Exploring and Controlling the Polymorphism in Supramolecular Assemblies of Carbohydrates and Proteins

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CONSPECTUS: In biology, polymorphism is a well-known phenomenon by which a discrete biomacromolecule can adopt multiple specific conformations in response to its environment. This term can be extended to the ability of biomacromolecules to pack into different ordered patterns. Thus, exploration and control of the polymorphism of biomacromolecules via supramolecular methods have been key steps in achieving bioinspired structures, developing bioinspired functional materials, and exploring the mechanisms of these self-assembly processes, which are models for more complex biological systems. This task could be difficult for proteins and carbohydrates due to the complicated multiple noncovalent interactions of these two species which can hardly be manipulated.

In this account, dealing with the structural polymorphisms from biomacromolecular assemblies, we will first briefly comment on the problems that carbohydrate/protein assemblies are facing, and then on the basis of our long-term research on carbohydrate self-assemblies, we will summarize the new strategies that we have developed in our laboratory in recent years to explore and control the polymorphism of carbohydrate/protein assemblies.

Considering the inherent ability of carbohydrates to recognize lectin, we proposed the “inducing ligand” strategy to assemble natural proteins into various nanostructures with highly ordered packing patterns. The newly developed inducing ligand approach opened a new window for protein assembly where dual noncovalent interactions (i.e., carbohydrate−protein interactions and dimerization of rhodamine) instead of the traditionally used protein−protein interactions direct the assembly pattern of proteins. As a result, various polymorphisms of protein assemblies have been constructed by simply changing the ligand chemical structure and/or the rhodamine dimerization.

Another concept that we proposed for glycopolymer self-assembly is DISA (i.e., deprotection-induced glycopolymer self-assembly). It is well known that protection−deprotection chemistry has been employed to construct complex oligosaccharide structures. However, its application in glycopolymer self-assembly has been overlooked. We initiated this new strategy with diblock copolymers. Such copolymers with a carbohydrate block having protected pendent groups exist as single chains in organic media. The self-assembly can be initiated by the deprotection of the pendent groups. The process was nicely controlled by introducing various protective groups with different deprotection rates. Later on, the DISA process has been proven practical in water and even in the cellular environment, which opens a new avenue for the development of polymeric glycomaterials.

Finally, the resultant polymeric glyco-materials, as a new type of biomimetic materials, provide a nice platform for investigating the functions of glycocalyx. The glycocalyx-mimicking nanoparticles achieved unprecedent functions which exceed their carbohydrate precursors. Here, the reversion of tumor-associated macrophages induced by glycocalyx-mimicking nanoparticles will be discussed with potential applications in cancer immunotherapy, where such a reversion effect could be combined with other methods (e.g., tumor checkpoint blockade).

INTRODUCTION

Great progress has been achieved in supramolecular chemistry in the last few decades, featuring the fact that small molecules can be arranged into complex and accurate structures under control.1,2 Nature presents amazingly complex self-assembly systems built from a broad range of raw materials, particularly various biomacromolecules. This inspires ambition from scientists to explore and control the self-assembly process of biomacromolecules in the laboratory. For the self-assembly of
biomacromolecules, it is crucial to deal with polymorphisms caused by their high molecular weight, complex interactions, and irregular hydrophobic and hydrophilic domains. Polymorphism is a common phenomenon in nature by which a discrete biomacromolecule can adopt multiple specific conformations in response to its environment change and thereafter pack into different arrangements. Such a phenomenon highly relates to biodiversity, genetic variation, and adaptation. Polymorphism usually functions to retain a variety of forms in a population living in a varied environment. Intuitive examples are microtubules which are supramolecular polymers of tubulins with different numbers of starting helices and different periodicities and the capsids of viruses which are highly symmetrical protein shells self-assembled by hundreds of protein units surrounding their genomes which is related to how viruses reproduce. Another example is the phenomenon of different glycan chains on the same modification site of protein called glycoform, which has a significant influence on cell functions including cell adhesion and signal transduction. Thus, exploration and control of the polymorphism of biomacromolecules on nanometer and micrometer scales via supramolecular methods turn out to be important steps for chemists in achieving bioinspired structures, developing bioinspired functional materials, and exploring the mechanisms of these self-assembly processes which could be models for complex biological systems.

