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Design, synthesis and biological applications of glycopolypeptides

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ABSTRACT

Carbohydrates play essential structural and biochemical roles in all living organisms. Glycopolymers are attractive as well-defined biomimetic analogs to study carbohydrate-dependent processes, and are widely applicable biocompatible materials in their own right. Glycopolypeptides have shown great promise in this area since they are closer structural mimics of natural glycoproteins than other synthetic glycopolymers and can serve as carriers for biologically active carbohydrates. This review highlights advances in the area of design and synthesis of such materials, and their biomedical applications in therapeutic delivery, tissue engineering, and beyond.

Contents

1. Introduction

Glycoproteins play essential roles in diverse biological processes including metabolism, homeostasis, lubrication, adhesion, infection, immunity, and cancer [1–3]. Glycosylation can affect protein lifetime by

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shielding from proteases, as well as induce structural changes by affecting folding or introducing charge. Diverse monosaccharide building blocks, in combination with multiple variations of sugar-sugar and sugar-protein linkages, result in an incredible range of structures. Relevant to human health, specific structures can induce cellular entry [4] and organelle targeting [5], bind to bacteria or viruses [6], or quiet [7] or stimulate [8] the immune system. Therefore, synthetic materials that can harness these properties are of great value for biomedical applications. Glycopolypeptides are one such class materials.

Glycopolypeptides are simply a polypeptide backbone with one or more pendant saccharide units. While there are many routes to access the peptide backbone, i.e. recombinant protein production or solid phase peptide synthesis (SPPS), polypeptides by N-carboxyhanhydride (NCA) polymerization have distinct advantages. Recombinantly produced glycoproteins suffer from variable glycosylation due to enzyme and substrate flux with metabolism and cell type, while SPPS is limited in chain length. Step-growth polymerizations of amino acids or short peptides are useful for preparation of polypeptides with a repeat sequence but are limited to low molecular weights and high dispersities [9]. By comparison, NCA polymerization is a high-yielding chemical route to polypeptides of high molecular weight and low dispersity [10].

There are two routes to prepare NCA-derived glycopolypeptides: polymerization of saccharide-bearing monomers and post-polymerization attachment of the saccharides. (Fig. 1) After considering design principles, this review will cover the advantages and limitations of both synthetic approaches along with recent developments in the field. Following discussion of synthesis, modern biomedical applications of glycopolypeptides will be examined with a particular focus on therapeutic delivery and tissue engineering applications.

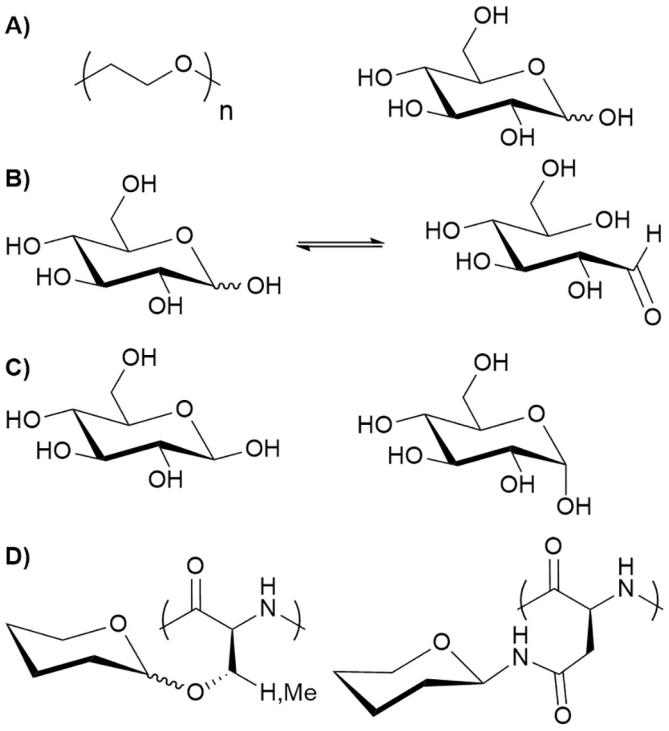

Fig. 2. A) Structure of PEG vs glucose; both are non-ionic and hydrophilic molecules. B) Equilibrium between ring-closed and ring-opened glucose; C) beta (left) versus alpha (right) form of glucose; D) Structures of glycan linkages to Ser and Asn, the two most common native glycoprotein linkages. αβ

2. Bioinspired design principles

Saccharide basic structures and stereoisomerism are thoroughly discussed elsewhere [11]. However, consideration of common mammalian linkage types and protein attachment sites are warranted when evaluating material design and synthetic approaches. In some cases, glycans may be desired for their ability to impart favorable properties to polypeptides due to their neutral hydrophilicity and high water-binding capacity. Poly(ethyleneglycol) (PEG) has been widely used for such purposes but is a non-natural substance that induces an immune response [12,13]. (Fig. 2A) Glycans are a natural alternative to PEG that may offer improved immunocompatibility and biodegradation. Beyond their simple physicochemical properties, researchers use glycosylation to achieve specific biological effects through receptor binding or similar phenomena. Such application requires careful consideration of glycan identity and display.

Monosaccharides consist of a chain of chiral hydroxymethylene units that terminate at one end with a hydroxymethyl group and with either an aldehyde or an α-hydroxy ketone group at the other. In solution, monosaccharides exist as an equilibrium mixture of acyclic and cyclic forms via condensation of the hydroxymethylene and the terminal carbonyl. (Fig. 2B) While the equilibrium percentage of the cyclized form does depend on the sugar, five- and six-membered rings are the most chemically stable, and therefore common, form of monosaccharides [14]. As such, it is important to consider that binding interactions with native partners are likely to depend upon the cyclic structure. However, the terminal carbonyl, or reducing end, of the sugar can be useful for conjugation chemistry. Further, structural and biological properties can be dictated by the stereochemistry of the saccharide linkages (α vs β) as shown in Fig 2C.

Glycosylation is among the most common of protein post-translational modifications and ranges from a single carbohydrate unit to chains that are hundreds of sugars long. Even the addition of a single sugar can have profound effects on protein structure and function [15,16]. In nature, monosaccharides are most commonly attached to proteins via N-linkage via asparagine (Asn) residues or O-linkages to serine or threonine (Ser or Thr) [17]. (Fig. 2D) Less common sites involve C-linkages to tryptophan, through phosphodiester bonds to Ser, or linking a phospholipid and a protein. After attachment of the first sugar via glycosyl transferases, additional enzymes can elongate and branch the structures into diverse glycan chains. Of special consideration are identity of the initial and terminal units. The initial sugar can affect protein conformation dramatically [15,18], while the terminal structures are most likely to be important for binding events [19]. It is also noteworthy that some monosaccharides will impart charge that may further affect protein conformation, solubility, and biology.

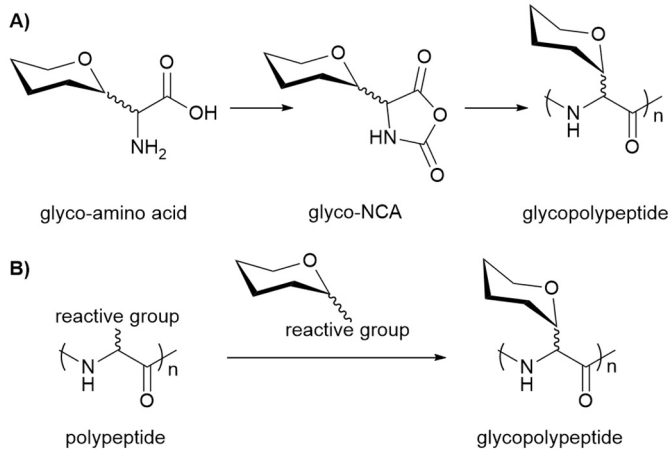

Fig. 1. Two routes to prepare glycopolypeptides. A) Polymerization of saccharide-bearing monomers; B) post-polymerization attachment of the saccharides via complementary reactive groups.

N-linked glycosylation is observed across all eukaryotes and archaea, but rarely in bacteria. Each species has unique characteristic core structures originating from enzymatic glycosylation of a conserved sequence, Asn-X-Ser/Thr [20]. O-linked glycosylation is much less well understood because a consensus sequence has not been discovered and glycan patterns are highly dynamic and variable. Therefore, tools to probe O-linked glycosylation are particularly valuable. Both types of glycosylation result in complex biology and are implicated in autoimmune diseases, cancer, cystic fibrosis, preterm birth, and more [21]. Proteoglycans are a subset of O-linked glycoproteins bearing high molecular weight polysaccharide chains at Ser resides within Ser-Gly-X-Gly- sequences. The polysaccharide chains are termed glycosaminoglycans (GAGs) due to their acetylated amino sugars. GAGs bear carboxylic acid or sulfated glycans which result in dense anionic charge, high water binding capacity, and lubricating functions. Proteoglycans are major components of connective tissue in the human body, loss of which is associated with decreased mobility and function.

3. Synthetic approaches

There are two routes for preparation of glycopolypeptides via NCA polymerization [22,23]. (Fig. 1) One route involves preparation of an amino acid glycoconjugate which is cyclized to a glyco-NCA and then polymerized. The second route involves post-polymerization functionalization, where saccharides or polysaccharides are chemically conjugated to pre-formed polypeptides. There are advantages and disadvantages that warrant consideration for each route. The glyco-NCA route has the distinct advantage of allowing production of polypeptides with precisely tunable glycosylation identity and density, up to 100% glycosylated if desired. Further, due to the scope of small molecule chemistry available, natural linkages can be utilized. Presentation of sugars in their native orientation can have profound effects on the conformation of the polypeptide and may be required for examination of the scope of biological effects. Challenges include developing new NCA monomers for each desired glycan and, to date, only monomers bearing up-to trisaccharides have been achieved. Larger glycans may present challenges with steric bulk and purification that could inhibit growth of large polymers.

NCA polymerization itself is scalable, and is in fact used industrially for an FDA approved drug [24,25]. However, commercial availability of glycoconjugates may currently limit industrial scale production. Desirable glyco-amino acid conjugates are typically not commercially available or are only available from specialty merchants are prices cost- prohibitive for large scale use. Researchers generally must undertake multi-step synthetic procedures to prepare glyco-conjugates, and separation of anomers is not trivial. This is a general challenge for glycopeptide synthesis by any method including NCA polymerization, SPPS, and other chemical routes. The authors have an optimistic outlook as the field of glycobiology continues to grow and consumer demand increases.

