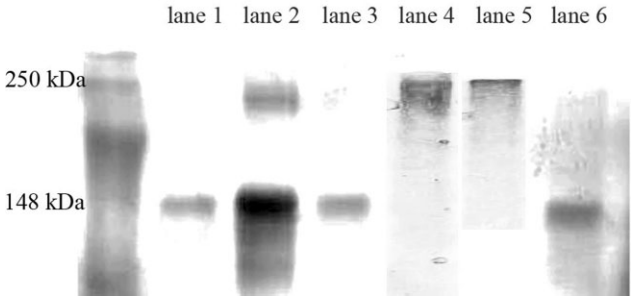


Figure 1. Conjugation of anti-RAGE antibody to **P1** followed by SDS-PAGE (silver staining); note: the anti-RAGE is monoclonal. Lane 1: free anti-RAGE; lane 2: anti-RAGE modified with Traut's reagent 2-Iminothiolan; lane 3: anti-RAGE modified with 2-iminothiolan and reduced with TCEP; lane 4: anti-RAGE-polymer conjugate; lane 5: polymer–antibody conjugate **PK1** after removal of free polymer; lane 6: mixture of maleimide-functionalized polymer **P1ref** with unmodified anti-RAGE antibody.

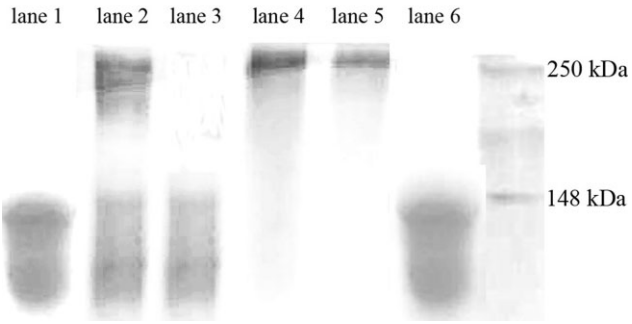


Figure 2. Conjugation of huIgG to **P2** followed by SDS-PAGE and Coomassie staining; note: huIgG is polyclonal; this is the reason for the broadened bands. Lane 1: free huIgG; lane 2: huIgG modified with Traut's reagent 2-iminothiolan; lane 3: huIgG modified with 2-iminothiolan and reduced with TCEP; lane 4: huIgG–polymer conjugate; lane 5: polymer–antibody conjugate **PK2** after removal of free polymer; lane 6: mixture of maleimide-functionalized polymer **P2ref** and unmodified huIgG.

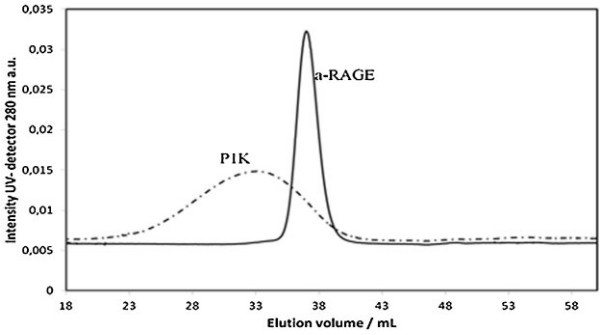


Figure 3. GPC elution profile in buffered aqueous solution of free antibody (aRAGE) and polymer–antibody conjugate P1K.

(no free antibody left, see lane 4 in Figure 1 and 2). This strategy is advantageous, because it is easier to remove the "relatively small" unbound polymer by centrifugal filtration from the conjugate than to remove the large unbound antibody. In addition, the polymers for conjugation **P1** and **P2** can be easily synthesized in 100-milligram to gram scale, while the amount of antibody is usually restricted.

Although the *Michael-like* addition between maleimide and thiol is known to be completed after 1 h for small molecules, we found that reaction times were elongated in the case of polymers to about 1 d to finalize the reaction between polymer and antibody. The prolonged reaction time is probably a result of the sterical hindrance during reaction between two macromolecular systems. The excess of polymer could easily been removed by repeating centrifugal dialysis. Both SDS and GPC measurements show that all unbound polymer is removed. In addition no residual free antibody could be observed (lane 5 in Figure 1 and 2). For comparison a simple mixture of unmodified antibody with maleimide functionalized polymers **P1ref** and **P2ref** was tested, but no unspecific interaction could be observed (lane 6 in Figure 1 and 2).