In the study of the self-assembly of biomacromolecules into regular structures driven by noncovalent interactions, how to control their noncovalent interactions in order to form well-controlled assembled structures becomes the most important mission. Biomacromolecules always go through complex self-assembly pathways due to various intra- and intermolecular interactions. When proteins become the target to be manipulated, the difficulties come from the complex chemical composition as well as protein–protein interactions: the 20 words as the natural amino acids make the assembly of proteins very complicated, while protein–protein interactions are contributed by not only hydrogen bonds but also hydrophobic interactions and electrostatic interactions. The situation can even be severe when carbohydrates are employed as building blocks since their numerous hydroxyl groups make both intra- and intermolecular hydrogen bonds possible. Moreover, the relatively nonpolar face of the pyranose ring increases the possibility of hydrophobic interactions. In short, with such complexities, both proteins and carbohydrates themselves are amphiphilic with irregular hydrophobic and hydrophilic domains but not as hydrophilic as one simply expects, which is the first obstacle to controlling the polymorphism of the assemblies.

This account will introduce our contributions in building up controllable carbohydrate/protein-containing self-assembled materials including carbohydrate-containing protein materials developed by using the “inducing ligand” strategy and glycopolymer assemblies induced by reactions and interactions on carbohydrates. Finally, the immunological functions of the glycocalyx-mimicking nanoparticles will be discussed.

**CARBOHYDRATE–PROTEIN INTERACTIONS AS THE SELF-ASSEMBLY DRIVING FORCE**

Carbohydrate–protein interactions, which widely exist in nature, are very important in cellular recognition and signal transduction processes in both plants and animals. Their significant contribution to the function of glycans inspires us to
utilize carbohydrate–protein recognition for building supramolecular complex structures based on carbohydrates. Driven by carbohydrate–protein interactions, supramolecular assemblies of carbohydrates containing a large portion of proteins could be constructed, making the new materials compatible to protein assemblies. In the field of protein self-assembly, scientists have spent decades to develop new approaches to achieve regular structures of protein assemblies. The majority of protein self-assembly methods are biotechnological strategies based on protein–protein interactions, which require the computational de novo design of the protein structure and interaction interface as well as laboratory work on protein expression and purification. Recent developments in chemistry and supramolecular chemistry open new avenues for protein self-assembly by introducing new interactions into the system such as metal coordination, host–guest recognition, receptor–ligand interactions, and cooperative receptor–ligand interactions. Inspired by the great successes of these methods, it is rational to introduce carbohydrate–protein interactions as new supramolecular interactions to control the polymorphism of carbohydrate-containing protein assemblies.

We proposed the use of native proteins as building blocks (Figure 1c) and dual noncovalent interactions as driving forces for constructing various regular structures. In our approach, a designer small-molecule inducing ligand, which contains a carbohydrate moiety and a rhodamine moiety connected by a short linker, was employed (Figure 1a). In short, this kind of inducing ligand was given a code of “RhnC”, where “C” represents the carbohydrate part of protein binding, “Rh” represents the rhodamine B (RhB) part which helps the carbohydrate-bound proteins to pack regularly with each other via π–π interactions, and “n” represents the number of ethylene oxide repeating units linking Rh and C together. A series of different carbohydrates which specifically bind with different lectins are listed in Figure 1b. By using this strategy, the self-assembly processes were controlled via the dual noncovalent interactions (carbohydrate–protein interaction and rhodamine dimerization), and different highly ordered protein assembly structures were achieved. It is worth mentioning that the lectin cross-linking of glycans has been known for quite a long time. About 20 years ago, Brewer et al. and Freeman et al. obtained cross-linked lattices of SBA (soybean agglutinin) and ConA (concanavalin A) via ditopic saccharides, respectively. The research field became...
relatively quiet afterward, until we found that the inducing ligands could achieve much more control over the assembly process than the previously employed ditopic saccharides, resulting in unprecedented precise protein assemblies with polymorphism.

