Polymers as Vaccines

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Water-Soluble Polymers Coupled with Glycopeptide Antigens and T-Cell Epitopes as Potential Antitumor Vaccines**

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Dedicated to Professor Manfred Reetz on the occasion of his 70th birthday

Tumor immunotherapy is considered a promising strategy for cancer treatment.[1] Many epithelial tumors are distinguished from healthy tissues by deficient glycosylation profiles of MUC1, a cell-surface mucin glycoprotein.[2–4] Due to altered glycosyltransferase activities, tumor-associated MUC1 lacks extended glycan motifs. Instead, short tumor-associated carbohydrate antigens (TACAs) are prevalent on the protein backbone.[2,4,5] As a consequence, peptide epitopes within the tandem repeat region of MUC1 shielded on healthy cells by polysaccharides are now accessible for the immune system. Therefore, tandem repeat glycopeptides with TACA side chains are promising targets for antitumor vaccines.[6] However, these endogenous antigens exhibit low immunogenicity. Thus, synthetic tumor-associated glycopeptides have been linked to immunostimulating components containing T-helper-cell epitopes to induce immune responses in mice.[7] For example, synthetic two- or three-component vaccines composed of tumor-associated MUC1 sequences, T-cell epitopes, and Toll-like receptor 2 (TLR2) ligands ($Pam_3CysSerK_4$) elicited strong immune responses.[8] Interestingly, when these glycopeptides were coupled to tetanus toxoid as the carrier, extraordinary antibody titers strongly binding to human cancer cell lines (e.g. MCF-7) were obtained.[9] The nanoscale structure of a protein-conjugated vaccine may resemble nanoparticulate fragments which the immune system naturally processes during an immune defense (e.g. bacterial compartments). Thus, it appeared attractive to investigate whether a combination of the MUC1 antigens with artificial nanometer-sized carriers also affords efficient vaccines.[10]

Nanoscaled particles have unique properties for medical applications.[11,12] For example, polymer–drug conjugates based on poly(*N*-(2-hydroxypropyl)methacrylamide), P(HPMA), were among the first that entered clinical trials.[13] Known as nonimmunogenic,[14] P(HPMA) may act as a multivalent carrier for the delivery of antigens and adjuvants.[15] In contrast to carrier proteins, it prevents undesired immune reactions against carrier epitopes and offers advantages due to its polymeric structure: It is based on Ringsdorf's concept of pharmacologically active polymers obtained by using orthogonally reactive functionalities for the coupling of drugs or ligands.[16] Moreover, additional structures can be attached to P(HPMA), inducing aggregation to nanosized objects which promote interaction with the immune system. These polymers are accessible in structurally defined architectures under controlled conditions.[17]

To our knowledge, synthetic polymer glycopeptide conjugates have not yet been reported. To obtain effective vaccines, a tumor-associated MUC1 tandem-repeat glycopeptide (PAHGVTSAPDTRPAPGSTAP) as the B-cell epitope and a T-helper cell epitope were coupled to a P(HPMA) polymer. Recent studies disclosed that anti-MUC1 autoantibodies in the sera of patients[18] preferentially bind to two immunodominant motifs (PDTRP[19] and GSTAP)[19,20] within the MUC1 tandem repeat. It has also been described that glycans within the STAPPA (Ser17, Thr18) sequence have a strong conformational influence.[21] Since the conformation of the glycopeptide is crucial for tumor selectivity,[22] the investigation of a 22-mer peptide, containing a coherent STAPPA sequence with T_N antigen at threonine-18 and both immunodominant motifs, is of particular interest.[9b] As the second component, the universal human and murine T-cell epitope P2 (QYIKANSKFIGITEL) of tetanus toxoid[23] was used for the vaccine design. Its conjugate with MUC1 glycopeptide and lipopeptide Pam_3CysSK_4 induced strong immune responses.[8e]

Well-defined functional P(HPMA) polymers are obtained by RAFT polymerization of pentafluorophenyl methacrylate and subsequent aminolysis with 1-amino-2-propanol in a post-polymerization modification.[17] Homo, statistic, and block copolymers with lauryl methacrylate have already been studied with regards to their nanodimensional self-assembling behavior[17a,24] and biodistribution.[17c,25] Polymer-analogous reactions of the reactive esters can be used to attach additional functional groups to the polymer through primary