Polypeptide post-modification has the distinct advantage of increased synthetic simplicity and avoids the need to separately prepare many different glycosylated monomers and individually optimize their polymerization conditions. NCA-derived polypeptides composed of natural and commercially available amino acids such as lysine, glutamate, cysteine, and methionine offer conjugation opportunities with minimal to no protecting group manipulations. However, such conjugations typically utilize unnatural linker groups or present attached sugars in non-native ring-opened forms. Due to limitations in amino acid sidechain reactivity and solubility the complete chemical conjugation toolbox is not available to polypeptides. High efficiency click-type reactions are popularly applied to glycopolypeptide preparation by this route, again resulting in unnatural linker groups. Depending on the application, this may be not of concern. Yet, detailed studies on the biodegradation and target-binding affinities of such groups has not been established and should be considered. Like complex glycans, click-group-functional amino acids are also typically synthesized by researchers since they are not commercially available or are only available from specialty merchants at high price points. Additionally, these methods require some synthetic steps to prepare the click-glycan partner. Control over glycosylation density is often inefficient and challenged by steric crowding resulting in low coupling yields and requiring an enormous excess of glycan reagent. This can be problematic particularly where presentation of multiple glycans is desired but where they have different conjugation rates.

While challenges remain in the availability of precursor glycans and amino acids, NCA-derived glycopolypeptides remain one of the most promising routes to such materials. NCA chemistry is inherently scalable, high-yielding, and can generate higher molecular weight structures than any other method. The popularity and applications of click reactions has steadily grown since their introduction in ca. 2000, as has the demand for glyco reagents and building blocks. The future outlook for this field is very promising. The following sections describe examples of recent advances in both the glyco-NCA and polypeptide modification strategies. Though not explicitly stated in each case, the saccharide alcohol groups are typically protected until after the conjugation reactions or polymerization chemistry is complete. acetate protecting groups have been preferred due to their ease of installation and removal, combined with stability under diverse reaction conditions, though benzyl protecting groups have been explored.

3.1. Polymerization of glycosylated monomers

Polymerization of glyco-NCAs has been an area of interest for nearly 60 years. In the late 1960's, in order to probe the immunological effects of glycosylation, Rüde and coworkers conjugated glucose (Glc) [26] and other sugars [27] to Ser and cyclized the conjugates to the NCAs. (Fig. 3A) For immunological studies, they prepared glycopolypeptides of 6–11% Glc by copolymerization with various other amino acid NCAs and by using peptide terminal amines as initiators. The obtained materials were poorly defined and low molecular weight; however, this initial study did indicate that addition of glucose to non-immunogenic polypeptide scaffolds did not result in an increase in immunological response.

Synthesis and polymerization of Rüde's glyco-NCAs using amine initiators was revisited in the mid 1990's by Okada and coworkers [28–31] who also obtained only short, oligomeric glycopolypeptides. Chain growth was proposed to be inhibited by steric and H-bonding interactions between the sugar substituents and the NCA rings. Nevertheless, linear sugar containing polypeptides and poly(amido amine) dendrimer structures were prepared. Glycopolypeptide copolymers were examined in hemagglutination inhibition assays with wheat germ agglutinin lectin (specific for *N*-acetyl-D-glucosamine, GlcNAc), and were compared to monovalent GlcNAc as a control. The multivalent glycopolypeptide showed a 103 times stronger affinity towards the lectin than monomeric N-acetyl glucosamine, illustrating the potential of glycopolypeptides in biological applications. In 2007, Cameron and coworkers reported an improved synthesis of the O-linked glyco-Ser and Thr conjugates that avoids the use of the highly toxic and environmentally damaging mercury salts used by Rüde and Okada [32]. (Fig. 3B) The conjugates were cyclized to NCAs but could not be sufficiently purified to allow polymerization.

The first controlled, living polymerizations of glycosylated NCAs were not achieved until 2010, when Deming and Kramer reported improved purification and polymerization conditions for these monomers [33]. They first reported *C*-linked Glc, galactose (Gal), and mannose (Man) amide conjugates to L-lysine (Lys), which were designed for improved stability against deglycosylation as compared to *O*-linked conjugates which are susceptible to acidic and basic hydrolysis and to enzymatic deglycosylation. (Fig. 4A) The side-chains of Lys were also intended to alleviate the steric hypothesis proposed by Okada. Employment of an anhydrous flash column chromatography purification

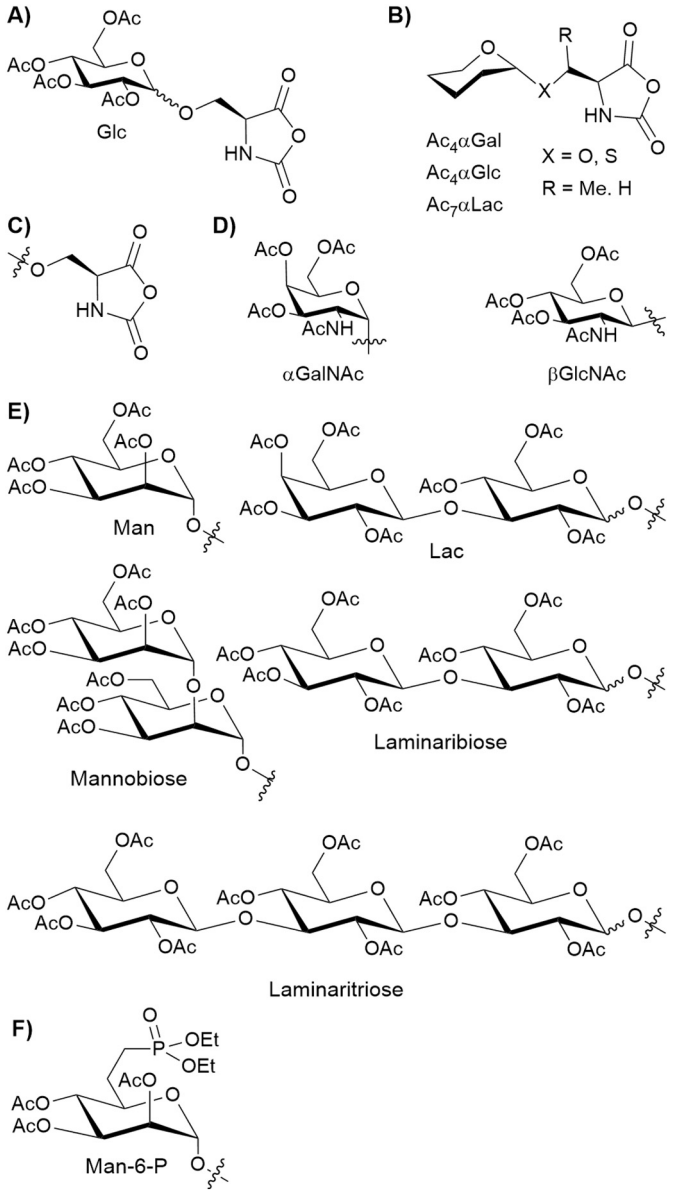

Fig. 3. Glycosylated Ser and Thr NCAs. A) The first published glyco-NCA, Glc-Ser, prepared by Rude et al. in 1966; B) glyco-Ser and Thr by Gibson and Cameron in 2007; C) glyco-Ser NCAs as prepared by D) Kramer et al. E) Zhou et al. and F) Banik et al.

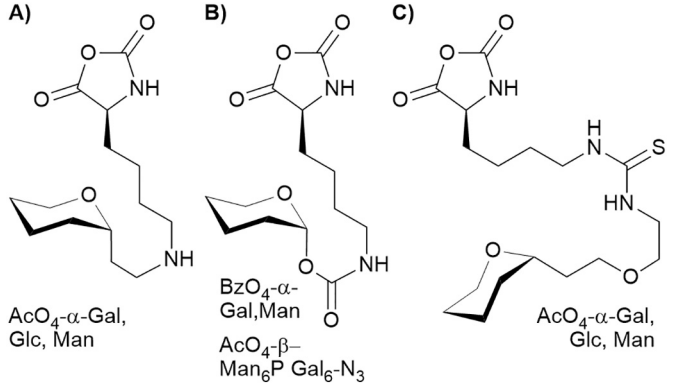

Fig. 4. Glycosylated Lys NCAs as prepared by A) Deming et al.; B) Gupta et al.; and C) Wenz et al.

technique developed by Deming and Kramer [34] resulted in highly pure monomers. Together with Deming's transition metal polymerization initiators [35], their work resulted in the first high molecular weight homo, block, and statistical glycopolypeptides. With this method, chain length could be precisely tuned up to ca. 400 residues with dispersities <1.2 and with control over glycan density and identity. It is also notable that the polymerization reaction time of ca. 1 hr is much swifter than amine polymerizations which are on the order of 3–6 days.

In 2011, both Gupta and Wenz contributed to the synthesis of glycopolypeptides and separately reported synthesis and polymerization of glyco-Lys NCAs. (Fig. 4B,C) Gupta's lab utilized carbamate linked Man or Gal L-Lys NCAs [36–38] and amine initiators, while Wenz and coworkers [39] utilized thiourea linkers to Glc, Man, or Gal and either tertiary amine or nickel initiators. Later, Gupta reported similar structures where the sugar had been modified with azide [40] or phosphate [41] groups. Basu's lab recently described routes to poly(glucosamininic acid) via NCA polymerization. These structures are not based on a natural amino acid side chain; rather the alpha carbon bears a ring-opened polyol chain [42,43].

Kramer and Deming reported thioether C-linked glycosylated poly (L-cysteine)s [44] (Cys, PLCs) and poly(homoCys)s [45]. (Fig. 5A) The thioether linkage was also oxidized to either the sulfoxide or sulfone. Conformational data reported in these studies indicated the various linkages resulted in glycopolypeptides with tunable structures from fully helical to partially helical to completely disordered. Schlaad and coworkers reported a thioether linked Gal-allyl-glycine (Gly) NCA conjugate prepared by in situ glycosylation and using thiol-ene chemistry and polymerization via primary amine initiators [46]. (Fig. 5B) As previously mentioned, amine initiated polymerizations typically result in low molecular weight materials with high dispersities, as compared to metal initiated reactions, due to multiple possible polymerization and termination pathways with variable, sluggish kinetics.