Our inducing ligand strategy succeeded first when three-dimensional protein crystalline frameworks (Figure 1d) were achieved from treating homotetrameric lectin ConA with the corresponding Rh3M inducing ligand in a dilute aqueous solution. Instead of protein–protein interactions, the dual supramolecular interactions between Rh3M and ConA mediated the formation of the protein lattice. The structure of crystalline frameworks revealed that mannopyranoside on Rh3M binds with the carbohydrate-binding sites of ConA in a ratio of 1:1 and RhB dimerization occurs between the moieties of RhnC, which had already been bound to the proteins (Figure 2a). We found that in distinct contrast with the traditional protein assembly and the previous lectin assemblies via ditopic ligands, our approach exhibited many new features including a two-step self-assembly process and the limited contribution of protein–protein interactions. The kinetic studies of crystalline framework formation revealed that, the carbohydrate (Man)–protein (Con A) binding occurred first, followed by RhB dimerization between the moieties of RhnC, which had already been bound to the proteins (Figure 2c). Thanks to this unique two-step mechanism, a higher ratio of inducing ligands can be used to increase the rate and yield of the crystallization compared to conventional methods of protein crystallization. We also found that, by using the same protein and inducing ligands, loose packing of proteins into the framework was achieved from the liquid–liquid diffusion method, while the condensed packing of proteins was found from the hanging-drop vapor diffusion method.

Figure 3. (a) Three possible packing patterns of LecA/RhnG and the MD simulation result of long–long packing. (b) Proposed mechanism of the assembly and disassembly of SBA/Rh3GN, SBA/Rh3G, and SBA/Rh5G with a pH change. (c) Typical equilibrium snapshots of the protein chains in different cases in the simulations. Reprinted with permission from refs 29 and 32. Copyright 2017 John Wiley and Sons and 2018 Royal Society of Chemistry.
protein microrings. A small molecule called RhYBio2 plays the
role of an inducing ligand, in which biotin instead of
biotin or streptavidin can be used. This method (Figure 2b). This difference can be explained by the
strong limitation of protein–protein interactions in liquid
diffusion and the promotion of protein–protein interactions in
the vapor diffusion method.

Afterward, this self-assembly approach was expanded to
other proteins and ligands to achieve polymorphism in protein self-assemblies. Besides crystalline frameworks, quite a number of
precise protein assemblies were observed in our research,
including nanoribbons, microtubules, microcrystalline aggregates,
and 2D patterns. Plant lectin SBA, a homotetrameric protein
that contains four identical N-acetyl-α-D-galactosamine carbohydrate-binding sites, was also studied. After incubating the
protein with the Rh3GN solution, uniform monolayer protein
microrings of 26 nm diameter and 4.7 nm thickness were
observed. With the help of cryo-EM 3D reconstruction and
computational modeling, we found that the microring was
constructed by the winding of three protofilaments. Each protofilament showed a helical structure with a periodicity of
approximately 19 nm. In each period, nine tetrameric SBA
units were observed (Figure 1e).

Moreover, typical research of polymorphism in protein self-assemblies was conducted using native protein LecA. With
the same method as mentioned above, a series of small
molecular ligands (i.e., RhnG (n = 1–5) with different lengths
were used to induce the formation of protein assemblies. Consequently, many different morphologies were observed,
including 1D nanoribbons of LecA/Rh1G, nanowires of LecA/
Rh3G (Figure 1f), 2D crystalline nanosheets, and 3D layered
structures of LecA/RhnG (n = 2, 4, 5) (Figure 1g). These
results proved that a slight difference in ligand length
significantly influences the structure morphologies, and therefore controllable self-assembly from the same protein
to into different dimensional structures in our method becomes
possible.