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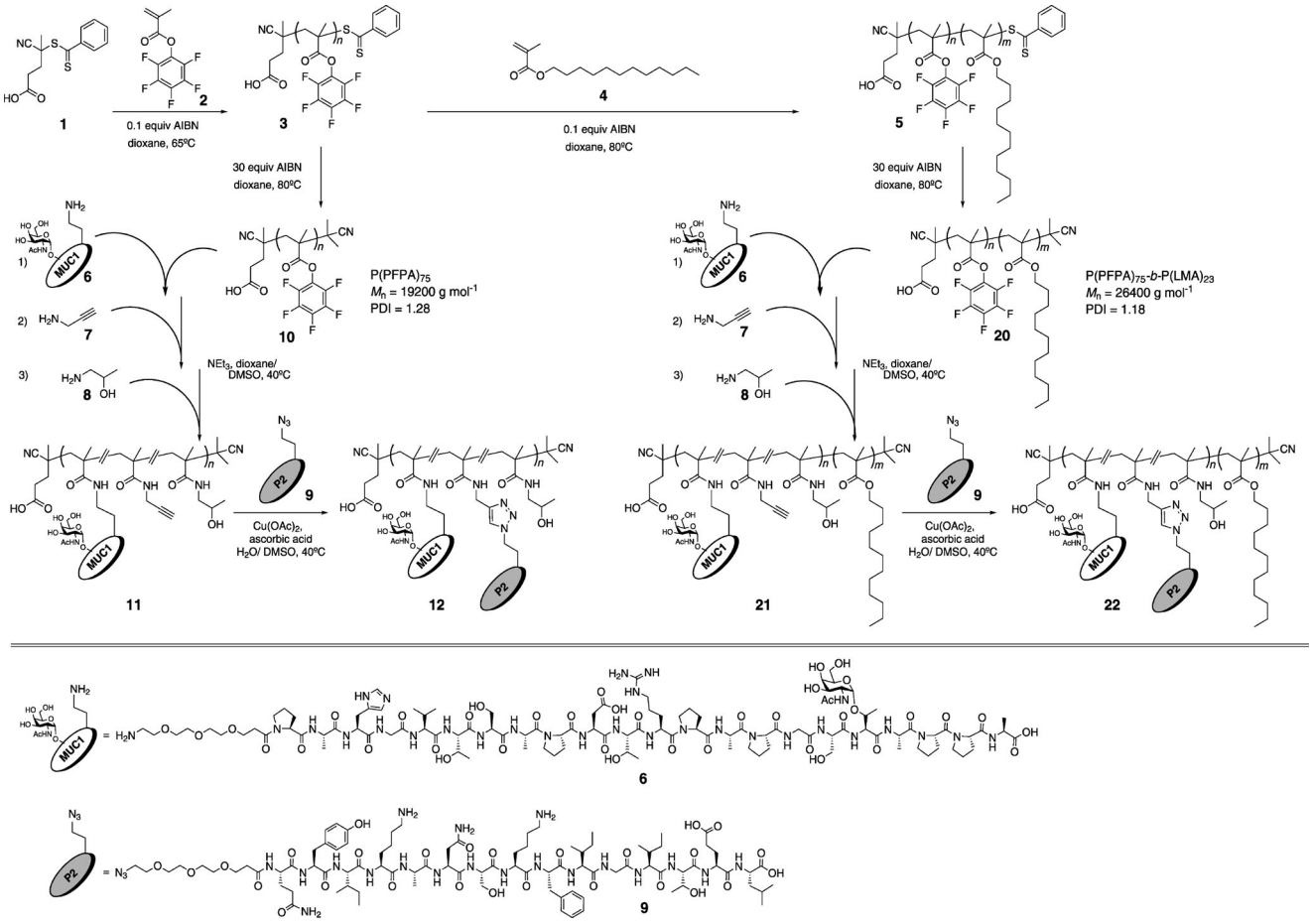

Scheme 1. Syntheses of the pentafluorophenyl methacrylate based reactive ester homopolymer **10** and block copolymer **20** and sequential modification with the tumor-associated MUC1 glycopeptide **6** and the T-helper-cell epitope P2 **9** affording HPMA-based polymer glycopeptide conjugate vaccines P(HPMA-MUC1-P2) **12** and P(HPMA-MUC1-P2)-*b*-P(LMA) **22**.

