Virus–glycopolymer conjugates by copper(I) catalysis of atom transfer radical polymerization and azide–alkyne cycloaddition†

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The Cu^{I}-catalyzed ATRP and azide–alkyne cycloaddition reactions together provide a versatile method for the synthesis of end-functionalized glycopolymers and their attachment to a suitably modified viral protein scaffold.

The polyvalent clustering of carbohydrate derivatives based on linear polymers[1] and dendrimers[2] has proven to be an effective tool in the study of carbohydrate-based cellular processes and is a useful strategy in the development of therapeutic agents.[3] Dense clusters of carbohydrates can be formed by arraying an end-functionalized glycopolymer to a biocompatible scaffold such as a protein. Such polymers have been recently prepared by cyanoxyl-mediated free radical polymerization (employing initiators bearing amine, carboxylic acid, hydrazide, or biotin moieties, with subsequent protein attachment by biotin–avidin binding[4]) and atom transfer radical polymerization (ATRP; side-chain poly(ethylene glycol) (PEG) or poly(2-hydroxyethyl methacrylate) polymers containing *N*-hydroxysuccinimide or pyridyl disulfide end groups, with protein attachment to lysozyme and bovine serum albumin).[5]

This worthy strategy can be further augmented by the use of the most active and selective bioconjugation reactions. Organic azides have achieved wide application due to their inert nature towards biological molecules and their participation in the Staudinger ligation with phosphine-esters[6] and the 1,3-dipolar cycloaddition reactions with alkynes.[7] We have found the latter process to be extraordinarily fast and versatile in demanding bioconjugation applications under dilute conditions.[7a,8] It has also been used in a wide variety of other applications, including the creation of small dendrimer-style polyvalent carbohydrate assemblies.[9] Since Cu(I) complexes catalyze both the ATRP[10] and azide–alkyne cycloaddition (AAC)[11] reactions, their combination is logical. We describe here the construction of azide-terminated glycopolymers by ATRP, their end-labeling with fluorophores, and the subsequent conjugation of these compounds to virus particles in high yield for purposes of polyvalent binding to cell-surface lectins.

Viruses are intriguing scaffolds for the polyvalent presentation of functional structures. Chemistry-based studies have included the organization of inorganic materials in or around virus cages,[12] the organization of viruses on surfaces,[13] and the chemical conjugation of organic compounds to virus coat proteins.[14] Our work has comprised a broad exploration of virus particles as chemical building blocks, focused on cowpea mosaic virus (CPMV) as a prototype. This plant virus can be produced and purified in large quantities, is structurally characterized to near-atomic resolution, is stable to a variety of conditions compatible with both hydrophobic and hydrophilic molecules, and can be manipulated at the genetic level to introduce mutations at desired positions. One of our goals is to bring new functions to virus particles by attaching functional molecules to the capsid protein, thereby generating novel species with diagnostic and therapeutic applications. Attachment of single carbohydrate compounds to CPMV residues produces a dendrimer-like display with polyvalent lectin-binding properties.[15] We have also derivatized CPMV with PEG to give well-controlled loadings of polymer on the outer surface of the coat protein assembly.[16] The resulting conjugates displayed altered physical properties and reduced immunogenicities, consistent with previous reports of PEGylated adenovirus vectors.[17]

The strength and selectivity of binding interactions between polyvalently displayed carbohydrates and target cells are likely to depend on the number and flexibility of the arrayed sugars. We desired to explore one extreme by covering a virion as densely as possible with carbohydrate groups. Increasing the degree of virus coverage requires the reactive polymer end group to be compatible with polymer synthesis and/or elaboration and yet reactive enough to accomplish a demanding subsequent connection to the virus coat protein—a union of two large molecules present in low concentrations.