This inducing ligand method was not restricted to using
ligands of the RhnC type, and other kinds of rigid ligands also
succeeded in constructing uncommon morphologies such as
protein microrings. A small molecule called RhYBio2 plays the
role of an inducing ligand, in which biotin instead of
hydrophobic provided the protein–ligand binding force
(Figure 1h). A fusion protein, GFP-SA containing green
fluorescent protein (GFP) and streptavidin (SA), was used as a
building block. Fluorescent microrings of GFP-SA/RhYBio2
were achieved, which have a micrometer-scale diameter and
a width of 30 nm. Our strategy was further expanded to other
rigid ligands by Yan and co-workers. They employed this
inducing ligand strategy with a ligand containing RhB and
phenothiazine. The latter bound with calmodulin to achieve
protein–ligand assemblies with tunable helical structures. The
successful introduction of biotin and phenothiazine demonstrates that this inducing ligand strategy could be extended to
many rigid ligands for proteins. Finally, such an approach via
dual noncovalent interactions is also helpful in building protein-containing materials such as protein hydrogel whose modulus was three orders of magnitude higher than that of a
similar network without the protein.

To explain the reason for achieving polymorphism in protein
self-assemblies, great effort has been dedicated in studying the
mechanism. Molecular simulation of the formation mechanism
of nanotubes, nanoribbons, and 2D patterns offered a deep
understanding of the effect of the spacer length of the inducing
ligands on protein assembly polymorphism. Results of all-atom
molecular dynamics simulation indicated that the relative
stable packing mode varies with different ligand lengths. For
example, when LecA was assembled with Rh1G or Rh2G in
diagonal–diagonal linkages, LecA/Rh2G was proven to be
more stable than LecA/Rh1G within the simulation time
scale. Different packing patterns were stable in different LecA
and RhnG assemblies, namely, short–diagonal (LecA/Rh1G),
long–long (LecA/Rh3G), and diagonal–diagonal (LecA/
Rh2G or Rh4G) styles, which were controlled by the ligand
length in experiments (Figure 3a). These results explained the
reason for polymorphism in LecA assemblies, and the
experimental and simulation results correlate to each other
quite well. In addition, other effects of inducing ligands could also be found in the case of microtubules. In this case,
not only the inducing ligand length but also the electrostatic interaction between RhB and the protein contributed to the
protein assembly (Figure 3b). Only with the attraction
between the negatively charged SBA and positively charged
RhB is the microtubule formation possible. Here the protein
protofilament constructed from 11 SBA with corresponding

Figure 4. (a) Gal-1-induced T-cell agglutination. (b) The formation of protein microribbons successfully inhibited T-cell agglutination. (c) An automatic cell count was performed to quantify the cell agglutination. Results are shown as means ± the standard error of mean (SEM) as
indicated. The statistical significance was calculated by one-way ANOVA (compared to the PBS group). *p < 0.05, **p < 0.01, and ***p < 0.001. Reprinted with permission from ref 26. Copyright 2018 Springer Nature.
ligands via a coarse-grained model was employed to demonstrate the initial state of protein assemblies. Results of Brownian dynamics simulations confirmed that, when attraction exists between RhB and SBA (case Rh3G/ATT), the protein protofilament is stable; when repulsion (case Rh3G/REP) exists or a longer ligand (cases Rh5G/ATT and Rh5G/REP) is used, the protein protofilament is unstable (Figure 3c). The above results proved that in our strategy of protein assembly a slight difference in the ligand length or surface charge has a significant influence on the resultant morphologies. In other words, polymorphism of the self-assemblies of proteins could be realized by simply adjusting the ligand structures.

