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Anti-CD20 multivalent HPMA copolymer-Fab′ conjugates for the direct induction of apoptosis

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ABSTRACT

A hybrid biomimetic system comprising high-molecular-weight, linear copolymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) grafted with multiple Fab′ fragments of anti-CD20 monoclonal antibody (mAb) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization followed by attachment of Fab′ fragments via thioether bonds. Exposure of human non-Hodgkin's lymphoma (NHL) Raji B cells to the multivalent conjugates resulted in crosslinking of CD20 receptors and commencement of apoptosis. Five conjugates with varying molecular weight and valence (amount of Fab′ per polymer chain) were synthesized. One of the copolymers contained enzyme degradable peptide sequences (GFLG) in the backbone. The multivalency led to higher avidity and apoptosis induction compared to unconjugated whole mAb. Time-dependent studies showed that the cytotoxicity of conjugates exhibited a slower onset at shorter exposure times than mAb hyper-crosslinked with a secondary Ab; however, at longer time intervals the HPMA copolymer conjugates achieved significantly higher biological efficacies. In addition, study of the relationship between the structure of conjugates and Raji B cell apoptosis revealed that both valency and polymer molecular weight influenced biological activities, while insertion of peptide sequences into the backbone was not a factor *in vitro*.

1. Introduction

The use of hybrid biomaterials composed of synthetic and biological macromolecules to design "smart" nanomedicines is an emerging field [1,2]. The goals include precise targeting to diseased sites, enhancing therapeutic efficiency, reducing adverse effects, and minimizing drug resistance. In particular, water-soluble HPMA copolymers are extensively used as delivery vehicles to conjugate anticancer therapeutic agents (*e.g.* small molecule drugs) and targeting moieties (*e.g.* antibodies) [3]. HPMA polymer and copolymers have favorable physicochemical and pharmacokinetic properties to provide a well-defined safety profile, increase circulation half-life of therapeutics, and provide a flexible (random coil) conformation of the polymer backbone in solution [4]. The design of macromolecular therapeutics has extended towards a unique paradigm where biomimetic strategies are used to trigger specific responses or facilitate therapeutic efficiency through innate biological processes [1,3,5,6]. For instance, an HPMA-based hybrid system has been used as a "drug-free" macromolecular platform to induce apoptosis via biorecognition and receptor crosslinking at the cell surface; a clinically relevant therapeutic efficacy was demonstrated *in vitro* [6] and *in vivo* [7].

Non-Hodgkin's lymphoma (NHL) is a prevalent cancer in the United States with a history of over a half-million incidences and projected 70,130 new cases diagnosed in 2012 [8]. Because about 85% of NHL is of B cell origin and more than 95% of B lymphomas bear the CD20 surface antigen [9], immunotherapies using anti-CD20 monoclonal antibodies (mAb) have revolutionized the treatment of NHL [10]. However, the overall response levels to clinically used mAb, mainly rituximab (Rituxan®), for treatments of relapsed/refractory low-grade or follicular NHLs are less than 50% [11]. Rare but lethal side effects such as progressive multifocal leukoencephalopathy (PML) and lung injuries observed in patients treated with rituximab or other anti-CD20 mAb also raised biocompatibility concerns [12–15]. Therefore, new therapeutic strategies are needed.

The clinical non-responsiveness and adverse effects of rituximab or other therapeutic mAb has been partly attributed to the Fc

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fragment-related biological events [14,16–18]. The inactivity of effector cells to hyper-crosslink bound rituximab on B cell surface via Fc results in failure of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), the main thrusts of anti-CD20 mAb's therapeutic effect [16,18–20]. In addition, Fc-mediated cellular events such as complement activation or the surge release of tumor necrosis factor-α (TNFα) upon mAb infusion are related to the severe side effects [12,14,17]. Consequently, approaches aiming at direct apoptosis induction through cell surface receptor clustering are becoming attractive [21–25]. In these previous studies, either multimeric Abs covalently linked to each other [24,25], bound to dextran [22], to lipid nanoparticles [23], or monomeric Ab lacking effector cell functions hyper-crosslinked by a secondary Ab [21] were used to specifically enhance apoptosis. In particular, Rossi *et al.* developed a hexavalent anti-CD20 Ab by covalently assembling 6 Fab′ to 1 Fc, and demonstrated that its anti-tumor efficacy in murine model was comparable to mAb monomer (Veltuzumab), but without any sign of CDC [25].