During conjugation, branching can occur due to the multifunctionality of polymer and antibody.[39] For an estimation of molecular weight as well as dispersity of the conjugates, we performed GPC in buffered aqueous solution (Figure 3, Superose^{TM}6 10/300 GL column, see the Experimental Section). The elution profile showed no traces of unbound antibody or unbound polymer. The number average molecular weight of the conjugates was determined to be 310 000 with a dispersity of 1.6. The antibody itself has a molecular weight of around 145 kg mol^{-1} and the polymer has an $\overline{M}_n$ of 20 kg mol^{-1}. Thus, an average molecular weight of 310 000 implies the formation of nano-aggregates of polymers attached to 1–3 antibodies. Despite this, the dispersity of the conjugates was still quite narrow (see Table 1) showing that a large excess of reactive polymer can prevent formation of large aggregates. Additionally the amount of antibody in the conjugate was measured by Bradford's assay (Figure S6 in the SI).

For further characterization we used fluorescence correlation spectroscopy to determine the size of the conjugate **P1K**. We observed a hydrodynamic radius of 2.4 nm for free polymer **P1/P2,** while about 5–6 nm can be expected for the antibodies.[42] 14 nm were determined for conjugate **P1K** (Figure S9 in the SI). This implies again that the conjugate consists of a small number of antibodies linked by several polymer chains.

3.1. Biological Activity of Anti-RAGE Antibody after Conjugation to Poly-HPMA (P1K)

To determine the functionality of **P1K** the conjugate was tested for its specific binding to RAGE, a transmembrane protein. For this purpose, human endothelial kidney cells (HEK), which overexpress RAGE, and SK-N-MC cells, which do not express RAGE at all, were incubated with the free antibody and the conjugate **P1K**. At first binding of free anti-RAGE antibody to RAGE can only be observed on cells expressing RAGE and not on control cells without the receptor (see Figure 4A and B). This visualization is achieved by probing the antibody with a second, Cy3-labeled antibody. The polymer-antibody conjugate **P1K** is functionalized with a fluorescent dye and does not need a second antibody for visualization. Again binding can only be observed on cells bearing RAGE (Figure 4C), and not on cells without the receptor (Figure 4D). The free antibody or antibody–polymer-conjugate is thereby located at the plasma membrane, since the receptor is a transmembrane protein and cells are immobilized. As reference, we synthesized the unconjugated polymer **P1ref** without