In the above-mentioned works, we mainly focused on the morphologies and mechanism of plant lectin self-assembly based on our inducing ligand strategy. Subsequent work proves that our approach was also widely applicable in animal lectins, which play important roles in organisms and provide broad opportunities for biological applications. Human galectin-1 (Gal-1), a kind of mammalian lectin, was chosen as our research object. Gal-1 is a homodimer protein and consists of two β-D-galactopyranoside recognition domains, which enabled it to assemble with Rh4L into protein microribbons. Gal-1 is known for inducing T-cell agglutination and apoptosis via cross-linking cellular glycans (Figure 4a). In addition, its overexpression in cancer cells has been known. After adding Rh4L to the system, we found that the formation of protein microribbons significantly inhibited T-cell agglutination with much higher efficiency than an equimolar amount of lactose (Figure 4b). This result clearly demonstrates that the formation of protein assembly via an inducing ligand is capable of dissociating the agglutinated T-cells (Figure 4c), indicating the potential of our approach in biological applications such as cancer treatment via blocking the effect of Gal-1.

### CONTROL OF THE POLYMORPHISM OF GLYCOPOLYMER ASSEMBLIES BY CHEMICAL REACTIONS AND SUPRAMOLECULAR INTERACTIONS

In previous sections, we introduced new carbohydrate–protein self-assembly strategies inspired by carbohydrate–lectin binding abilities, which is an indispensable property of carbohydrates in nature. However, considering the multiple existing forms of carbohydrates in organisms, their properties in nature are not limited to carbohydrate–lectin recognition. Since great quantities of carbohydrates exist as oligosaccharides or polysaccharides with a high density on the surface of cells, besides carbohydrate–lectin assemblies, it is also important to prepare bioinspired carbohydrate-containing assemblies based on carbohydrate-containing polymers as a simplified mimic to natural glycans. In previous studies on the molecular assembly of carbohydrate-containing macromolecules, the carbohydrates were mainly simply treated as hydrophilic components, while few research has studied the complexity of carbohydrates intensively. Considering these problems, it is crucial to control the interactions between the multiple hydroxyl groups by chemical reactions and supramolecular interactions. Therefore, rather than just as hydrophilic components, hydroxyl groups of carbohydrates become one of the elements we can operate to control the self-assembly process.

For this goal and inspired by the almost unavoidable protection–deprotection procedure in oligosaccharide synthesis, we proposed DISA (deprotection-induced glycopolymer
self-assembly) as a new strategy for the assembly of carbohydrate-containing polymers. In this approach, block copolymers having one glycopolymer block with protected hydroxyl groups and one hydrophobic block serve as starting materials. The block copolymers exist as single chains in organic solvents because the hydroxyl groups are protected. Then, upon deprotection the polarity change of the glyco-block makes the block insoluble, inducing the formation of new self-assembled structures. Taking advantage of this feature, control of the self-assembly process was achieved and the polymorphism of glyco-nanoparticle morphologies was obtained. Since almost every carbohydrate unit contains several hydroxyl groups, DISA is suitable for nearly any kind of glycopolymer, polysaccharide, or glycan. Moreover, this process and related morphology transition could take place in organic solvents, water, and even inside cells, which ensures broad applications of DISA in biological studies.

In the initial studies of DISA, the most common protective group (i.e., the acetyl group (Ac)) was employed and polystyrene was chosen as the hydrophobic block in GP-Ac (Figure 5a). Since the hydroxyl groups were all protected by Ac groups, these diblock copolymers were first dissolved in THF. As soon as tetrabutylammonium hydroxide (TBAOH) was added to induce deprotection, the molecularly dissolved polymer chains aggregated into unique glyco-inside self-assemblies very quickly.

Despite the success of DISA in achieving various morphologies using acetyl groups as the protective groups, the rapid deprotection prevented kinetic studies and good control of morphology. This problem was overcome by using benzoyl instead of acetyl groups for protection. The results of the same polymers protected by benzoyl groups (GP-Bz) or acetyl groups (GP-Ac) are shown in Figure 5 for comparison. We found that Ac-protected polymers self-assembled into small vesicles via a quick process (Figure 5c), while those with Bz groups self-assembled with three-stage kinetic character: fast initiation, steady growth, and fast decrease to a stable state. The mechanism was explained as follows (Figure 5d): the initial aggregation took place as soon as TBAOH was added, and then only a small number of Bz groups had been removed (Figure 5b), resulting in unstable assemblies. Then, with an increasing number of deprotected hydroxyl groups, the assemblies grow into larger but loose and kinetically trapped aggregates. Finally, these unstable aggregates reorganize into stable vesicles. These results uncovered the relationship between the degrees of protection of carbohydrates units and the self-assembly behavior. These findings are in accordance with the results of another study in which the carbohydrate containing temperature-sensitive copolymers self-assembled into different morphologies according to different degrees of protection on the carbohydrate.