In 2015, Kramer and Bertozzi described the synthesis of mucin mimic glycopolypeptides based on α*N*-acetylgalactosylated (GalNAc) poly(L-Ser) (PLS) which is the linkage found in the native protein [15]. (Fig. 3C,D) Using an azide functionalized nickel initiator, they achieved controlled molecular weight GalNAc-PLS up to ca. 400 residues which is within the size range of native mucins. The chain length could be precisely varied, and glycan density tuned up to 100%. These data refute Okada's hypothesis that Ser-based monomers can't be polymerized due to steric or hydrogen bonding limitations. A small library of GalNAc-PLS copolypeptides of varied length, charge and glycan density were prepared via copolymerization with Glu, Lys, or Ala NCAs. Their conformations were studied by AFM and CD. Their CD spectra indicated a very rigid extended conformation with high glycosylation density, regardless of length or charge, and the polyproline II type conformation was similar to that of native mucins. AFM studies were used to visualize these conformations and calculate persistence lengths and revealed that native glycosylation indeed results in a rigidification of the peptide

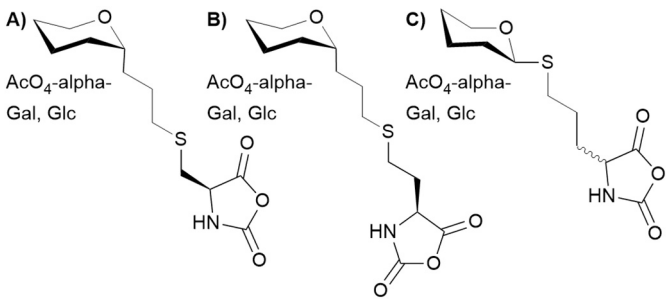

Fig. 5. Thioether-linked glyco NCAs as prepared by A, B) Deming et al. from Cys and homoCys; and C) Schlaad et al. from allyl-Gly.

backbone. Later, Kramer, Bertozzi and coworkers reported similar PLS conjugates with natural linkages to mono-, di-, and tri- Man, Glc and lactose (Lac) structures that mimic pathogen associated molecular patterns that activate immune cells [47]. (Fig. 3E) The trisaccharide monomers are the largest glycan-bearing monomer to date. More recently, similar methods utilizing Ni^0 initiators was applied to prepare and polymerize mannose-6-phosphate (Man6P) bearing Ser NCAs [48]. (Fig. 3F) These were utilized in targeting ligands for the Man6P receptor as described in Section 6.

3.2. Post polymerization glycosylation

Strategies to conjugate glycans to polypeptides have mainly utilized amide linkages, azide-akyne cycloadditions, or photoinitiated thiol-ene reactions. Efforts have focused on addition of monosaccharides as well as long, polysaccharide chains.

3.2.1. Amide, amine, and amidine linkages

L-Lys, L-glutamic acid (Glu) and L-aspartic acid (Asp) are natural residues that are amenable to functionalization via amide bond formation due to their pendant amine or carboxylic acid groups. Commercially available poly(Lys) (PLL) (Fig. 6A) has frequently been utilized as a substrate in applications where precise molecular weights are not essential. Early work in the 1990s focused on PLL conjugations where Man, Gal, or Lac were attached via amine [49–51], amidine [52], and amide [53–56] linkers. For most of these studies, only fractional glycosylation of the PLL backbone was desired or attempted. Amine linkages were generated via simple reductive amination of PLL with the reducing end of free sugars and sodium cyanoborohydride. (Fig. 6C) Amide linkages were prepared via conversion of PLL `to its chloroacetamide followed by reaction with various sugar-containing or other hydrophilic thiols [53]. (Fig. 6D) Feng and coworkers prepared glyconamidated polypeptides using amphiphilic triblock copolymer scaffolds prepared using bisamine-terminated polytetrahydrofuran poly[THF] macroinitiator to polymerize N-ε-benzyloxycarbonyl-L-Lys NCA (Z-Lys) [55,57]. (Fig. 6B) After removal of the Z groups, the Lys amines were conjugated to D-gluconolactone or lactobionolactone under basic conditions to attach the ring-opened sugars via amide linkages. Substitution densities higher than 47–75% could not be reached presumably due to steric crowding.

A different approach to prepare amide-linked glycopolypeptides using amino sugars and poly(L-Glu) (PLG) carboxylic acids has more recently been utilized by Menzel and Heise [58,59]. (Fig. 6E) The coupling reactions were promoted using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), which is a mild and efficient coupling agent. Conjugation of amino-glucose units to the polypeptide was found to be efficient at low loading ratios (<40% amino sugars per carboxylate), but decreased at higher loadings, with the maximum being ca. 80% glycosylation. Menzel and Heise later used similar methodology to form glycosylated star-shaped polypeptide functional dendrimers [60]. Benzyl-L-Glu (BLG) NCA polymerization was initiated from various generations of polypropyleneimine (PPI) dendrimers to afford a series of star-shaped derivatives that, after polypeptide deprotection, were conjugated with amino sugars.

3.2.2. Thiourea linkages

Thiourea linkages have also been utilized by multiple labs to generate glycopolypeptides. (Fig. 7A) Li and coworkers [61] partially glycosylated PLL homopolymers via thiourea linkages using isothiocyanate functionalized Man (Fig. 7B) while Midoux [62–64] et al. used 4–isothiocyanatophenyl glycosides to attach Lac, Man, or Gal through thiourea linkages. (Fig. 7C) In both cases, even with excess glycosylating reagent, only 16%–36% glycosylation density was achieved. Li reported interesting pH and surfactant dependent self-assembling behavior where aggregation was postulated to be driven by intermolecular hydrogen bonds from sidechain thiourea N–H groups to amide carbonyls on the PLL backbone. Circular dichroism (CD) spectra indicated the glycosylated PLL took on a random coil conformation under aqueous neutral conditions, but addition of an anionic surfactant at pH 4 initiated a shift to a β-sheet secondary structure coincident with formation of rod-like micelles. At pH 10, where the PLL amines became deprotonated an α-helical conformation was observed accompanied by a morphology shift to 45–80 nm vesicular assemblies. Typically, PLL is poorly soluble when deprotonated; however, even at low grafting densities the pendant mannose units facilitated solubility indicating advantageous properties of glycosylation.

Lecommandoux and Gillies prepared thiourea linked glycopolypeptide dendrons [65]. Alkyne-terminal polyester dendrons of varied generation and bearing amines groups were conjugated under basic conditions to *C*-linked α-Gal moieties functionalized with isothiocyanate groups. The alkyne-Gal-dendrons were then conjugated to azide-terminal-(polybenzyl-L-Glu) (PBLG) of varied lengths to give amphiphiles with hydrophilic mass fractions ranging from 0.07–0.54. These were self-assembled in water using a solvent exchange method. Copolymers composed of lower generation dendrons tended to aggregate, while those of a 4th generation dendron and with PBLG degree of polymerization of 28 formed micellar nanoassemblies ca. 40 nm in size. Size could be tuned by choice of organic cosolvent during self-assembly, or by addition of homo PBLG.

3.2.3. Click-type linkages

Click reactions have had an enormous impact on conjugations involving macromolecules, particularly polymer chemistry. Mild conditions, selectivity, and high yield are attractive qualities for polypeptide glycosylations. The Huisgen [3 + 2] cycloaddition between organic azides and alkynes and the radical mediated thiol-ene couplings are popular and highly efficient approaches. Additional conjugation reactions with click-like characteristics have emerged and will also be discussed here.

In 2009, Hammond and coworkers published the first example of a click coupling onto NCA-derived polypeptides [66]. They used acid catalyzed esterification of L-Glu with propargyl alcohol to prepare γ-propargyl-L-Glu NCA, (PrLG) which was utilized in PEG-azide conjugations. (Fig. 8A) PrLG NCA was subsequently used by many labs for click glycosylations since it is relatively straightforward to synthesize. Chen and coworkers utilized poly(PrLG) (PPrLG) for coupling to three different azide-functionalized monosaccharides using copper catalysis [67]. The glycosylations proceeded with near complete conversion, yielding α-helical glycopolypeptides with very high glycan densities. Brougham and Heise initiated PLG NCA polymerization from surface bound amines on iron oxide nanoparticles, followed by click to prepare glycopolypeptide decorated magnetic nanoparticles [68]. Gupta's lab used PPrLG for click reactions with azide and 6-alkyl- glycans. The glyco-PLG formed multimicellar aggregates in aqueous solution likely due to hydrophobic interactions of the aliphatic chains on the sugar moieties [38].

Though PPrLG is a substrate for high yielding click glycosylation, a potential limitation of this method is the hydrolytic instability of ester linkages. Hydrolysis could lead to loss of the attached glycan leading to reduced activity over time. Additionally, the ester bonds are incompatible with many common deprotection techniques used polypeptide chemistry. Therefore, several labs investigated clickable structures based on propargyl functionalized lysine and glycine, as well as azide-bearing NCAs.

The first NCA-derived alkyne polypeptide, poly(propargyl-glycine), (PPrG), was published in 1960 [69]. (Fig. 8D) In 2010, Heise and coworkers utilized commercially available racemic D,L-PG to prepare PPrG. Post-polymerization click galactosylation was achieved using azide functionalized-Gal and copper catalysis [70]. Block copolypeptides of Gal-PPrG-*b*-PBLG could successfully undergo deprotection and removal of the benzyl groups under highly acidic conditions while leaving the Gal groups intact. Heise and Lecommandoux later explored self-assembly of related block glycopolypeptides [71]. Because glycine is