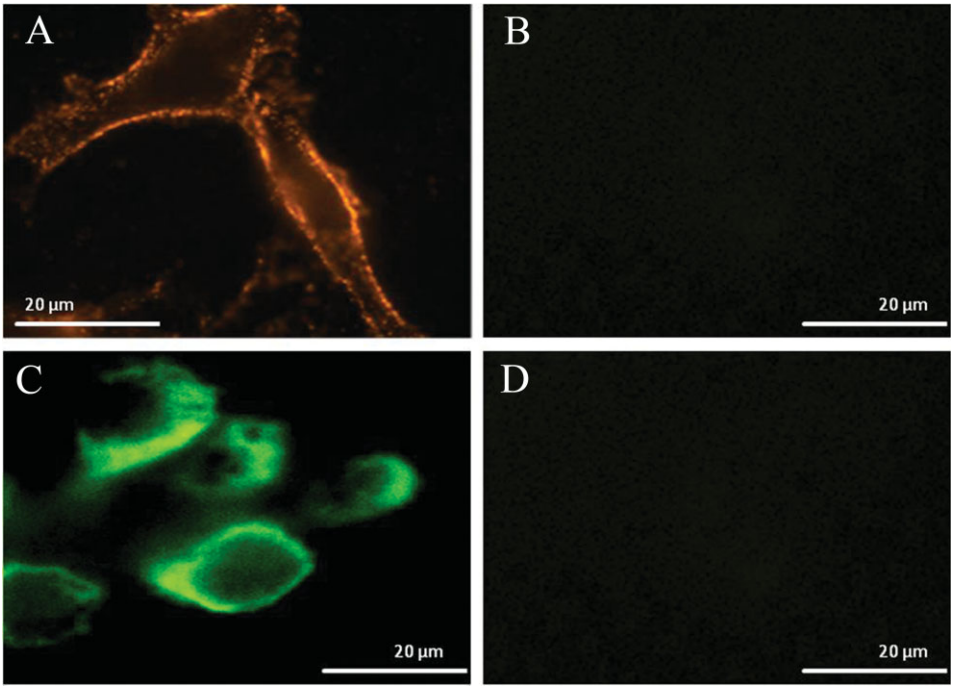


Figure 4. Fluorescent microscopy: A) HEK Flp-In cells, overexpressing RAGE, treated with anti-RAGE antibody and probed with a secondary antibody labeled with Cy3; B) SK-N-MC cells, not expressing RAGE, treated with anti-RAGE antibody; C) HEK Flp-In cells, over-expressing RAGE, treated with **P1K**; and, D) SK-N-MC cells, not expressing RAGE, treated with **P1K**.

maleimide groups (Scheme 1). This polymer did not bind to cells with or without RAGE.

Moreover, to prove binding specificity, "Western blotting" was performed (Figure 5). First the RAGE (transmembrane protein with a size of 55 kDa) present in cell lysate is separated by gel electrophoresis. After transferring the proteins to a nitrocellulose membrane RAGE was detected using unmodified anti-RAGE antibody followed by treatment with an anti-mouse antibody labeled with horse radish peroxidase and ECL-substrate (Figure 5, lane 2). One can distinguish two protein bands due to the glycolysated (upper band) and unglycolysated (lower band) form of the receptor.[32]

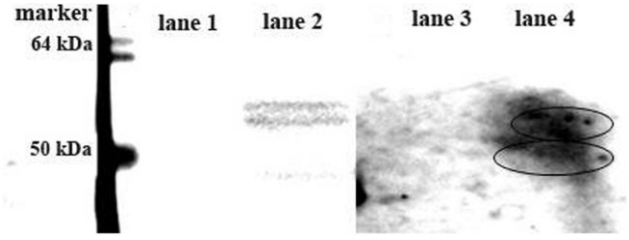


Figure 5. Western blotting: i) SK-N-MC cell lysates, not expressing RAGE, separated by SDS-PAGE and blotted to nitrocellulose membranes and probed with: lane 1 anti-RAGE antibody, probed with a second anti-mouse antibody labeled with horse radish peroxidase and ECL-substrate; and, lane 3: anti-RAGE antibody-polymer conjugate **PK1** (detection by fluorescent dye of the polymer). ii) HEK Flp-In cell lysates, over-expressing RAGE separated by SDS-PAGE and blotted to nitrocellulose membranes probed with: lane 2 anti-RAGE antibody, probed with a second anti-mouse antibody labeled with horse radish peroxidase and ECL-substrate; and, lane 4 anti-RAGE antibody-polymer conjugate **PK1** (detection by fluorescent dye of the polymer).

Alternatively, the receptor is blotted to a nitrocellulose membrane, incubated with anti-RAGE-antibody conjugate **P1K** (detection by fluorescent dye of the polymer) and washed. The fluorescence image shows intensity only at the position of the size of RAGE (Figure 5, lane 4) and again two different bands for the glycolysated and the unglycolysated form of the protein. This proves that the antibody, which is conjugated to several polymers shows still specific interaction with its receptor and no unspecific binding.

3.2. Biological Activity of huIgG after Conjugation to Poly-HPMA (P2K)

Nanoparticle-based immunotherapy of cancer requires that cells of the immune system be addressed to stimulate a direct response. Thus, targeting mediated by specific antibodies is of great interest. As a first approach we tested the huIgG conjugate (**P2K**). HuIgG binds to Fc-receptors expressed mainly by monocytes and significantly less by B or T cells. Consequently, conjugation of copolymers to

huIgG should result in enhanced binding to these cell populations. Furthermore for analyzing the unspecific interaction of polymers with distinct lymphocyte populations, HPMA-polymers with deactivated maleimide groups (**P2ref)** were used. To determine the difference between unspecific and targeted uptake peripheral blood mononuclear cells (PBMCs, composed of human T cells, B cells, and monocytes) were incubated either with huIgG-conjugated (**P2K)** or unconjugated (**P2ref)** polymers. In PBMCs, subpopulations of human lymphocytes can be differentiated and characterized upon expression of specific surface molecules (Figure 6A).