In addition to organic solvents, DISA was also feasible under physiological conditions. To achieve this goal, enzymes instead of organic or inorganic base should be used in studies. Lipase type I from wheat germ was chosen as the model enzyme for lipase-catalyzed DISA. When lipase is captured in vesicles of protected glycopolymer peg-GP-Ac, it can be self-released along with the morphology transition of the polymersomes triggered by the deprotection initiated by itself (Figure 6a). This kind of enzyme-responsive polymersome was...
also able to release a model protein antigen such as ovalbumin (OVA), which implied the potential application of our self-assemblies in immunotherapy. As shown in Figure 6b, dendritic cells (DCs) are an important antigen-presenting cell type, which can stimulate the activation of T cells. In order to get involved in the antigen presentation process, OVA were loaded into glyco-vesicles which were assembled by protected glycopolymer. After these glyco-vesicles were incubated with DCs, lysosomal lipases of DCs triggered the deprotection process, leading to the morphology transformation and the release of OVA. Compared to the control groups, OVA encapsulated in glyco-vesicles exhibited higher cellular uptake and more efficient antigen presentation to T cells (Figure 6c). These positive results are due to the dual function of the glyco-vesicles, which not only acted as a delivery vehicle but also promoted the maturation of T cells through the carbohydrates on the surface.

Furthermore, the concept of DISA can be expanded to a much broader range, in which other dynamic chemical reactions but also included CCIs (carbohydrate-carbohydrate interactions) led by abundant carbohydrates themselves, since the shells of the self-assemblies contain a high density of carbohydrates in most cases. Considering the distinct role of CCIs between cell surfaces in cell adhesion and recognition in nature, scientists have built models to reveal the thermodynamic evidence that carbohydrate aggregation was an enthalpically favorable process in polyvalent systems. In our previous studies, it was found that glycopolymer, even with both blocks of the polymer being hydrophilic, can form some aggregates in water because of the strong CCIs between carbohydrate pendants with high density. Inspired by this phenomenon, we believe that with CCIs, more precise control of self-assembly polymorphism and many complicated structures, including some hierarchical assemblies, can be achieved. A library of glycopeptide brushes containing oligomannosides and oligophenylalanines as pendant groups was synthesized. These glycopeptide brushes self-assembled in water and gave a series of morphologies including micelles, nanowires, and nanoribbons. We found that the self-assembly behavior was controlled by the saccharide to phenylalanine (S/F) ratio, and a phase diagram was drawn to describe the results. Among these results, the nanowires formed by P2tM-F4 (Figure 7a) were the most attractive ones because they exhibited a hierarchical self-assembly behavior (Figure 7b−g) which was rarely achieved in former studies. It was found that single-molecule micelles appeared first, induced by their amphiphilicity. Since the shell of the micelles was
covered by carbohydrates at high densities, the strong associations between carbohydrates coming from CCIs caused the following steps: the alignment of the micelles into filaments, the adhesion of the micelles, the formation of nanowire I, the fusion of micelles into nanowire II, and the formation of nanowire III. These results revealed the significant influence of CCIs on controlling polymorphism in self-assembly processes.

IMMUNOLOGICAL APPLICATIONS OF GLYCOCALYX-MIMICKING SELF-ASSEMBLIES

In previous sections, we gave a detailed introduction of how we controlled the self-assembly process to achieve a library of different glyco-nanoparticles, which gave us the chance to explore the immunological functions of the assembled glyco-nanoparticles with polymorphism. Considering the fact that the surface of cells contains a layer of glycocalyx, which plays an indispensable role in cell–cell and cell–matrix interactions, our glyco-nanoparticles covered with a carbohydrate surface may mimic glycocalyx, thus providing a promising platform for studying carbohydrate–protein interactions and subsequent biological events. It was found that although our glyco-
nanomaterials were assembled from simple carbohydrate molecules with limited functionalities, they exhibit unprecedented properties contributed by polymorphism and the multivalent effect, beyond the functions of carbohydrates themselves.