We have reported a hybrid biomimetic system composed of branched HPMA copolymer and multiple Fab′ fragments of the anti-CD20 mAb (1F5) which targets and crosslinks CD20 on the surface of B cells [26,27]. We hypothesized that the crosslinking would lead to clustering of (non-internalizing) CD20 antigens and induction of apoptosis via CD20-mediated signaling pathways. The design features the absence of Fc fragment and multimeric interactions with targets. Superior binding affinity [26] and apoptosis induction [27] when compared to unconjugated mAb were observed in several B cell lines. Here we aimed to improving this system using high-molecular-weight linear HPMA copolymers synthesized by controlled radical polymerization. This provided tailor-made multivalent conjugates with narrow molecular weight distribution, precise control of valences (Fab′ content per polymer chain), well-defined and reproducible architectures, and potentially longer circulating half-lives. The improved design permitted the study of the relationship between the structure of conjugates and their biological activities, which facilitated the understanding of processes involved in CD20-crosslinking mediated apoptosis induction.

2. Materials & methods

2.1. Materials

N-(3-Aminopropyl)methacrylamide hydrochloride (APMA) was purchased from Polysciences (Warrington, PA). 4,4′-azobis(4-cyanopentanoic acid) (V-501) was from Wako Chemicals (Richmond, VA). Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and sulfo-SMCC were purchased from Soltec Ventures (Beverly, MA). o-Phthalic dicarboxaldehyde (OPA) and 3-mercaptopropionic acid (MPA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-(2-Hydroxypropyl) methacrylamide (HPMA) [28] and 4-cyanopentanoic acid dithiobenzoate (CPDB) [29] were prepared as previously described. All solvents were obtained from Sigma-Aldrich as the highest purity available.

2.2. Cell line, hybridoma, and Fab′ fragment preparation

Human Burkitt's B cell non-Hodgkin's lymphoma Raji cell line (ATCC, Bethesda, MD) was used for biological evaluations. Cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and grown at 37 °C in a humidified atmosphere with 5% CO_2 (v/v). All experiments were performed using cells in exponential growth phase. Murine 1F5 anti-CD20 IgG2a antibody was prepared from the hybridoma clone 1F5 in a CellMax bioreactor (Spectrum Laboratories, Rancho Dominguez, CA) according to the manufacturer's instructions. Cells were initially cultured in aforementioned conditions and adapted to chemically defined, serum-free medium (Invitrogen, Carlsbad, CA). Anti-CD20 mAb was purified on a Protein G Sepharose 4 Fast Flow column (GE Healthcare, Piscataway, NJ) from bioreactor harvest supernatant. Preparation of Fab′ fragment from the whole Ab was achieved using a previously reported procedure [6,26]. Briefly, 1F5 mAb was digested into $F(ab')_2$ with 10% (w/w) pepsin (Sigma, St. Louis, MO) in 0.1 M citric buffer (pH 4.0) and labeled with Rhodamine Red™-X succinimidyl ester (R6010) (Molecular Probes®, Invitrogen). Immediately before use, 5 mg/mL of $F(ab')_2$ was reduced to Fab′ with 5 mM tris(2-carboxyethyl)phosphine (TCEP) (Thermo Scientific, Waltham, MA) in 0.1 M phosphate buffered saline (PBS) (pH 6.5).

2.3. HPMA copolymers and polymer precursors

Synthesis of HPMA copolymer with pendent amino groups (P-NH_2) and its conversion into maleimide-derivatized polymer precursor (P-mal) are depicted in Fig. 1. P-NH_2 was synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization of HPMA and APMA in deionized (DI) water at 70 °C using CPDB as chain transfer agent (CTA) and V-501 as initiator. For the backbone degradable polymer precursor, a bifunctional dithiobenzoate containing enzyme cleavable oligopeptide Gly-Phe-Leu-Gly (GFLG) was synthesized [29] and used as CTA (N^{α},N^{ε}-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl)lysine, abbreviated peptide2CTA) (Fig. 1A). A typical polymerization was as follows: HPMA (134.6 mg, 0.94 mmol) and APMA (10.7 mg, 0.06 mmol) were added into an ampoule attached to a Schlenk-line. After three vaccum-nitrogen cycles to remove oxygen, 0.46 mL degassed DI H_2O was added to dissolve monomers, followed by addition of CPDB solution (0.35 mg in 60 μL methanol) and V-501 solution (0.12 mg in 60 μL methanol) via syringe. The mixture was bubbled with nitrogen for 15 min before sealing the ampoule; the copolymerization was performed at 70 °C for 20 h. The copolymer was isolated by precipitation into acetone and purified by dissolution-precipitation in methanol-acetone twice and dried under vacuum. Yield of P-NH_2 was 127 mg (87.3%). The molecular weight (Mw) and molecular weight distribution (Mw/Mn) were determined by size-exclusion chromatography (SEC) on ÄKTA FPLC system (GE Healthcare, Piscataway, NJ) equipped with miniDAWN and OptilabREX detectors. Superose 6 HR10/30 column (GE Healthcare) was used with sodium acetate buffer and 30% acetonitrile (v/v) (pH = 6.5) as mobile phase. The content of amino groups in the copolymer was determined by ninhydrin assay [30].