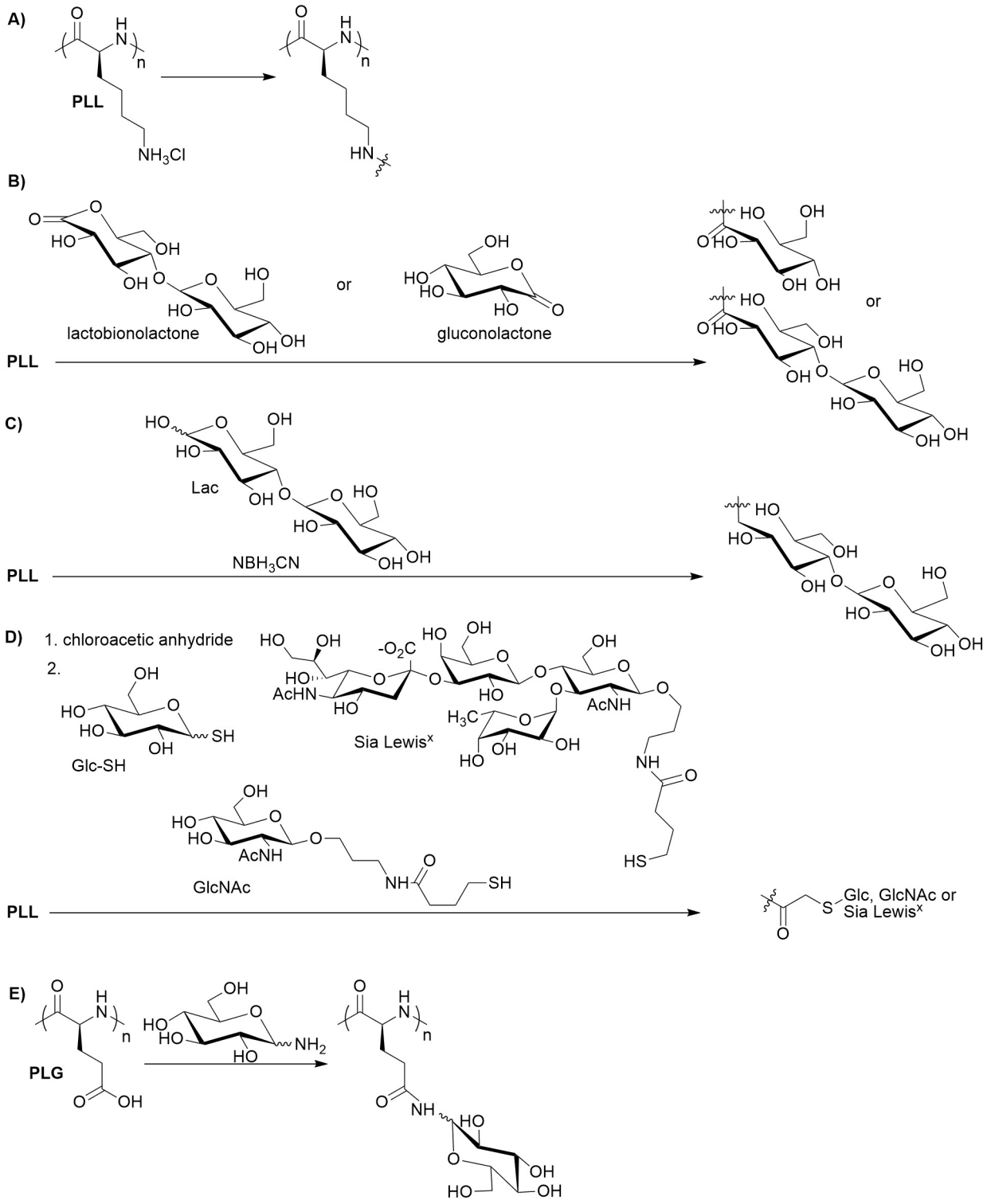

Fig. 6. Post-polymerization modification of PLL and PLG to form amide linked glycopolypeptides. A) Structure of PLL and modified PLL; B) reaction with glycosyl lactones to generate amide bonds; C) reductive amination with native sugars to generate amine linkages; D) chloroacetylation followed by nucleophilic substitution to generate alpha thioester amide linkages; E) PLG coupling to amino-Glc.

racemic, Gal-PPrG segments adopt disordered conformations while PBLG segments were helical and rod-like. The resulting rod–coil amphiphiles self-assembled via a nanoprecipitation method to yield spherical and wormlike micelles, or vesicles depending on the block ratios and assembly conditions. In related work, Heise, Compain, and Lecommandoux clicked iminosugars to the same amphiphile scaffold [72].

Heise, Lecommandoux, and coworkers also used PPrG and copolypeptides of PPrG as substrates for appending of mono-, oligo- and poly- saccharides. Tree-like oligosaccharide-grafted-polypeptides were prepared via Huisgen cycloadditions between PBLG-*b*-PPrG and either azido-Lac, azido-Gal, azido-dextran or azido-hyaluronan (HYA) [23,73,74]. HYA is a polymer of repeating disaccharide units of

D-glucuronic acid and N-acetyl-D-glucosamine, while dextran is polymer of glucose units. In aqueous solution, the conjugates spontaneously formed 50 nm spherical micelles in water. Self-assembly properties are proposed to be induced by the curvature of the grafted oligosaccharide segments and the favorable hydrophilic to hydrophobic volume ratios. Blends incorporating PBLG homopolymers afforded a transition to lamellar structures. The Lecommandoux lab also synthesized clickable-PLL-b-PBLG copolypeptides by amide formation of the Lys amines with pentynoic acid. Gal, Lac, or EG_3 moieties were clicked onto the chain via copper click chemistry and after nanoprecipitation, spheres, pearl-necklace, and wormlike structures were obtained depending on the hydrophilic block functionalization and the hydrophobic block length [75].

The use of azide functional polypeptides is relatively less explored as compared to alkyne functional polypeptides. In 2013, Deming and Rhodes reported the synthesis and polymerization of azide-bearing NCAs [76]. (Fig. 8C) These were prepared in from N-α-carboxybenzyl (Cbz) L-ornithine or N-α-Cbz-L-Lys, and the resulting NCAs were polymerized to give azidopolypeptides where the linkage was hydrotically stable. The polypeptides were subsequently glycosylated by copper catalyzed reaction with an alkyne bearing monosaccharide. The azide groups were found to be quantitatively converted to the corresponding triazole derivatives, yielding water soluble, helical glycopolypeptides. Polypeptides bearing a single terminal azide group have also been synthesized by the Lecommandoux lab via an azide bearing amine initiator. Polypeptide-polysaccharide hybrids were prepared via click conjugation of the terminal PBLG azide group to an alkyne-bearing HYA [77,78].

An alternate route to azide functional polypeptides was reported by Zhang and coworkers [79]. They reported the preparation of γ-3-chloropropyl-L-Glu NCA by esterification of 3-chloropropanol with L-Glu followed by cyclization. (Fig. 8B) Polymerization of the NCA was initiated by hexamethyldisilazane (HMDS) and α-helical alkyl chloride functional polypeptide was further modified by near quantitative conversion of chloro to azide groups. The azide groups were then coupled to alkyne functionalized D-Man using copper catalysis. Grafting proceeded with high conversions yielding water soluble, α-helical mannosylated PLG.

Thiol-ene click chemistry has also emerged as a useful method to synthesize glycopolypeptides. Schlaad and coworkers prepared poly (D/L-allyGly) utilizing commercially available racemic allylGly, conversion to NCAs and treatment with amine initiators to generate polypeptides that display alkene groups [80,81]. (Fig. 9A) Radical thiol-ene couplings using ester-thiols and Glc-thiols gave polymers with degrees of functionalization that varied greatly with reaction conditions. Near quantitative glycosylation could be achieved using UV irradiation. Follow up studies used the same methodology to modify poly(Glu-co-allylGly) and conformational studies indicated the polypeptides were disordered at neutral and basic pH, but switched to helical structure at acidic pH where the Glu is protonated. Dong's lab used an opposite thiol-ene strategy where the thiols were displayed on polypeptides via natural Cys residues [82]. Lac and PEG were grafted to the Cys thiols by thiol−ene click chemistry after UV photolysis of the thiol protecting groups.

3.2.4. Methionine-derived linkages and other conjugation strategies

Kramer and Deming reported highly efficient alkylations of methionine (Met) residues in peptides and polypeptides, including applications toward glycosylations. (Fig. 9B) These reactions have many of the defining qualities of click chemistry such as quantitative conversation, lack of by products, wide substrate scope, mild conditions, and high chemoselectivity under acidic conditions [83,84]. High molecular weight, homo, diblock, and random Met-containing polypeptides were prepared with controlled lengths using $(PMe_3)_4Co$ initiator. These were alkylated directly with activated alkyl halides or alkyl triflates, including a Gal-triflate. Alkylations with unactivated alkyl halides, including Glc functionalized with an iodoethyl group proceeded upon addition of silver tetrafluoroborate. These well-defined, fully glycosylated polypeptides were readily prepared in high yield. Follow up work by Deming and Kramer described the reversible modification of polypeptides with sugars via reversible methionine sulfonium salt formation. More recently, Deming and coworkers expanded this methodology to include the reaction of Met with epoxide substrates, including examples of glycosyl epoxides [85].

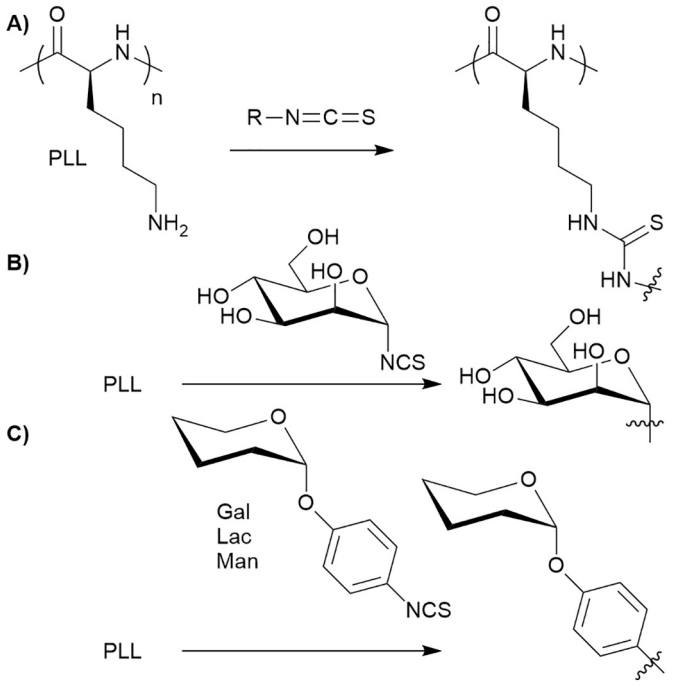

Fig. 7. A) Reaction of PLL with isothiocyantes to generate thiourea linkages. B) Man- PLL by Li and coworkers; C) Various glyco-PLLs by Midoux et al.

Wollenburg, Perlin, and Deming recently described a series of glycopolypeptides based on Met derivatives [86]. (Fig. 9C) They prepared *N*-methylaminooxy functionalized homocysteine derivative NCAs, which are Met analogs in the sense that the methyl group has been extended. These were polymerized with transition metal initiators and then hydrophilicity was increased by conversion to the sulfoxide or methyl sulfonium salt. These were then reacted with a panel of mono- and di- saccharides at varied pH and temperature. Aniline was found to improve the conjugation efficiency. Extended sidechains between the peptide backbone and the *N*-methylaminooxy were also explored. In these reactions, saccharide conjugation efficiency and number of isomers formed was highly dependent upon these conditions and ranged from 15–96%. The Met analogs formed disordered structures while those with extended sidechains had some alpha helical character. Several samples were studies for their aqueous stability and remained intact at pH 7 for 1 week at 37°C.

Zhao and coworkers prepared CO_2-switchable supramolecular block glycopolypeptide assemblies via an interesting noncovalent approach [87]. Poly(valine) was conjugated to benzimidazole, which can participate in host-guest assemblies with cyclodextrin. Polypeptide-polysaccharide conjugates were prepared using host-guest assembly of a dextran-cyclodextrin conjugates. When CO_2 was added in the solution of this complex, the benzimidazole species became protonated and converted into the charged form, which is excluded out of the host.