To mimic glycocalyx, we managed to utilize phase separation to control carbohydrate distribution on glycocalyx-mimicking particles. The modification of crystalline polymers with high-density Man and Gal leads to different crystallization abilities of polymer backbones. Due to these properties, we synthesized a series of polymers with PCL backbones and carbohydrate pendants (i.e., P-CP-Man, P-CP-Gal, and P-CP-MG) (Figure 8a). Four glyco-nanoparticles with similar sizes and carbohydrate contents but different micelle architectures were obtained. CP-Man and CP-Gal were prepared directly from their corresponding polymers containing only one kind of carbohydrate. CP-M/G was prepared in the same way but contained both Man and Gal with a uniform distribution on the surface of the particles. CP-M/G was formed by blending P-CP-Man with P-CP-Gal, and it had the same Man/Gal ratio as CP-MG. Since the crystallization abilities of polymer chains were different in P-CP-Man and P-CP-Gal, phase separation

Figure 8. (a) Chemical structures of glycopolymers and the self-assembly of glyconanoparticles. (b) Illustration of the endocytosis of glyconanoparticles by macrophages: MG-type nanoparticles lead to faster internalization, and M/G-type nanoparticles lead to slower internalization. (c) Time dependence of the endocytosis of glyconanoparticles with RAW264.7 macrophages. (d) Cytokine (iNOS) release under equal amounts of endocytosis. Data are shown as the mean ± SEM of three independent experiments. Key: *p < 0.05 and **p < 0.01. Reprinted with permission from ref 44. Copyright 2017 American Chemical Society.
occurred in the shell architecture of the nanoparticles, leading to a nonuniform distribution of carbohydrates on the surface of the particles. Although these particles share the same shape and size, the polymorphism of shell architectures are significantly affected by high-density carbohydrates. It is well known that nanoparticles can be internalized by macrophages through different receptor-mediated pathways. The different distributions of carbohydrates on the surface of the above particles significantly affected the biofunctions of these nanostructures. When treating macrophages with these particles, via binding with galactose lectin (MGL, CD301) or a mannose receptor (MR, CD206) on the cellular surface, the receptor-dependent uptake of GNPs by macrophages was achieved. Results indicated that although CP-MG and CP-M/G have the same shape and size and the same ratio of two kinds of carbohydrates on their surfaces, they exhibited different internalization behavior (Figure 8b). CP-MG was internalized more effectively by RAW 264.7 macrophages (Figure 8c), which led to more significant inflammatory responses (Figure 8d) than for other particles because both Man and Gal on CP-MG can bind to the receptors on cell surfaces while only one kind of carbohydrate is effective in CP-M/G cases due to phase separation.

Besides the shell architecture of spherical GNPs, the polymorphism from the morphologies of GNPs also plays an important role in immunological research. We built nanoparticles covered with high-density mannose whose shapes can be controlled from spherical shapes to short cylinders and long cylinders. These GNPs were used in endocytosis experiments, and spherical particles exhibited a higher uptake efficiency than cylinders because spherical particles were taken up through both clathrin- and caveolin-mediated pathways while cylinders were taken up only through a clathrin-mediated pathway. On the other hand, we also observed that these GNPs induced different extents of inflammatory response in macrophages. In contrast to what was observed in endocytosis experiments, longer cylinders showed higher activity in inflammatory response than shorter cylinders and spheres. These findings convince us that the polymorphism of glyconanoparticles has a significant influence on immunological behavior, thus providing us with a new method for controlling GNPs-related immunotherapy.