Flow cytometry was performed after different incubation times to assess the rate of polymer-positive cells (Figure 6B). Additionally, these measurements demonstrated that the HPMA-polymers are not cytotoxic to PBMCs at the applied concentration of 10 $\mu g\,mL^{-1}$, as no changes in levels of vital cells were detected by SSC and FSC (see Figure S7 in the SI).

First, it is important to look at the cell associated fluorescence of reference polymers **P2ref**. The highest mean fluorescence intensity could be detected in monocytes (MFI 65) at all time points up to 16 h. Noteworthy B cells and T cells showed almost no binding of **P2b** even after 16 h. This changes dramatically after conjugation of huIgG. Polymer–antibody-conjugates **P2K** increased the cellular associated polymer fluorescence in all cases including B cells and even T cells. The increase in cell associated fluorescence in B cells and T cells is small, but since they had shown almost no uptake for the unmodified polymer **P2ref**, the relative increase is rather high. It is remarkable that it is possible to increase the interaction with B cells and T cells so strongly despite their well-known low expression levels of Fc-receptors. Again the highest fluorescence intensity could be detected in monocytes at all time points. After 16 h an order-of-magnitude higher amount of polymer positive cells were observed than for the control polymer **P2ref**.

These results clearly indicate that huIgG conjugated to polymers mediates selective targeting of Fc-receptor expressing lymphocytes in human peripheral blood, resulting in increased amounts of polymer positive cells. The ratio of polymer positive cells is noticeable smaller in B cells and T cells with low Fc-receptor expression,