4. Drug delivery

Glycopolypeptides hold great promise as materials for applications in drug delivery. Not only are they typically biodegradable and biocompatible since the components are based on natural substances,

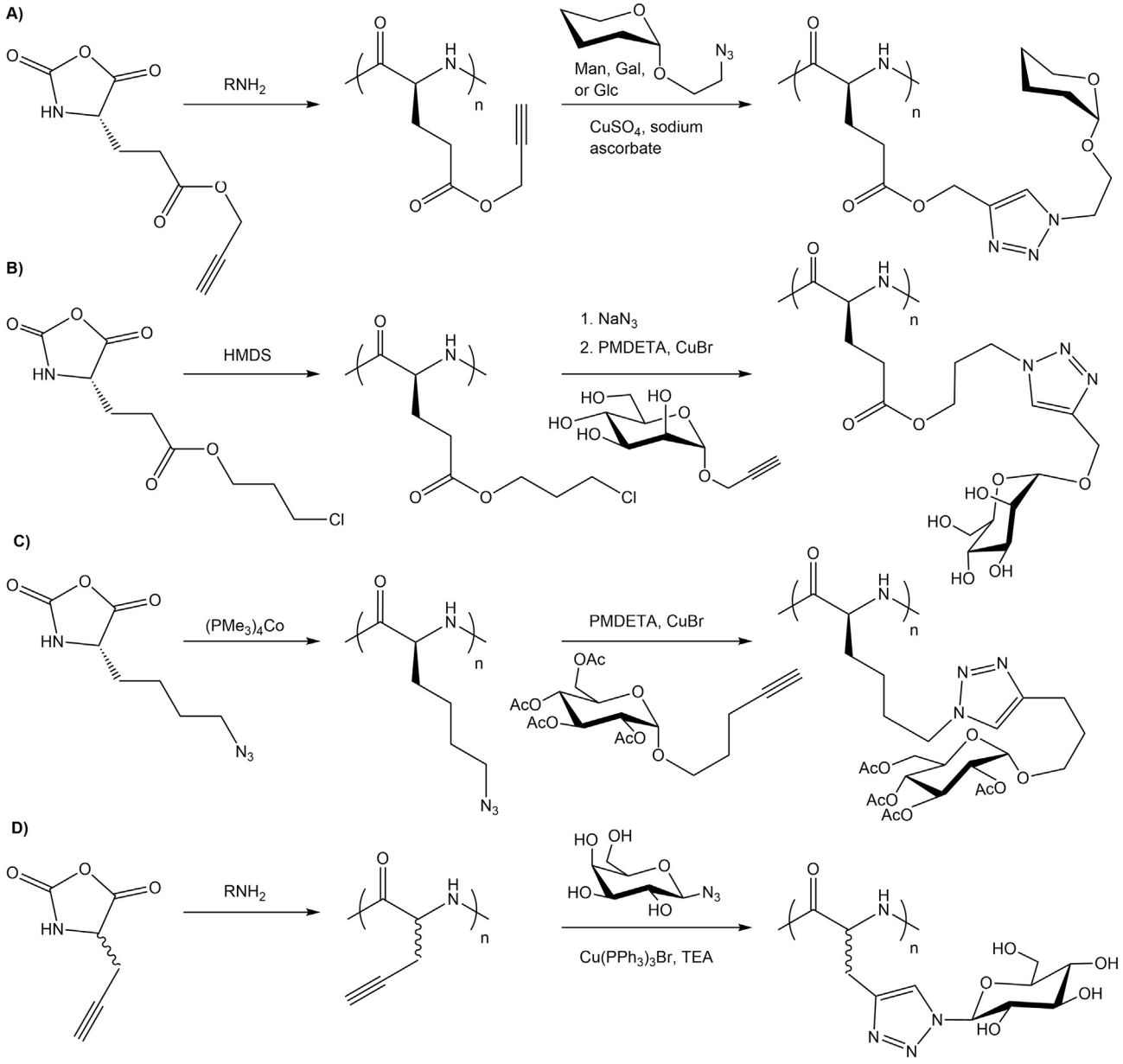

Fig. 8. A) Polymerization of PrLG NCA to yield PPrLG as reported by Hammond and coworkers, followed by click glycosylation with various azido-glycans as utilized by others. B) Polymerization of chloropropyl-LG NCA followed by Sn2 displacement with N_3 and click mannosylation. C) Hydrolytically stable azidoalkyl NCA prepared by Deming et al. and click glucosylation. D) PPrG prepared from PrG NCA, followed by reaction with azido-Gal.

the glycans can be utilized for their biological and biochemical properties. Glycan-binding cell surface receptors can be used for internalization and tissue-specific targeting. (Fig. 10) Mono-, oligo-, and poly- saccharides have all been utilized for their receptor binding properties. Typically, binding interactions between carbohydrates and receptor proteins are highly specific but weak compared to other biological interactions [88]. Binding efficiency can be improved by the multi-valent display and clustering that polymeric materials offer [89].

One of the most widely utilized glycan-binding receptors for targeting applications is the asialoglycoprotein receptor (ASGPR) which is present in high concentration on hepatocytes. This receptor binds Gal and GalNAc [4] and potentially α2,6-sialic acids [90]. Many classes of polymeric materials have been conjugated with these structures to promote accumulation in the liver [91–93]. Though relatively less studied, mannosyl and fucosyl bearing structures are reported, in rats, to preferentially accumulate in the reticuloendothelial systems such as those in the liver, spleen, and bone. Xylosyl conjugates accumulated mainly in the lung and liver [54].

Lac, which bears a terminal Gal, has been used for glycopolypeptide targeting via ASGPRs. A series of studies in the 1990's utilized lactosylated-PLL, prepared as described in section 3.2.1 and conjugated to antiviral agents. Lac-PLL was conjugated to adenine arabinoside monophosphate (ara-AMP) and administered to mice via intramuscular (i.m) injection [49]. The conjugate was found to accumulate in the liver and was devoid of acute toxicity even at high dose (1.3 mg/g). I.m. injections for 20 days did not induce detectable antibodies. Follow up work by Fiume et al. coupled 3 antiviral nucleoside analogs (ara-AMP, ribavirin, and azidothymidine) to Lac-PLL [51]. The conjugates were administered to mice via i.m. injection and were selectively taken up by the liver and eliminated by the kidney only in minute quantities. Corroborating the previous work, they found the ara-AMP conjugate to be devoid of acute toxicity and did not induce detectable antibodies after repeated injections.

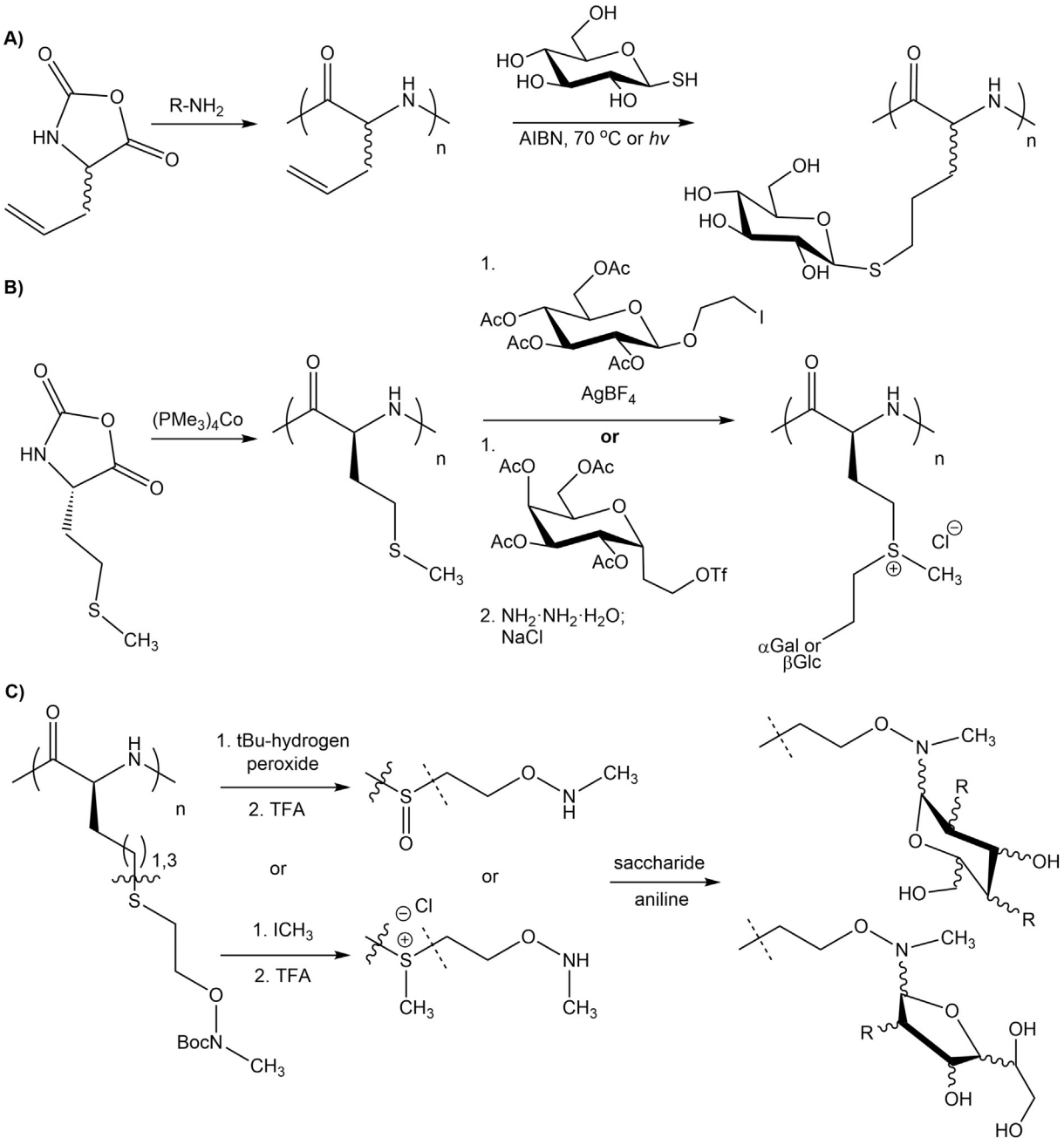

Fig. 9. Post-polymerization glycosylation via thioether linkages. A) Preparation of poly(allyl-Gly), followed by radical thiol-ene conjugation of thio-Glc. B) Polymerization of Met NCA followed by sulfonium formation with Gal-triflate or iodoalkyl-functionalized Glc. C) Reaction of *N*-methylaminooxy polypeptides with free saccharides.

Related work indicated that only five Lac residues per PLL molecule (M_r 38,000) was sufficient for high affinity competition in *in vitro* ASGPRs competition assays and for 70–80% of the injected dose to accumulate in the liver *in vivo* [50]. The Lac-PLL was derivatized with antiviral drug 5-iodo 2′-deoxyuridine, 5′-monophosphate and was determined to be serum stable. The drug-polymer conjugate was rapidly cleared from the bloodstream within 1 min and ca. 90% of the injected dose could be recovered in the liver due to parenchymal liver cell uptake. Using parenchymal liver cells, kinetics of endocytosis was observed and revealed that the drug-Lac-PLL conjugate was immediately internalized.