amines.[26] Within the vaccine design presented here, the tumor-associated glycopeptide fulfills this function. It bears only one primary amino group at the N-terminus which can undergo direct aminolysis of the pentafluorophenyl esters of the polymer.[8b,c] In contrast, the P2 sequence contains lysine ε-amino groups. Therefore, the copper-catalyzed alkyne–azide cycloaddition[27] was applied as an alternative ligation technique. To this end, the P2 sequence was elongated by an azide-modified spacer[28] and the reactive ester polymer was modified by propargylamine to provide alkyne functions on the P(HPMA) (Scheme 1). Accordingly, pentafluorophenyl methacrylate (**2**) was subjected to RAFT polymerization using 4-cyano-4- (phenylthiocarbonylthio)pentanoic acid (**1**) as a chain-transfer agent, affording the reactive ester polymer **3** with narrow dispersity. Due to its dithiobenzoate end group, it can undergo additional RAFT block copolymerization with lauryl methacrylate (**4**), installing an alkyl block on polymer **5** and, thus, a hydrophobic domain that causes self-assembly to micellar nanosized objects. To avoid side reactions during polymer-analogous reactions, the dithiobenzoate end group was removed with excess AIBN affording reactive ester polymers **10** ($M_n = 19200\ g\,mol^{-1}$, PDI = 1.28) and **20** ($M_n = 26400\ g\,mol^{-1}$, PDI = 1.18) which were characterized by NMR spectroscopy and size exclusion chromatography (SEC) in THF.

MUC1 glycopeptide **6** and T-helper-cell epitope P2 **9** were synthesized on solid phase according to the established Fmoc protocol.[9a,29] The couplings of the glycosyl amino acid and of the two functional spacers were performed under modified conditions (see the Supporting Information). As for glycopeptide **6**, release from the resin and removal of the amino acid protecting groups was achieved using trifluoroacetic acid (TFA)/triisopropylsilane (TIS) and water. After purification by semipreparative HPLC the protecting groups of the carbohydrate were removed using NaOMe/MeOH at pH < 10. For peptide **9** release from the resin and protecting-group removal were performed without TIS in order to avoid reduction of the azide. Prior to conjugation to the polymer, both deprotected peptides were purified by semipreparative HPLC affording well-characterized **6** and **9** in multi-milligram scale.

In a stepwise synthesis glycopeptide **6** was conjugated to homopolymer **10** and block copolymer **20**. Selective aminolysis of the pentafluorophenyl esters with 0.15 equiv of the glycopeptide was performed in anhydrous dioxane/dimethyl sulfoxide and triethylamine. After the reaction mixture had been stirred at 40 °C for five days, 0.1 equiv of propargylamine

7 was added, and one day later the remaining ester groups were reacted with 1-amino-2-propanol. The stepwise conversion was monitored by ^{19}F NMR spectroscopy which indicated the sequential attachment of all components onto the polymer chain (see the Supporting Information). After semipreparative SEC the obtained glycopeptide polymer conjugates **11** and **21** were characterized by NMR spectroscopy and SEC with hexafluoroisopropanol (HFIP) as eluent. The alkyne moieties on the polymers were subsequently conjugated with azide-containing P2 peptide **9** using copper acetate and ascorbic acid. The reaction was conducted in degassed water/DMSO at 40 °C for four days and yielded after semipreparative SEC bifunctionalized polymer conjugates **12** and **22**, which were characterized by NMR spectroscopy and SEC in HFIP. In the ^{1}H NMR spectrum, for instance, conjugate **11** features the aromatic histidine proton signals of peptide **6** (Figure 1). The spectrum of conjugate **12**, in addition, displays aromatic phenylalanine and tyrosine signals of peptide **9**. The elution volume of **12** corresponds to higher molecular weights (by SEC) than that of the monofunctionalized polymer **11**. Moreover, all HSQC spectra indicate that the α-glycoside (^{1}H: $\delta = 4.89$ ppm, $J_{H1\text{-}H2} = 3.9$ Hz; ^{13}C: $\delta = 98.6$ ppm) is stable during all conjugations (see the Supporting Information).