Among these results, the most important phenomenon we observed during the immunotherapy studies using GNPs is that in both in vitro and in vivo cases, the internalization of glyco-nanoparticles resulted in the polarization of primary mouse peritoneal macrophages (Mφs) from an immunosuppressive phenotype (M2) to an inflammatory type (M1) (Figure 9a). This was the first time that synthetic nano-assemblies were reported to achieve such functions. Thanks to these amazing features, we can use glyco-nanoparticles to improve the immune checkpoint blockade in cancer immunotherapy, which was often inhibited by tumor-associated macrophages (TAMs). To achieve this, GNP were internalized by TAMs through a lectin receptor-mediated pathway leading to the reversion of TAMs, which was controlled by STAT6 and NF-κB signaling pathways. As a result of TAM reversion, the expression of pro-inflammatory IL-12 increased while that of immunosuppressive IL-10, Arg1, and CCL22 decreased, which reversed the microenvironment in tumor tissues (Figure 9a). Since TAMs shows extremely strong inhibition toward T cells in antitumor immunity, the reversion of TAMs also exhibited a significant promotion to T cells in both proliferation and activation. The same results were also observed in vivo when we injected GNPs into tumors in LLC tumor xenograft mice (Figure 9b). The combined use of αPD-L1 and GNPs exhibited a remarkable improvement in the immune checkpoint blockade in immunotherapy, in which around 70% of the tumor growth was suppressed compared to that in the control group.

**OUTLOOK**

This account summarizes our contributions to controlling the polymorphism of self-assembled proteins and carbohydrate-containing polymers. In the Carbohydrate–Protein Interactions as the Self-Assembly Driving Forces section, we proposed and developed a new strategy called the inducing ligand strategy. Furthermore, we expect this strategy to be even more widely adopted than we previously demonstrated because the dual noncovalent interactions from the inducing ligand could be extended to other rigid ligands of proteins and other supramolecular interactions. With complex protein structures and the aid of computer design, we should expect that much more complex nano- and microstructures can be designed and achieved. These types of structures might be very attractive in different research aspects: They could demonstrate new functions at the cellular level and even in vivo. For example, with the combination of fused proteins and sequence mutation, functional moieties including enzymes for cascade reactions or fluorescent labels can be integrated to the assemblies with...
possible enhanced functionalities. Moreover, the obtained structures with precise protein packing and helices are feasible templates for the preparation of various hybrid materials with astonishing properties.

In the Control of the Polymorphism of Glycopolymer Assemblies by Chemical Reactions and Supramolecular Interactions section, we focused on dealing with reactions and interactions on carbohydrates to control the polymorphism of glyco-assemblies. From our current immunological studies, one may find that the self-assembled glyco-structures demonstrated valuable properties that were not found in their carbohydrate precursors. Considering the development in macromolecular self-assembly, introducing other widely used self-assembly approaches such as PISA (polymerization-induced self-assembly), CDSA (crystallization-driven self-assembly) into our research may lead to better control of the self-assembly process. The research on DISA can also be expanded by absorbing new results of other researches such as using chemoenzymatic methods in order to control the self-assembly morphologies by the saccharide structures. By introducing natural glycans into glyco-assemblies, we may expect not only new self-assembled materials but also new applications in biology. These materials may even help us to understand the natural carbohydrate moieties better. Utilizing glycopolymer assemblies to design nanodrugs and monitoring the dynamic processes mediated by carbohydrate interactions in vitro and in vivo can also be an important issue.

In short, by using proteins and carbohydrates as our building blocks, our new self-assembly approaches opened a new avenue not only for controlling the structure of polymorphism but also for biological functions. The combination of supramolecular chemistry and glyco-science provides us a new route to highly ordered carbohydrate and/or protein structures with controllable patterns and functions, which deserves attention from more and more scientists.

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■ ACKNOWLEDGMENTS

G.C. thanks NSFC/China (No. 51721002, 21861132012, 91956127 and 21975047) for financial support. The authors thank Prof. Ming Jiang and Dr. Kongchang Wei for helpful discussions.

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