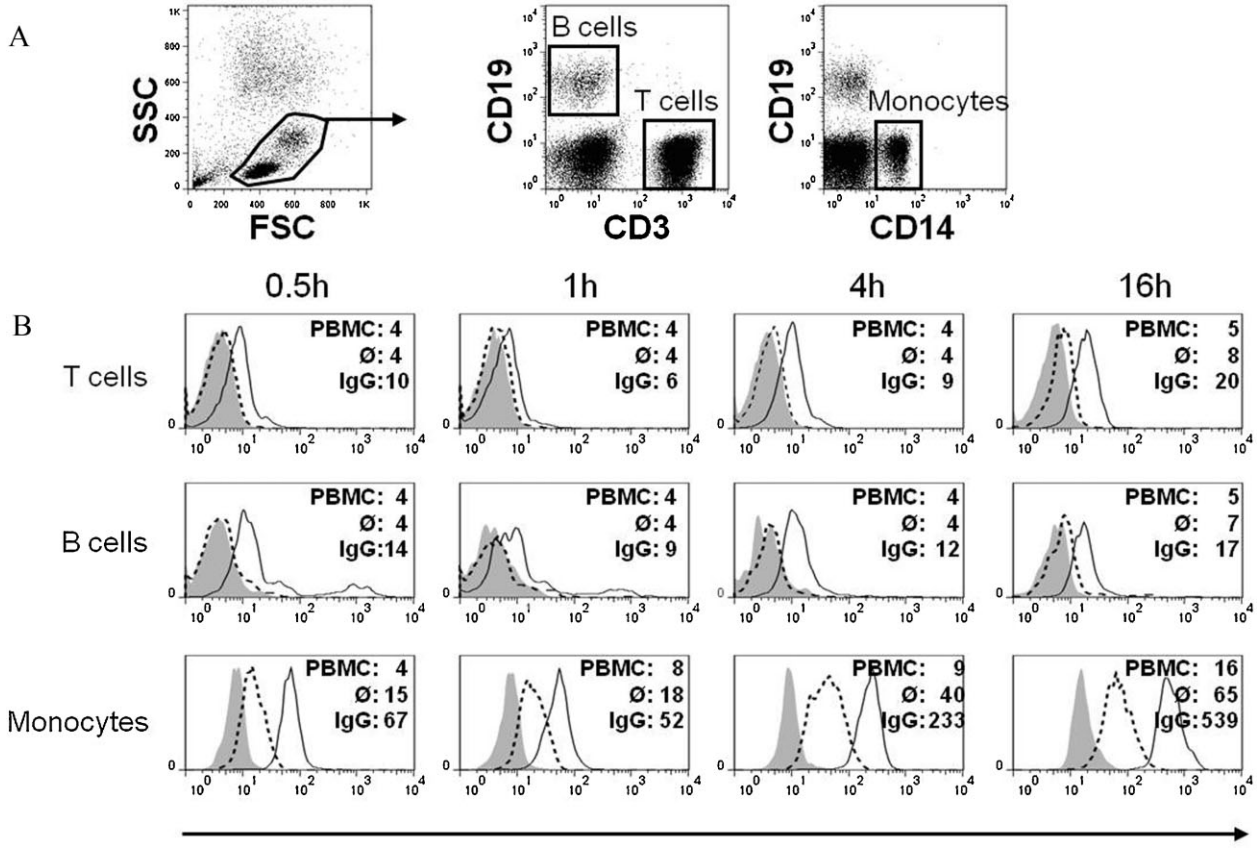


Figure 6. A) Setup of gates for sorting of PBMCs based on flourochrome-conjugated mAb anti-CD3 (T cells), anti-CD14 (monocytes), and anti-CD19 (B cells) monoclonal antibodies. B) Intensity of cell associated fluorescence: mean fluorescence intensities of PBMC alone (grey), PBMC + HPMA-polymer **P2ref** (dotted histogram), and, PBMC + huIgG-conjugated HPMA-polymer **PK2** (lined).

compared to monocytes. Strong phagocytotic activity of monocytes facilitate their uptake of control polymers in vitro, but their high expression of Fc-receptors also further enhance the binding of conjugated polymers **P2K**. Usage of monoclonal antibodies recognizing cell-specific molecules of immune cells are warranted and currently under evaluation.

4. Conclusion

In summary, a protocol for the controlled attachment of antibodies to HPMA copolymers has been established, which does not require a complex purification scheme for the polymer-antibody conjugate. RAFT-polymerization of pentafluorophenol methacrylate and subsequent post-polymerization modification allows the synthesis of narrowly distributed, dye-labeled HPMA copolymers with maleimide groups. The combination of Traut's reagent and reduction with TCEP allowed the activation of lysine residues and produced antibodies with an average of 8 thiol groups; a number which turned out to be sufficient for polymer binding. The functional conjugates were found to have a hydrodynamic radius of about 14 nm, and a number average of molecular weight of 310 000. This implies the formation of nano-aggregates of polymers attached to 1–3 antibodies. The dispersity of 1.6 shows that a large excess of reactive polymer can prevent formation of large aggregates. Furthermore the conjugates are free of residual unmodified antibody or polymer.

The balance between loss of binding specificity and maximized amount of bound polymer is always an issue when dealing with modified antibodies. For the conjugates **P1K** and **P2K**, however, specific binding of the FAB-fragment with its receptor was demonstrated by "Western blotting" of **P1K** (Figure 5). In addition the Fc-fragment of huIgG can selectively bind to distinct CD receptors presented on immune cells, as shown by FACS analysis of **P2K** (Figure 6). This shows that in both cases binding sites are still active. Furthermore the developed protocol is designed in a way that it is applicable to more complex polymeric nanoparticles.[43]

Acknowledgements: The authors would like to acknowledge financial support by the university centers SAMT and COMATT. We thank M. Doroshenko and K. Koynov of the Max Planck Institute for Polymer Research for the FCS measurements. Furthermore, we thank R. Duncan for fruitful and inspiring discussion and advice.