After polymerization, P-NH_2 copolymers were reacted with 2,2′-azobis(2,4-dimethyl valeronitrile) (V-65) (Wako Chemicals) to remove the terminal (active) dithiobenzoate groups. Briefly, HPMA copolymer (45 mg, Mn = 105 kDa, 0.43 μmol) and V65 (20× excess, 2.13 mg, 8.57 μmol) were added into an ampoule. After three vacuum-nitrogen cycles to remove oxygen, 0.4 mL methanol was added. The solution was bubbled with nitrogen for 15 min, sealed and reacted at 50 °C for 3 h. The end-modified copolymer was purified by precipitation into acetone twice and then dried under vacuum (yield 42 mg).

The side chain amino groups of P-NH_2 were converted to maleimides by reaction with SMCC or sulfo-SMCC in DMF in the presence of triethylamine (TEA). A mixture of 42 mg P-NH_2 (12.6 μmol NH_2) and SMCC (12.7 mg, 37.8 μmol) was dissolved in 0.5 ml DMF followed by dropwise addition of TEA (ratio of [NH_2]:[SMCC]:[TEA] = 1:3:3), then kept at room temperature overnight. The product was precipitated into acetone/ether (2:1, v/v), filtered, and redissolved in methanol, precipitated into acetone again, filtered and dried under vacuum. The amount of maleimide in copolymer was determined by a modified Ellman's assay [31]. The conversion of amine into maleimido groups was 54%-59% with SMCC and >80% when sulfo-SMCC was used.

2.4. Preparation of multivalent conjugates

The polymer precursors P-mal were conjugated with reduced 1F5 Fab′ fragments via thioether bonds following a previously established protocol [32]. In brief, 10 mg of P-mal were dissolved in 100 μL of DMSO, and the solution was added to Fab′ (5 mg/mL) in PBS (pH 6.5) (ratio of [mal]:[Fab′] = 5:1). The products were purified on a Superose 6 HR16/60 column (GE Healthcare) to remove unbound Fab′, if any. The HPMA copolymer-Fab′ conjugates (P-Fab′) containing varying amounts of Fab′ per macromolecule were collected and analyzed on a Superose 6 HR10/30 column (Supplementary Data Fig. S1). Fab′-equivalent concentration of conjugates was determined by UV spectroscopy, measuring absorbance at 280 nm on a Varian Cary 400 Bio UV-visible spectrophotometer, and confirmed by bicinchoninic acid (BCA) protein assay (Thermo Scientific).

2.5. Determination of valences and effective diameters of P-Fab′ conjugates

A modified amino acid analysis procedure was utilized to determine the concentrations of both amino acid residues from Fab′ and 1-amino-2-propanol derived from HPMA polymer backbone. This enables the calculation of valence (number of Fab′ per polymer chain) of P-Fab′ conjugates. In practice, after hydrolysis in 6 N HCl (125 °C, 24 h), samples were pre-column derivatized with o-phthalic dicarboxaldehyde in the presence of 3-mercaptopropionic acid, and analyzed by HPLC (Agilent Technologies, Santa Clara, CA) equipped with an Eclipse XDB-C8 column and fluorescence detector (excitation 229 nm, emission 450 nm). Free 1F5 Fab′ and HPMA homopolymer were used for calibration (Supplementary Data Fig. S2).

The effective diameters of HPMA copolymer-Fab′ conjugates were analyzed by dynamic light scattering using a Brookhaven BI-200SM goniometer and BI-9000AT digital correlator equipped with a He–Ne laser (λ = 633 nm) at room temperature in