Hashida and coworkers used partially galactosylated PLL of various molecular weights (1,800, 13,000 or 29,000) as polycations to complex plasmid DNA for targeted delivery to hepatocytes [52]. As expected, a larger ratio of Gal-PLL1800 was required for complex formation as compared to the higher molecular weight species. Increasing the number of Gal units on the PLL resulted in reduced binding to plasmid DNA. After intravenous (i.v.) injection of [^{32}P] plasmid DNA complexed with Gal-PLL13K or Gal-PLL29K ca. 80% of the radioactive species accumulated in the liver due to preferential uptake by parenchymal cells. Both these *in vivo* and *in vitro* studies indicated higher gene expression was achieved via the higher molecular wight PLL species and with higher % Gal functionalization.

More recently, Dong's lab synthesized gold conjugated Lac-PEG-PLC nanoparticles (NPs) as described in section 3.2 for chemo- and photothermal therapy applications [82]. Gold NPs are generally bioinert and have been widely investigated for photothermal cancer therapy and theranostics due to their excellent optical properties, high surface-

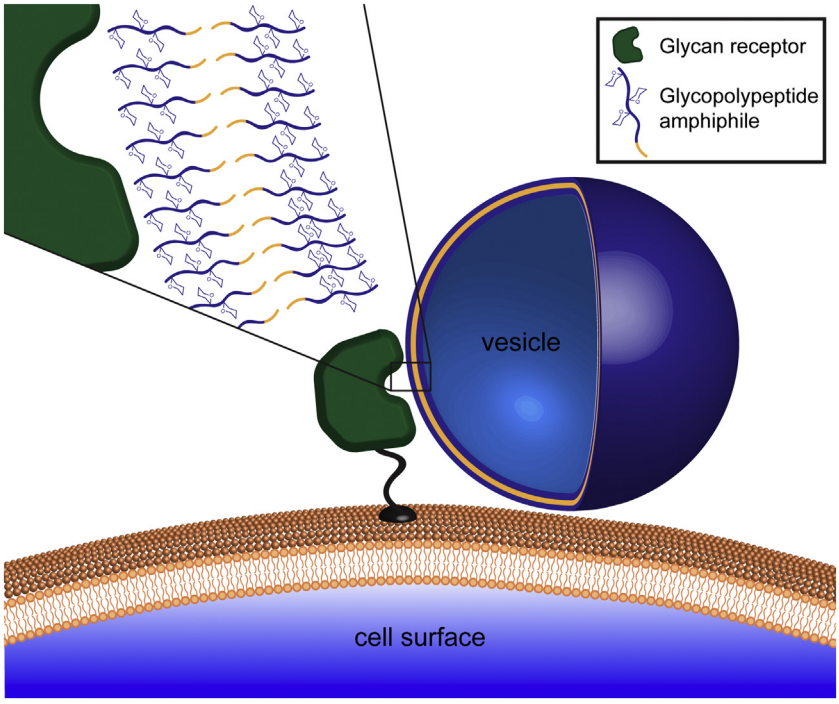

Fig. 10. Cell-surface glycan receptors can be used for delivery of encapsulated cargo via glycan bearing vehicles such as polymersomes. Such vehicles are typically composed of amphiphilic diblock polymers with a hydrophobic domain that drives assembly and a hydrophilic domain displaying glycans.

volume ratio, and facile surface modification. Thiols in the PLC block were used as sites for generation of plasmonic gold NPs. Chemotherapeutics doxorubicin (DOX) and 6-mercaptopurine were loaded into the micelles through physical interactions and Au—S bonding, respectively. The NPs absorbed in the near-infrared (NIR) at 650–1100 nm and increased the temperature of phosphate buffer solution by 30.1 °C upon continuous-wave laser irradiation. Further, the gold-Lac-PEG-PLC NPs were internalized more efficiently by HepG2 cancer cells that overexpress ASGPRs as compared to HeLa cells that do not, and NIR-mediated heating completely killed HepG2 cells *in vitro*, indicating promise for these NPs in combination chemo-photothermal therapy.

Mannose receptors (MR) are concentrated on the surface of professional antigen presenting cells involved in immune responses, such as macrophages and dendritic cells and to some extent endothelial cells [94]. These cells play crucial roles in recognition of viral and bacterial pathogens, as well as within the cancer microenvironment. Many malignant human tumors over express the MR, and therefore, Man-conjugates may allow targeting for delivery applications [95,96]. Gupta's lab prepared Man-PLL conjugates as described in section 3.1 and formulated nanorods, and polymersomes [97]. Man-PLL was conjugated to a branched poly(ε-caprolactone) (PCL) by Huisgen cycloaddition to form self-assembling miktoarm star amphiphiles. Changing the length of the hydrophilic and hydrophobic blocks varied the self-assembled architectures. These structures were investigated in MDA-MB-231 cells which are MR expressing breast cancer cells and were found to be non-cytotoxic and selectively and efficiently taken up. Treatment of the cells with free Man decreased polymersome and rod uptake suggesting an MR-dependent endocytotic mechanism, whereas treatment with Glc or Gal had negligible effects.

The Man-6-phosphate receptor (Man6PR) is a cell-surface protein whose major function is to bind and transport M6P-enzymes to lysosomes, but also modulates the activity of a variety of extracellular M6P-glycoproteins [5]. Man6PR functionalized materials have been used for directed delivery to the lysosome which could be applied for lysosomal storage disease therapy. Gupta's lab explored the properties of oligomeric Man6P functionalized glycopolypeptides, either as homopolymers [41] or as conjugates to PCL or poly(propylene oxide) (PPO) [98]. The diblock polymers self-assembled into micelles and could encapsulate a hydrophobic dye. Assays indicated the polymers were nontoxic in murine fibroblast L929 cells and 2 human breast cancer cell lines, MDA-MB-231 and MCF-7. Internalization into these cells over 2–6-hour incubations was observed and attributed to M6PR mediated endocytosis as evidenced by free Man6P competition assays and colocalized with lysotracker, though no control polymers were utilized.

A recent example of utilization of the Man6PR was for the purpose of targeted degradation of extracellular and membrane-associated proteins [48]. In this work by Bertozzi and coworkers, lysosome-targeting chimeras consisting of a small molecule or antibody conjugated to NCA-derived Man6P-bearing glycopolypeptides were prepared. The glycopolypeptides were composed of Ala and Man6P-Ser and ranged from 8-37kDa. Degradation of a scope pf therapeutically relevant proteins was demonstrated, including apolipoprotein E4, epidermal growth factor receptor, CD71 and programmed death-ligand 1.

Pandy et al. [99] made a later generation of these star copolymers by including a block of PPrG between the PCL and glycopolypeptide blocks. These were initiated from an amine-terminated branched PCL to form the PPrG and glycopolypeptide blocks from their corresponding NCAs. The alkyne block served as a site of crosslinking after self-assembly by treatment with a bisazide disulfide. Reduction of the disulfide in the intracellular environment is expected to cause the release of the loaded drug. Crosslinking significantly increased the particle stability compared to uncrosslinked assemblies (UCLs) loaded with RBOE dye, especially at low concentrations where UCLs would exist as individual polymer chains. The authors showed an increase in DOX release under reducing conditions with DOX-loaded crosslinked assemblies (DOX-ICLs), compared to DOX-UCLs. Treatment of HepG2 cells with DOX-ICLs led to lower cell viability as compared to treatment with DOX-UCLs, likely due to increased intracellular DOX delivery, as UCLs can dynamically assemble and disassemble before they are endocytosed.

Li, Wenz and coworkers explored Glc-, Man-, and Gal- PLL conjugates for their toxicity and uptake in human T lymphocytes [39]. In vitro toxicity assays indicated cells were viable at polymer

concentrations up to 0.33 mg/mL and the cells did not change morphology after 3 days exposure. Uptake by human T lymphocytes was investigated by flow cytometry and both the Man and Gal glycopolypeptides were readily taken up, while the Glc polypeptide was not. The uptake mechanism is unknown but was theorized to be dependent upon L-selectin or a galectin, which binds terminal βGal and βGalNAc.

To probe the effect of chain conformation on self-assembly, Kramer and Deming prepared α-helical Gal-PLL-*b*-leucine (Leu) and Gal-PLC-Leu where the Cys residues were oxidized to the sulfone rendering the chain disordered [100]. Self-assembly of the amphiphilic diblock copolypeptides in water was investigated and different assembly morphologies were observed. Glycopolypeptides with disordered segments favored vesicle formation, likely due to their flexible backbones that could accommodate vesicle curvature. The α-helical glycopolypeptides formed irregular aggregates and plate-like structures. The vesicles could be extruded to ca. 100nm and were able to encapsulate model drugs. A comparison of the cytotoxicity of the glyco-vesicles with previously studied cationic polypeptide vesicles indicated greatly improved cytocompatibility in HeLa cells since the glyco-vesicles were non-toxic at all concentrations tested while compatibility of the cationic formulations was low. Though the glyco-vesicles were not tested in vivo, they could specifically bind to native sugar binding lectins indicating the C-linked sugars were bioactive.

HYA is a major component of synovial fluid and the extracellular matrix, and also plays a role in immunity. HYA is a ligand for CD44 receptor and has been widely used to target CD44-overexpressing cancer cells [101–106]. In several studies, the Lecommandoux lab investigated the use of PBLG-HYA conjugates for their ability to selectively target, and deliver cargo to, CD44+ C6 glioma cells [107] and MCF-7 cells [77] while using low CD44-expressing U87 cells as a control. PBLG-*b*-HYA was formulated into polymersomes and loaded with chemotherapeutic doxorubicin (DOX) via nanoprecipitation methodology. *In vitro* drug release was observed at pH 5.5 and 7.4 over 10 days. Polymersomes without DOX were non-cytotoxic over a range of examined concentrations. Also, the polymersome formulation was less cardiotoxic than free DOX. Uptake was observed in all cell lines, though cytoplasmic vs. nuclear localization of DOX varied. In *in vivo* work, the DOX-PBLG-*b*-HYA particles suppressed breast tumor growth in female rats as compared to the control group. PBLG-*b*-HYA polymersomes were labeled with technetium-99m radionuclide and loaded with DOX for *in vivo* biodistribution studies [78,108]. Selective tumor accumulation in Ehrlich ascites tumor bearing mice was observed, presumably due to a combination of passive accumulation and CD44-mediated endocytosis. Compared to free DOX, PBLG-*b*-HYA polymersomes enhanced DOX circulation time, increased life span 6x, and controlled tumor growth by delaying doubling time of ascites tumor cells over 30 days post-treatment.