The side-chain neoglycopolymer **3** was prepared by ATRP of methacryloxyethyl glucoside (**2**) using azide-containing initiator **1** (Scheme 1).[18] The presence of the azide chain end in the polymer was confirmed by colorimetric testing[19] and by the presence of the characteristic peak at 2100 cm^{-1} in the infrared spectrum. GPC analysis established the clean nature of the material and an average molecular weight (M_n) of 13 000 with a polydispersity of 1.3, consistent with the initiator : monomer ratio used and within expectations for ATRP of acrylates in water.[18b,20,21]

Azide-terminated polymer **3** was elaborated to the alkyne-terminated form **5** by reaction with fluorescein dialkyne **4** (Scheme 1). The excess dye was removed by filtration and the polymer products were further purified by size-exclusion chromatography (Sephadex G-15). The complete conversion of the azide to the alkyne end group was confirmed by the observation of a negative colorimetric test and by the disappearance of the azide IR resonance (the corresponding alkyne resonance is much less intense and therefore not visible). The chromophore thus installed serves as a spectroscopic reporter for subsequent manipulations. The dimeric polymer, formed as a minor byproduct from the

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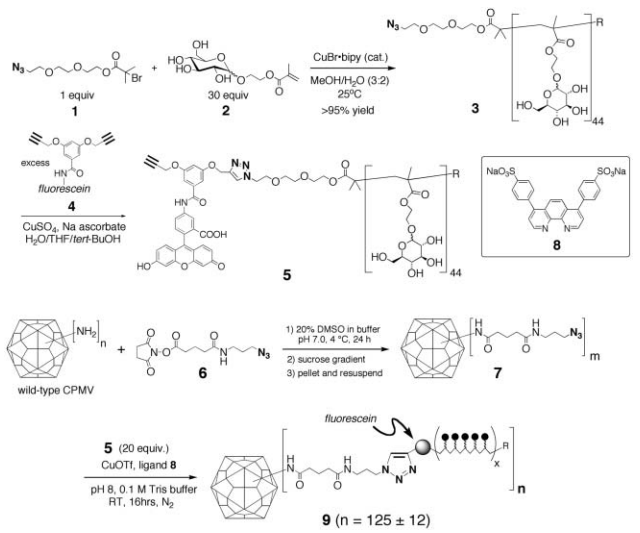

Scheme 1 Synthesis of glycopolymers and virus–polymer conjugates.

reaction of two molecules of **3** and one of **4**, was not separated from **5** as it cannot participate in bioconjugation.

CPMV was derivatized with *N*-hydroxysuccinimide **6** to install azide groups at lysine side chains of the coat protein (Scheme 1). NHS esters have been previously established to acylate lysine residues over the external surface of the capsid, with loadings controlled by overall concentration, pH, and reaction time.[14b] In this case, conditions were employed which result in the derivatization of a substantial fraction of the approximately 240 solvent-accessible lysine side chains (m = approximately 150 in Scheme 1). The resulting azide-labeled virus (**7**) was then condensed with 20 equivalents of polymer–alkyne **5** in the presence of copper(I) triflate and sulfonated bathophenanthroline ligand **8**[8a] under inert atmosphere to produce the glycopolymer–virus conjugate **9** in excellent yield after purification by sucrose-gradient sedimentation to remove unattached polymer. By virtue of the calibrated dye absorbance, the number of covalently bound polymer chains was found to be 125 ± 12 per particle, representing the addition of approximately 1.6 million daltons of mass to the 5.6 million Da virion. This procedure, the general application of which will be described elsewhere, is far more efficient than the previous Cu^{I}-mediated method,[7a] which required 100 equivalents of **5** with respect to azide to achieve similar results.

Covalent labeling of the vast majority of CPMV protein subunits with glycopolymer was confirmed by denaturing gel electrophoresis (Fig. 1C). The intact nature of the particle assembly and its larger size was verified by size-exclusion fast protein liquid chromatography (FPLC, Fig. 1A) as well as transmission electron microscopy (TEM, Fig. 1D). TEM images revealed the virus conjugates to be more rounded in shape, to take on uranyl acetate stain differently, and to be 12–15% larger in diameter than the wild-type particle. The hydrodynamic radius and molecular weight of **9** were found by multi-angle dynamic light scattering (DLS) to be dramatically larger as well: 30.3 ± 3.4 nm and 1.4 ± 0.4 × 10^7 Da, compared to 13.4 ± 1.3 nm and 6.1 ± 0.3 × 10^6 Da for wild-type CPMV. That both radius and molecular weight values are substantially greater than expected reflects the uncertainties of calibration and interpretation of light scattering data for these unique polymer–virus hybrid species.