Received: September 20, 2012; Revised: October 20, 2012; Published online: December 13, 2012; DOI: 10.1002/mabi.201200344

Keywords: antibody attachment; HPMA copolymers; RAFT-polymerizations; targeting moieties

[1] M. Ferrari, *Nat. Rev. Cancer.* **2005**, *5*, 161.
[2] R. Duncan, *Nat. Rev. Drug Discov.* **2003**, *2*, 347.
[3] a) H. Ringsdorf, *J. Polm. Sci.* **1975**, 135; b) R. Haag, F. Kratz, *Angew. Chem., Int. Ed.* **2006**, *45*, 1198; c) K. Miyata, N. Nishiyama, K. Kataoka, *Chem. Soc. Rev.* **2012**, *41*, 2562; d) H. Cabral, N. Nishiyama, K. Kataoka, *Acc. Chem. Res.* **2011**, *44*, 999.
[4] a) C. P. Leamon, P. Low, *Drug Discovery Today* **2001**, *6*, 44; b) M. Barz, F. Canal, K. Koynov, R. Zentel, M. J. Vicent, *Biomacromolecules* **2010**, *11*, 2274.
[5] C. Mamot, D. Drummond, U. Greiser, K. Hong, D. Kirpotin, J. Marks, J. W. Park, *Cancer Res.* **2003**, *63*, 3154.
[6] a) R. Duncan, L. C. W. Seymour, L. Scarlett, J. Lloyd, P. Gejmanova, J. Kopecek, *Biochim. Biophvs. Acta* **1986**, *880*, 62; b) A. David, P. Kopečková, A. Rubinstein, J. Kopeček, *Bioconjugate Chem.* **2001**, *12*, 890.
[7] G. Köhler, C. Milstein, *Nature* **1975**, *256*, 495.
[8] M. Jain, N. Kamal, S. K. Batra, *Trends Biotechnol.* **2007**, *25*, 307.
[9] R. K. Jain, *Science.* **2005**, *307*, 58.
[10] T. Waldmann, *Nat. Med.* **2003**, *9*, 269.
[11] P. Carter, *Nature* **2001**, 118.
[12] a) K. Ulbrich, T. Etrych, P. Chytil, M. Jelínková, B. Říhová, *J. Controlled Release* **2003**, *87*, 33; b) D. Wang, P. Kopečková, T. Minko, V. Nanayakkara, J. Kopeček, *Biomacromolecules* **2000**, *1*, 313; c) J. H. Lee, G. Sabnis, A. Nan, *Macromol. Biosci.* **2012**, *12*, 55.
[13] M. Calderon, M. A. Quadir, S. K. Sharma, R. Haag, *Adv. Mater.* **2010**, *22*, 190.
[14] B. Obermeier, F. Wurm, C. Mangold, H. Frey, *Angew. Chem., Int. Ed.* **2011**, *50*, 7988.
[15] a) J. Kopeček, P. Kopečková, *Adv. Drug Delivery Rev.* **2010**, *62*, 122; b) K. Ulbrich, V. Šubr, *Adv. Drug Delivery Rev.* **2010**, *62*, 150.
[16] A. Godwin, M. Hartenstein, A. Müller, S. Brocchini, *Angew. Chem.* **2001**, *113*, 614.
[17] a) C. W. Scales, Y. A. Vasilieva, A. J. Convertine, A. B. Lowe, C. L. McCormick, *Biomacromolecules* **2005**, *6*, 1846; b) P. Chytil, T. Etrych, J. Kříž, V. Šubr, K. Ulbrich, *Eur. J. Pharm. Sci.* **2010**, *41*, 473.
[18] R. M. Broyer, G. N. Grover, H. D. Maynard, *Chem. Commun.* **2011**, *47*, 2212.
[19] a) K. L. Heredia, H. D. Maynard, *Org. Biomol. Chem.* **2007**, *5*, 45; b) J. Nicoas, G. Mantovani, D. M. Haddelton, *Macromol. Rapid Commun.* **2007**, *28*, 1083; c) H.-A. Klok, *Macromolecules* **2009**, *42*, 7990; d) B. Le Droumagent, J. Nicolas, *Polym. Chem.* **2010**, *1*, 563; e) B. S. Sumerlin, *ACS Macro Lett.* **2011**, *1*, 141.
[20] M. Eberhardt, P. Théato, *Macromol. Rapid Commun.* **2005**, *26*, 1488.
[21] M. Barz, M. Tarantola, K. Fischer, M. Schmidt, R. Luxenhofer, A. Janshoff, P. Theato, R. Zentel, *Biomacromolecules* **2008**, *9*, 3114.
[22] a) M. Barz, R. Luxenhofer, R. Zentel, A. V. Kabanov, *Biomaterials* **2009**, *30*, 5682; b) M. Allmeroth, D. Moderegger, B. Biesalski, K. Koynov, F. Rösch, O. Thews, R. Zentel, *Biomacromolecules* **2011**, *12*, 2841.