Cell uptake can also be promoted by the physical properties of glycopolypeptides. Cell penetrating peptides (CPPs) are a class of molecules that have received much attention as transporters for intracellular therapeutic delivery. Most CPPs contain a critical density of cationic charged residues, typically Arg or Lys, in combination with hydrophobic residues. Efficient cellular entry often comes at the penalty of increased cytotoxicity. A recent study by Kramer and Deming examined glycopolypeptides containing Gal and Glc functionalized cationic derivatives of Met [109]. As described in section 3.2, cationic Met sulfonium salts can easily be prepared by reaction of Met with alkyl halides, triflates, and epoxides. They found that glyco-Met polypeptides were highly efficient CPPs and could enter cells at ca. 7x lower concentration as compared to an equivalent Arg polypeptide. Importantly, these structures also possessed much low cytotoxicity than the cationic amine polypeptides. X-ray analyses were performed to understand mechanism of entry and was proposed to be a combination of the sterically demanding and hydrophilic cationic groups interactions with lipid membranes. These structures have not yet been used to deliver therapeutic cargo.

Yang and Zhang synthesized [110] amphiphilic block copolymers from dextran and PBLG that self-assemble into polymersomes. First, a PBLG block was synthesized from the corresponding NCA with an alkyne disulfide primary amine initiator. The alkyne was "clicked" onto an azide-functionalized dextran block. The authors anticipated that under reducing conditions the disulfide would reduce, separating the hydrophilic and hydrophobic blocks, resulting in disassembly and drug release. Polymersomes loaded with methotrexate released the drug significantly faster in reducing conditions compared to nonreducing conditions. Experiments in cell culture were not undertaken.

5. Tissue engineering

High molecular weight glycoproteins are major constituents of the cellular glycocalyx and extracellular matrix (ECM), serving mechanical, hydration, lubrication and biochemical signaling functions. Mucin glycoproteins are the major component of mucus [111], and proteoglycans [112,113], which bear long glycosaminoglycan (GAG) polysaccharide chains, are essential to the ECM and are in connective tissue. Mucins, proteoglycans, and GAGs have all demonstrated biochemical functions beyond their bulk physical roles, affecting processes such as cell growth and differentiation, morphogenesis, inflammation, and healing. (Fig. 11)Therefore, these biomaterials and their mimics have desirable properties for engineering of tissues for therapeutic applications.

Ren et al. [114] synthesized PPrLG that was conjugated to azide-modified Man and 3-(4-hydroxyphenyl) propanamide (HPPA) as injectable hydrogels for articular cartilage regeneration (PPrLG-g-Man/HPPA). The HPPA allowed for crosslinking and hydrogel formation after mixing with horseradish peroxidase (HRP) and H_2O_2 solutions. Time-to-gel occurred within minutes and depended upon the solutions' concentrations. *In vitro* cytocompatibility assays in L929 mouse fibroblasts showed >90% cell viability up to 100 mg/mL glycopolypeptide. Interestingly, incorporation of Man increased cell viability when compared to a PPrG modified with 1-(2-(2-methoxyethoxy)ethoxy)-2-azidoethane instead of Man (PPrG-g-MEO_3/HPPA).

PPrLG-g-Man/HPPA hydrogels formed within 15-minutes post-injection in a subcutaneous rat model. Complete degradation occurred within 4-weeks post-injection, which was attributed to hydrolysis of PPrLG esters and polypeptide amides rather than the *in situ*-formed crosslinks. An acute inflammatory reaction was observed 1-week post-injection. However, after hydrogel degradation, histology of the surrounding tissue appeared nearly normal, suggesting high biocompatibility. Chondrocytes cultured within 3D PPrLG-g-Man/HPPA hydrogels exhibited a spherical morphological and high viability over 21 days. On the other hand, chondrocytes cultured in PPrLG-g-MEO_3/HPPA hydrogels exhibited lower viability and lower proliferation, emphasizing the benefit of glycosylation. Increases in GAG content and gene expression of aggrecan, a cartilage-specific proteoglycan, and type II collagen were observed. Such molecules are important for articular cartilages mechanical properties. 3D PPrLG-g-Man/HPPA hydrogels seeded with chondrocytes (Fig. 11A) were implanted subcutaneously into mice. Again, total GAG content, aggrecan, and type II collagen levels increased. H&E staining showed increased chondrocyte proliferation. Overall, these glycopolypeptide hydrogels showed high biocompatibility in both *in vivo* and *in vitro* models.

Dhaware et al. [115] developed a biopolymer/glycopolypeptide composite hydrogel for the culture of hepatocytes. The glycopolypeptide component was formed from an amine-terminated PPO which initiated β-Gal-L-Lys NCA to form a PPO-*b*-Gal-PLL block copolymer. The PPO block was incorporated as a thermoresponsive segment to prevent leaching at physiological temperature by acting as noncovalent crosslinks above PPO's LCST. The authors chose Gal because hepatocytes express ASGPRs which bind galactose and induce spheroid formation. Spheroid formation plays important roles in hepatocyte viability, differentiation, and proliferation. (Fig. 11B)

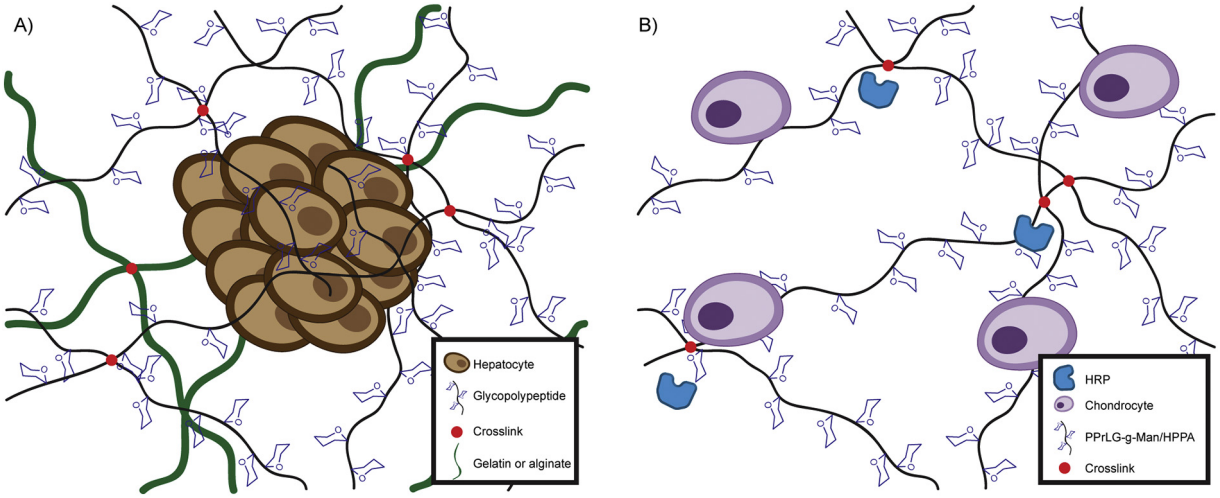

Fig. 11. The cellular glycocalyx and extracellular matrix are composed mainly of glycoproteins such as GAGs and mucins. Synthetic glycopolypeptides are excellent mimics of these structures and have applications in tissue engineering. Cells can be seeded throughout a glycopolypeptide matrix as in A) or via spheroid formation as in B), which is typical for hepatocytes.

PPO-*b*-Gal-PLL was blended with either gelatin or chitosan which were chosen since they are both biocompatible polymers that are easily crosslinked with glutaraldehyde. Characterization of the crosslinked gel scaffolds showed PPO-*b*-Gal-PLL evenly distributed across the gel with pore sizes of 20–120 microns, which the authors note is important for proper spheroid formation. Interestingly, the gelatin-based gel exhibited significant leaching of PPO-*b*-Gal-PLL whereas the chitosan-based gel did not. The authors attributed this to the higher number of hydrogen bond donors in chitosan as compared to gelatin.

Cell proliferation increased over a seven-day experiment with hepatocytes. Spheroids formed in PPO-*b*-Gal-PLL gelatin and chitosan gels were larger, rounder, and more connected as compared to gelatin or chitosan hydrogels lacking the glycopolypeptide. Confocal microscopy also showed actin filaments were less spread out in the PPO-*b*-Gal-PLL-containing scaffolds, supporting the authors' hypothesis that glycosylated structures aid in hepatocyte spheroid formation.

Kramer and Bertozzi utilized mucin-mimic GalNAc-PLS to engineer the glycocalyx of live cells [15]. HEK293T cells were simultaneously transfected with vectors encoding the *Methanosarcina mazeii* (Mm) aminoacyl-tRNA, the Mm pyrrolysyl-tRNA synthetase, and a membrane protein containing the amber stop codon (epidermal growth factor receptor-green fluorescent protein, EGFR-GFP fusion gene).Transfections in the presence of a norbonene-bearing pyrrolysine mimic produced full-length EGFR-GFP as determined through visualization of GFP by fluorescence microscopy. GalNAcPLS polymerization was initiated using an azide-bearing species, and the termini bore an amine. The azide was reacted with an alkyne-fluorophore and the amine was conjugated to a tetrazine-isothiocyanate. The modified GalNAcPLS was incubated with the EGFR-GFP bearing cells to allow covalent conjugation of the norbornene and tetrazine groups. Display of the mucin-mimic on the cell surface was observed by colocalization of GFP and the glycopolypeptide fluorophore.

6. Broader applications

6.1. Imaging

Multifunctional materials with, for example, imaging and therapeutic activity are highly desirable for biomedical applications [116]. Such systems with both diagnostic and therapeutic potential have been coined theranostics. Superparamagnetic iron oxide nanoparticles (MNPs) are frequently used in theranostics because of their magnetic resonance imaging (MRI) properties. However, they require surface functionalization for aqueous stability. Additionally, functionalization with biologically active groups, such as sugars, offers the possibility of organ or cell targeting. (See Fig. 12)

Heise's lab [117] used amine-terminated MNPs as macroinitiators to grow statistical or block copolymers of PLL-*s*-PPrLG and PLL-*b*-PPrLG, respectively. Azido-Gal was then conjugated to the PrLG groups to generate glycopolypeptide-coated MNPs. Charged Lys amino groups were incorporated for polyelectrolyte complexation of therapeutic small interfering RNA (siRNA) for loading to delivery to cancer cells. The block structure was anticipated to provide distinct compartments for siRNA polyplexes close to the MNPs surface surrounded by a glycosylated periphery. The particles retained superparamagnetic activity as evidenced by fast field-cycling NMR relaxometry, and no major aggregation was observed. The Gal residues were shown to be biologically active through lectin binding assays. SiRNA loading capacity was examined alongside colloidal stability and aggregation propensity. PLL-*s*-PPrLG

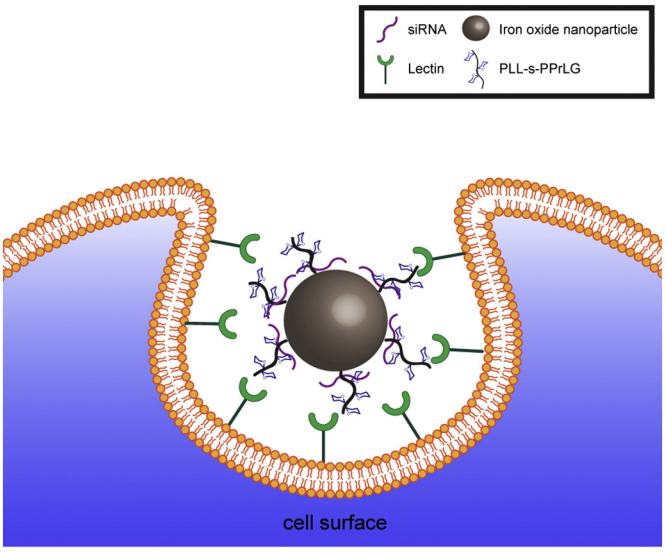

Fig. 12. Superparamagnetic iron oxide nanoparticles have applications as theranostics. The iron particles provide contrast during MRI and can be formulated with therapeutic cargo and targeting structures such as glycopolypeptides.

demonstrated over 7x higher siRNA loading capacities as compared to PLL-*b*-PPrLG and colloidal stability was higher. The authors hypothesize that for PLL-*s*-PPrLG MNPs, the PLL component is more accessible to the siRNA since it could be accommodated in the outer part of the polymer shell, leading to improved chain disruption and resulting in lower aggregation at higher siRNA content. The systems have yet to be evaluated in cell culture or *in vivo*.