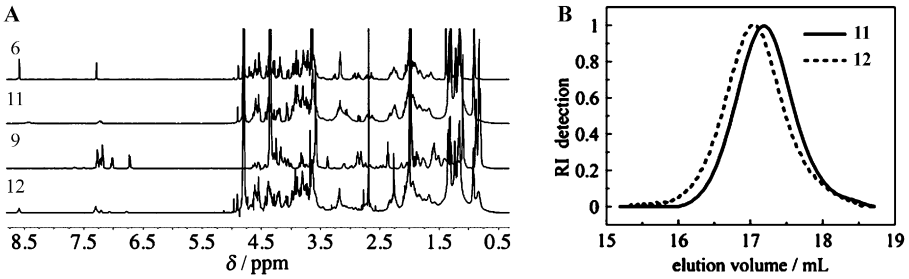

Figure 1. A) ^{1}H NMR spectra of **6**, **9**, **11**, and **12**. B) SEC elugrams of the homopolymers **11** and **12** (eluent: HFIP).

Dynamic light scattering (DLS) of the polymeric vaccines in phosphate-buffered saline (PBS) revealed for the monofunctionalized homopolymer **11** a *z*-averaged hydrodynamic radius of 5.5 nm and narrow dispersity ($\mu_2 = 0.04$) representing molecularly dissolved polymeric structures (Table 1). Its block copolymer derivative **21** bearing a hydrophobic lauryl methacrylate block, however, self-assembles to narrowly distributed superstructures with sizes of 64.5 nm ($\mu_2 = 0.06$). Similar results have been reported for P(HPMA)-*b*-P(LMA) copolymers where the hydrophobic block effectuated well-defined micelle-like nanoobjects.[17a,24]

Surprisingly, conjugation of T-helper-cell epitope P2 **9** to the homopolymer **11** also induced self-assembly. The DLS data of difunctionalized homopolymer **12** reveal a *z*-averaged hydrodynamic radius of about 52.1 nm ($\mu_2 = 0.08$), which is in a similar regime as that for the monofunctionalized block copolymer **21**. The combination of P2 sequence and P(LMA) block in the difunctionalized block copolymer **22** enhanced this aggregation to a radius of about 86.2 nm (with broader dispersities of $\mu_2 = 0.11$).

Table 1: Dynamic light scattering data for HPMA-based homopolymer and block polymer glycopeptides conjugates.

	Composition	$R_h = <1/R_h>_z^{-1}$	μ_2
11	P(HPMA-MUC1)	5.5 nm[a]	0.04[a]
12	P(HPMA-MUC1-P2)	52.1 nm[b]	0.08[b]
21	P(HPMA-MUC1)-*b*-P(LMA)	64.5 nm[b]	0.06[b]
22	P(HPMA-MUC1-P2)-*b*-P(LMA)	86.2 nm[b]	0.11[b]

[a] Determined at 1.0 g L^{-1} in PBS. [b] Determined at 0.1 g L^{-1} in PBS.

For immunological evaluation, the nanosized polymer vaccines **12** and **22** were each administered to three wild-type (Balb/c) mice together with complete Freund's adjuvant (CFA). At intervals of 21 days two booster immunizations were performed with incomplete Freund's adjuvant. Five days after the second boosting, blood was collected from the tail vein. Each obtained serum was used for ELISA studies to quantify vaccine-induced antibodies against the tumor-associated MUC1 epitope. The microtiter plates were coated with glycopeptide **6**–BSA conjugate (see the Supporting Information). The sera of all mice revealed immune responses with significant antibody titers. Due to the structural similarity of both homopolymer vaccine **12** and block copolymer vaccine **22**, no difference in titer level was observed (only mouse 3 vaccinated with **12** produced titers above average; Figure 2). Subtype analysis of the induced antibodies showed prevailing IgG types. This indicates MHC-II mediated immune responses. Interestingly, mouse 3 vaccinated with **12** showed a higher titer and a prevalence of IgG2a antibodies.

In order to evaluate the binding of the induced antibodies to tumor cells, human breast cancer cells of cell line MCF-7[30] were incubated with the induced antisera, and the recognition of the tumor cells was recorded by flow cytometry (FACS) as described.[9] All antisera showed notable binding to the tumor cells (Figure 2C and the Supporting Information). The flow cytometry showed that the serum induced by block copolymer conjugate **22** caused the strongest binding to the tumor cells within this set of polymer-based vaccines. While in the homopolymer **12** the P2 epitopes are responsible for the nanoparticulate aggregation (Table 1) and, thus, are fixed inside, in the block copolymer **22** the lauryl methacrylate domains mainly stabilize the micellar aggregation. As a consequence, both the MUC1 and the P2 epitopes are more accessible and flexible. This may have an influence on the preferred conformation of the MUC1 epitope, which in **22** can more easily adopt structures related to those present on the tumor cell surface.