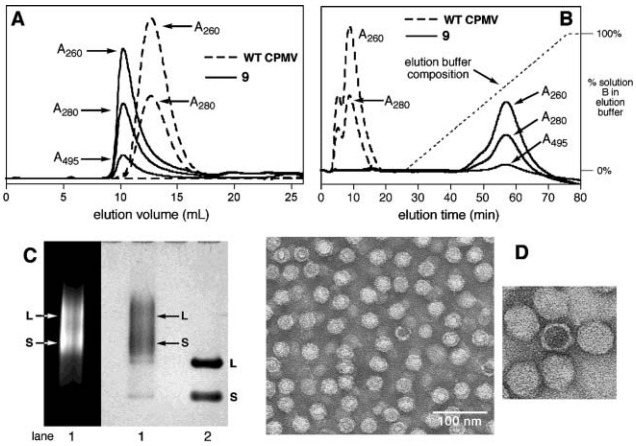

Fig. 1 (A) Size-exclusion FPLC (Superose 6) of wild-type CPMV and glycopolymer conjugate **9**. Protein from disassembled particles would appear at longer retention times than the peaks observed here, and the A_{260} : A_{280} ratios are characteristic of intact, RNA-containing capsids for both samples. The more rapid elution of **9** is indicative of a substantial increase in the size of the particle, as 10 mL is the void volume of the column. Dye absorbance at 495 nm appears only for **9**. (B) FPLC on concanavalin-A Sepharose column of wild-type CPMV and virus–polymer conjugate **9**. The elution buffer was the indicated gradient mixture of 20 mM Tris-HCl, pH 7.4, with 0.15 M NaCl, 0.1 mM Ca^{2+}, and 0.1 mM Mn^{2+} (solution A) and 1 M glucose (solution B). (C) SDS-PAGE of **9** (lane 1) and WT-CPMV (lane 2). On the right (light background) is the gel visualized after Coumassie blue staining; note that almost all of the protein is converted to a slower-eluting form, expected for protein–glycopolymer conjugation. On the left (dark background) is the gel illuminated by ultraviolet light before staining (lane 2 shows no emission and is omitted). The arrows mark the center of the bands derived from the small (S) and large (L) subunits; their broad nature derives from the polydispersity of the polymer and the possibility for more than one attachment of polymer per protein subunit. (D) (*Left*) Negative-stained TEM of **9**. (*Right*) Enlarged TEM image of a WT-CPMV particle surrounded by **9**.

The glycosylated particles interacted strongly with both an immobilized form of the glucose-binding protein concanavalin A (Fig. 1B) and with tetrameric conA in solution. The latter process resulted in the formation of large aggregates, the rate of which was monitored by light scattering at 490 nm. At a concentration of 0.7 mg mL^{-1} in **9** (approximately 0.1 μM in virions) and 0.3 mg mL^{-1} in conA, aggregation occurred within seconds (see Supporting Information†), as expected for the efficient formation of a network by a large and polyvalent particle.

In conclusion, we have demonstrated an efficient strategy for end-functionalization of glycopolymers, using an azide-containing initiator for a living polymerization process followed by click chemistry elaboration of the unique azide end group. Azide–alkyne cycloaddition with a chromophoric dialkyne served to label the polymer with a single dye molecule, allowing for convenient monitoring of further manipulations. The copper-catalyzed cycloaddition reaction provides very efficient coupling of such polymers to a functionalized viral coat protein. In our hands, this method outperforms bioconjugation procedures previously used for polymer attachment to proteins such as acylation of lysine amine groups by activated esters and reaction of cysteine thiols with 2-thiopyridyl disulfides.[22] To the best of our knowledge, this

is the first time a well-defined side chain neoglycopolymer possesing a single activated chain end has been chemically conjugated to a protein or bionanoparticle in such a "bioorthogonal" fashion.[23]

We have found particles such as **9** to have extraordinarily high binding affinities for lectins in the canonical hemagglutination assay, as will be reported in full elsewhere. We are using the ATRP/AAC methodology to synthesize a range of glycopolymer–CPMV conjugates targeted towards overexpressed carbohydrate receptors in cancer cells.

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