Yang et al. [118] synthesized block copolymers from PCL and poly(2-azidoethyl-L-Glu) with a disulfide linker between the blocks. Propargyl-Lac or propargyl-Gal were separately conjugated to form amphiphilic polypeptides that self-assembled into micelles. Lectin binding assays indicated the sugars were biologically active. MNPs and DOX were loaded into the hydrophobic core of the particles via dialysis, resulting in increased micelle size particularly for the Lac conjugate. *In vitro* stimuli responsive drug release was explored using native reductant glutathione (GSH). The assemblies rapidly released drug release under reductive GSH conditions, in contrast to only 35% release over 24 hours in control conditions. Fluorescent imaging and flow cytometry demonstrated that HepG2 cells were able to traffic both Lac- and Gal- DOX-loaded MNPs, and both exhibited significant cytotoxicity. Application as MRI contrast agents was explored via observation of T_2 relaxivity, which increased with glycopolypeptide-MNP concentration. Higher r_2 values were observed for Gal-MNP micelles as compared to the Lac-MNP micelles presumably due to differences in particle size.

6.2. Immunomodulation

Modification of the immune response is a rapidly growing field of great promise for medical applications. Materials that activate or suppress its function can dampen destructive activities resulting in inflammation or tissue degradation, or can mark diseased sites for destruction by immune cells and proteins [119,120]. Cell-surface glycans play important roles in determination of self from non-self, and many immune receptors specific for glycans have been documented [121]. Glycobiology and immunology are both fields in relatively early stages, and therefore examples of materials at their intersection are rare. However, both fields are experiencing a research boom and glycomaterials that can play such roles are sure to emerge.

One such example are glycopolypeptides designed by Zhou et al. [47], who synthesized the first chemically defined ligands for dectin-1 and dectin-2. Dectin-1 (Dec-1) and dectin-2 (Dec-2) are C-type lectin receptors (CLRs) expressed on antigen-presenting cells such as dendritic cells and macrophages and are best known for their roles in anti-fungal and anti-bacterial immunity. High molecular weight mannoside- and glucoside-containing particulates from fungal extracts were shown to bind these receptors and activate the immune cells, while soluble low molecular weight structures bound but did not activate. Such data indicates that multi-valency and receptor clustering may be required for activation.

Zhou and coworkers prepared Ser NCAs bearing mono-, di-, and tri- Man or di-Glc structures as well as Lac-NCA as a control. The NCAs were copolymerized with Glu and Ala NCAs to generate glycopolypeptides of varied glycan density and identity. These polymers were conjugated to polystyrene beads to mimic pathogen surface presentation and used to identify structures that elicit a pro-inflammatory response in Dec-1 and Dec-2 positive antigen-presenting cells. The glycoPLS-coated beads stimulated cytokine production, while PEG coated beads did not. Cytokine production was lower as compared to polysaccharides isolated from native fungal and bacterial extracts, which is hypothesized to be due to on-bead vs off-bead presentation and potential contaminants in the polysaccharides. Activation was receptor dependent and could be abrogated by pre-treatment with blocking antibodies.

6.3. Diabetes

Rapid-release, glucose-sensitive insulin delivery systems are the next generation of insulin delivery devices for treating diabetes. These systems rely on phenylboronic-acid-incorporated depots loaded with insulin and sometimes glucose oxidase (GOx). GOx oxidizes glucose to gluconic acid and produces H_2O_2 as a byproduct. H_2O_2 can oxidize the phenylboronic acid leading to polymer degradation and insulin release from within the depot. Phenylboronic acid and its derivatives form reversible boronate esters with cis-diol compounds such as sugars. The dynamic nature of boronate ester formation imparts a glucose sensitivity to boronic-acid-based delivery depots: as glucose concentrations rise, the hydrogel will swell, increasing pore size and releasing insulin.

Zhao et. al [122] used an amine-terminated PEG to produce mPEG-b-(PBLG-s-PPrLG). Azidoethyl glucose was conjugated to the alkyne groups via a Huisgen reaction. The resulting glycopolypeptide was crosslinked with adipoylamidophenylboronic acid. The insulin loaded nanogel showed glucose-dependent rapid insulin release. The nanogel showed good biocompability when evaluated in hemolysis and cell viability assays. In a similar approach, Wang and coworkers [123] partially modified an mPEG-b-poly(L-asparate) (mPEG-b-PLD) with glucosamine using EDC as the coupling reagent. A boronic-acid-containing polyacrylate was blended with the resulting glycopolypeptide to form nanogels. GOx/insulin-loaded nanogels swelled in response to increasing glucose concentrations. Incorporation of GOx increased insulin release kinetics compared to controls. An *in vivo* rat model using dual GOx/insulin-loaded nanogels maintained normoglycemic levels for nearly 15 hours, longer than the free insulin control. In addition, the GOx/insulin nanogels showed satisfactory antihyperglycemic activity when evaluated in a diabetic rat model compared to controls.

6.4. Lectin binding

Lectins are carbohydrate-binding proteins with a high specificity for sugars presented on cells or macromolecules. Binding events typically cause agglutination or precipitation. Lectins have been utilized as diagnostics for cancer screening [124,125] and microbial infection [126,127] and have been applied as chemical warfare agents [128]. Due to the inherently heterogenous nature of biological glycoconjugates [11], synthetic glycopolypeptides have great promise as well-defined materials that can display specific glycans from ordered scaffolds that mimic authentic glycoproteins.

In an effort to study the role of multivalency in glycan-galectin binding, Heise and Lecommandoux prepared PBLG-b-galactan and PBLG-b-PPrG copolymers which were click glycosylated with Lac or Gal [74]. Galactan is a polysaccharide of repeating Gal units, while Lac is a Gal-terminal disaccharide. Polymeric NPs with sizes < 50 nm were assembled in water and the bioactivity of the outer surface sugar units was evaluated by relative binding affinities to human galectins 1 and 3. The NPs displaying galactan had an increased binding affinity of two orders of magnitude as compared to the linear display of monosaccharides. Such insights are useful to fine tune the design of novel biomimetic nanocarriers for interaction with specific receptors and tissue types.

The labs of Heise, Lecommandoux, and Compain have collaborated to prepare glycopolypeptide nanocarriers and used *in vitro* lectin binding and glycosidase inhibition as a metric of bioactivity before *in vivo* experiments. Binding assays of Gal-PPrG polymersomes to lectin RCA_{120} as compared to ConA indicated specific binding to RCA_{120} [71]. Man-mimic iminosugars, where the sugar ring oxygen is replaced with a nitrogen atom, were click conjugated to PPrG and nanoparticles were formulated [72]. These structures demonstrated remarkable multi-valency properties when inhibiting α-mannosidase activity. Heise's lab also used amide-linked Gal PLGA or PLL as macromolecular surfactants in the emulsion polymerization of styrene to produce uniform 100−150 nm size NPs with a polystyrene core and glycopolypeptide periphery. The

particles could also specifically bind lectins and bound to the surface of Chinese hamster ovary (CHO) cells [129]. Similarly, Kramer and Deming prepared Gal-Cys-*b*-Leu conjugates that were formulated into vesicles and could encapsulate model therapeutics [100]. These nanocarriers were shown to bind specifically to RCA_{120} but not ConA.

Gupta and coworkers showed that various Man-PLL conjugates will specifically bind ConA [37]. Helical glycopolypeptides based on enantiomerically pure L-lysine were compared to the corresponding racemic glycopolypeptides with no ordered secondary structure. Binding studies with the lectin ConA were performed using isothermal titration calorimetry (ITC) and precipitation and hemagglutination assays. Overall, the binding affinity was found to be nearly equivalent for both secondary structures. Polyionic complex vesicles Man-PLL grafted on the external surface were also examined and found to bind ConA [130]. Gupta's lab also prepared Gal-PLL polymersomes and these specifically bound RCA_{120} [131].

Selectins are a class of cell adhesion proteins that bind to terminal sialic acid (Sia) glycans. These lectins are widely distributed throughout various tissue types and are thought to play roles in extravasation, immunity, and cancer [132,133]. As described in section 3.2.1 Thoma and coworkers [53] prepared PLL-Sia Lewis^X and PLL-Sia Lewis^A polymers for application in selectin ligand binding assays. Binding was evaluated in both *in vitro* and cell culture assays. A cell adhesion assay using a parallel plate flow chamber coated with E-selectin-expressing human umbilical vein endothelial cells was used to study the rolling of polymorphous neutrophils in flow conditions. Cell interactions were imaged in the presence or absence of various PLL-Sia conjugates of varied glycan structure and density and in combination with carboxylic acid and sulfonate groups. The PLL-Sias served as competitive binders of E-selectin. Polymers containing 5–30% Sia Lewis^X with the remainder of the PLL glycerol functionalized were inactive at the concentrations examined. However, polymers of the same Sia Lewis^X concentration but with 25% carboxylate or sulfonate functionality displayed 30–35% reduction in adhesion. Similar competitive binding assays were conducted with surface-bound purified E-selectin. In this assay, the PLLs with 70–95% glycerol and 5–30% Sia Lewis^X did show inhibition of E-selectin.

7. Conclusions

Glycopolypeptides have generated much interest in recent years for their excellent physical and biological properties for biomedical applications. Such structures can impart non-ionic aqueous solubility while offering simultaneous biochemical interactions through native sugar-binding proteins. Such binding events have been utilized for tissue targeting, cellular internalization, activation of immune cells, and to study the binding events themselves. Advances in synthetic approaches have streamlined production of such materials and opened doors to expanded structural variation. There is still much work to be done in this emergent and exciting field.

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