In conclusion, first examples of nanosized polymer-linked vaccines have been synthesized by coupling tumor-associated MUC1 glycopeptides and T-cell epitope peptides to water-soluble methacrylamide polymers through the application of active ester aminolysis and Huisgen cycloaddition as orthogonal conjugation methods. The attachment of the tetanus toxoid T-cell epitope P2 onto the hydrophilic polymer vaccines causes their self-assembly to micelle-like nanoobjects. The novel vaccine concept was extended to block

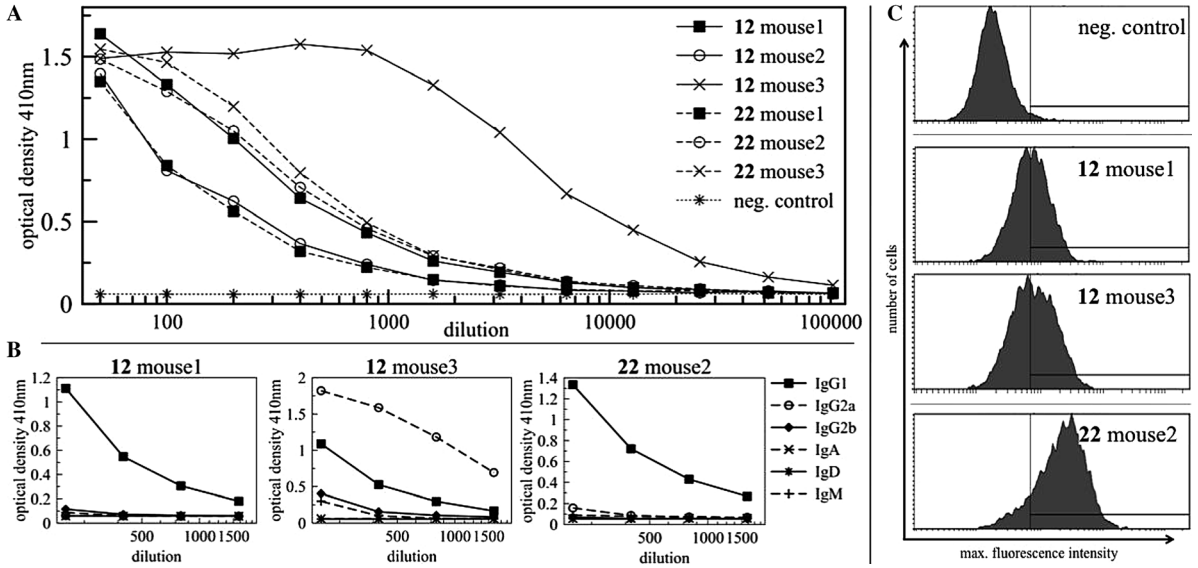

Figure 2. A) ELISA binding studies of the antisera induced by vaccines **12** and **22** binding to immobilized BSA conjugates derived from tumor-associated MUC1 glycopeptide **6**. B) Antibody subtype analysis of the antisera induced by vaccine **12** (mouse 1 and mouse 3) and vaccine **22** (mouse 2) after binding to immobilized glycopeptide **6**–BSA conjugates. C) FACS analysis of the binding of the antisera induced by vaccines **12** and **22**; top: MCF-7 cells treated with PBS buffer (neg. control); middle: MCF-7 tumor cells treated with antisera of mouse 1 and mouse 3 immunized with **12**; bottom: MCF-7 cells treated with antiserum of mouse 3 immunized with **22**.

copolymers consisting of hydrophilic and hydrophobic domains, in which the latter reinforce the formation of nanoparticles as well. The novel polymer-based glycopeptide vaccines induce significant MHC-II-mediated immune reactions in mice and elicit IgG antibodies, which recognize MCF-7 breast tumor cells. In particular, the block copolymer **22** containing additional nanostructure-promoting domains induced antibodies that exhibit high affinity to the tumor cells. Considering the numerous degrees of freedom for structure modification, this novel vaccine concept may open up new ways for the construction of efficient immunotherapeutics, for example antitumor vaccines.

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