[23] M. Hemmelmann, C. Knoth, U. Schmitt, M. Allmeroth, D. Moderegger, M. Barz, K. Koynov, C. Hiemke, F. Rösch, R. Zentel, *Macromol. Rapid Commun.* **2011**, *32*, 712.
[24] M. A. Gauthier, M. I. Gibson, H.-A. Klok, *Angew. Chem., Int. Ed.* **2009**, *48*, 48.
[25] M. M. Herth, M. Barz, D. Moderegger, M. Allmeroth, M. Jahn, O. Thews, R. Zentel, F. Rösch, *Biomacromolecules* **2009**, *10*, 1697.
[26] Y. K. Chong, T. P. T. Le, G. Moad, E. Rizzardo, S. H. Thang, *Macromolecules* **1999**, *32*, 2071.
[27] M. Eberhardt, R. Mruk, R. Zentel, P. Théato, *Eur. Polym. J.* **2005**, *41*, 1569.
[28] R. Zuckermann, E. Martin, D. Spellmeyer, G. B. Stauber, K. Shoemaker, *J. Med. Chem.* **1994**, *37*, 2678.
[29] T. Oishi, A. Kamori, M. Fujimoto, *J. Macromol. Sci., Part A* **1992**, *29*, 231.
[30] M. Green, J. Berman, *Tetrahedron Lett.* **1990**, 5851.
[31] L. Zhang, M. Bukulin, E. Kojro, A. Roth, V. V. Metz, F. Fahrenholz, P. P. Nawroth, A. Bierhaus, R. Postina, *J. Biol. Chem.* **2008**, *283*, 35507.
[32] W. Wray, T. Boulikas, W. Wray, R. Hancock, *Anal. Biochem.* **1981**, 197.
[33] M. A. Gauthier, H.-A. Klok, *Chem. Commun.* **2008**, 2591.
[34] a) A. P. Chapman, *Adv. Drug Delivery Rev.* **2002**, 531; b) R. N. Johnson, P. Kopečková, J. Kopeček, *Biomacromolecules* **2012**, *13*, 727.
[35] S. Matsui, H. Aida, *J. Chem. Soc., Perkin Trans. 2* **1978**, *12*, 1277.
[36] a) N. Donoghue, P. T. W. Yam, X.-M. Jiang, P. J. Hogg, *Protein Sci.* **2000**, *9*, 2436; b) B. Sahaf, L. H. K. Heydari, L. A. Herzenberg, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 4001.
[37] a) A. Bierhaus, P. P. Nawroth, *Diabetologia* **2009**, *52*, 2251; b) R. Deane, R. D. Bell, A. Sagare, B. V. Zlokovic, *CNS Neurol. Disord. – Drug Targets* **2009**, *8*, 16.
[38] R. Jue, J. Lambert, L. Pierce, R. Traut, *Biochemistry* **1978**, *17*, 5399.
[39] T. Etrych, T. Mrkvan, B. Říhová, K. Ulbrich, *J. Controlled Release* **2007**, *122*, 31.
[40] K. Kafi, D. J. Betting, R. E. Yamada, M. Bacica, K. K. Steward, J. M. Timmerman, *Mol. Immunol.* **2009**, *46*, 448.
[41] T. Etrych, J. Strohalm, L. Kovář M. Kabešová, B. Říhová, K. Ulbrich, *J. Controlled Release* **2009**, *140*, 18.
[42] A. San Paulo, R. García, *Biophys. J.* **2000**, *78*, 1599.
[43] M. Barz, R. Luxenhofer, R. Zentel, A. V. Kabanov, *Biomaterials* **2009**, *30*, 5682.