Macromolecular Engineering by Surface-Initiated ATRP: New Nanomaterials for Bioapplications

Jiang, Lingdong

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Macromolecular Engineering by Surface-Initiated ATRP:

New Nanomaterials for Bioapplications

Lingdong Jiang

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DOCTORAL DISSERTATION
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Faculty opponent

Prof. Cameron Alexander, Division of Molecular Therapeutics and Formulation, Faculty of Science, University of Nottingham, UK.

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Title and subtitle

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SI-ATRP is an excellent controlled radical polymerization (CRP) method for the synthesis of polymer nanohybrid by growing polymer brushes (chains) from an interface, which allows precise control over polymer composition, topology, and functionality. Polymer brushes have proven to be attractive platforms for applications spanning drug delivery, tissue engineering, biosensors as well as bioseparation.

Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) click reaction has been widely applied for the design, fabrication and post-polymerization modification of polymer nanohybrid due to some important features: high reaction yields, benign reaction conditions, and tolerance to diverse functional groups.

One aim of this thesis is to grow polymer brushes from inorganic nanoparticles via SI-ATRP in organic solvent followed by post-polymerization functionalization of the polymer brushes via click reaction for different applications. Specifically, thermo-responsive polymer brushes composed of poly(N-isopropylacrylamide) (pNIPAm) and poly(glycidyl methacrylate) (pGMA) were grafted from silica nanoparticles via SI-ATRP. A high amount of boronic acid ligands and iminodiacetate (IDA) ligands were introduced into the polymer brushes through the high-efficiency click reaction for the enrichment of glycoproteins and histidine-tagged proteins, respectively. The polymer nanohybrids were characterized to determine the particle size, morphology, organic content, densities of polymer chains and the affinity ligands via techniques including dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FT-IR), elemental analysis, thermogravimetric analysis (TGA), and gel permission chromatography (GPC). The nanocomposites showed high adsorption capacity and selectivity towards the target proteins due to the dense ligands immobilized on the long and flexible polymer brushes that are able to provide rapid protein transport to binding sites. The synthetic approaches developed in this thesis have a great potential for the development of more efficient adsorbents for biological samples.

Another focus of this thesis is to investigate the possibility of growing polymer chains from biological interface via SI-ATRP in an aqueous solvent. Specifically, ATRP initiators were first selectively immobilized on amelogenin (AMEL), which is a pH-responsive protein, followed by growing thermo-responsive pNIPAm chains in an aqueous solution via SI-ATRP, leading to a pH and temperature dually responsive bioconjugate. The bioconjugate was characterized in terms of particle size, molecular weight of polymer, and self-assembly behavior in response to pH and temperature. The bioconjugates may serve as a promising platform for bioapplications such as drug delivery and biosensing.

Key words: Nanohybrid, Polymer brush, ATRP, Bioseparation, Bioconjugate

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Lingdong Jiang

LUND
UNIVERSITY

Division of Pure and Applied Biochemistry
Department of Chemistry
Lund University

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To my family

Contents

Popular summary

Polymer nanohybrid materials have attracted ever-increasing attention in the fields of materials science, biomedical engineering and bioseparation due to its unique structural characteristics and distinct properties provided by the individual components.

Protein purification is of great importance in producing proteins for therapeutic or research purposes. The selective isolation of a target protein from a complex biological sample is a challenging task. Until now, various types of materials including molecularly imprinted polymers, core-shell magnetic beads, monolithic macroporous gels, and lectin/antibody-based molecular tools have been utilized for the enrichment of specific proteins. However, these protein adsorbents have some drawbacks such as low binding capacities, severe steric constraint due to rigid structures, high cost and poor stability. Researchers have been exploring new methods and materials that can enrich and recover proteins efficiently and facilely.

In recent years, polymer nanohybrids with well-defined structure have emerged as novel protein adsorbents due to their unique structures. The controlled radical polymerization (CRP) technique has provided a powerful avenue towards engineering the structure and properties of synthetic polymers. Atom transfer radical polymerization (ATRP) is among the most efficient and robust CRP processes for the synthesis of polymer nanohybrid, which allows precise control over the polymer in terms of chemical composition, topology and functionality.

Glycoproteins play a vital role in numerous biological activities including molecular recognition, protein folding, signal transduction, immune response, and so on. Lectins and antibodies as molecular tools for selective enrichment of glycoprotein are often associated with high cost and poor stability. Hydrazide chemistry, another separation method, is generally time-consuming and involves side reactions. As an alternative method for glycoprotein separation, boronate affinity materials have attracted great interests in recent years. Boronic acid can react with the cis-diol moiety of glycoproteins to form boronate ester bonds under basic conditions. The bound proteins can be eluted under acidic conditions via the hydrolysis of boronate ester bonds. This unique property makes boronic acid an ideal affinity ligand for glycoprotein separation.

Using recombinant technology, various kinds of proteins can be expressed in large quantities, and a wide range of affinity tags can be easily introduced into the proteins of interest. Immobilized metal ion affinity chromatography (IMAC) is an effective approach for selective capture of recombinant proteins containing engineered affinity tags. Due to its low immunogenicity, small size, and general applicability, histidine tag (His-tag) has been the most widely used affinity tag that can reversibly coordinate with transition metal ions via chelating agents such as iminodiacetate (IDA) and nitrilotriacetate (NTA), allowing the bound proteins to be recovered. Nowadays, many kinds of IMAC adsorbents are available in the market for His-tagged protein separation. Most of these are magnetic beads that have limited binding capacity.

In this thesis, polymer nanohybrids with well-defined structure were prepared via the ATRP technique, which could be converted into different affinity adsorbents via further modification. Specifically, starting from the initiator-functionalized silica nanoparticles, poly(N-isopropylacrylamide) (pNIPAm) and poly(glycidyl methacrylate) (pGMA) brushes were grown from silica surfaces. After opening the epoxide ring on PGMA brushes, clickable boronic acid ligands or clickable IDA-Cu ligands were introduced into the polymer brushes via the click reaction. The resulted nanohybrids with boronate affinity and metal affinity were applied for glycoprotein and His-tagged protein separation, respectively. The experimental results demonstrated that the novel polymer nanohybrids could efficiently and specifically isolate and enrich target proteins from complex samples. For the purpose of protein separation, the polymer nanohybrids are superior to other types of adsorbents. The long and flexible polymer brushes could provide local proximity and multiple affinity ligands for protein binding, facilitating stable immobilization. Moreover, the swollen polymer brushes could decrease steric hindrance, providing sufficient accessible space for protein binding, thus leading to higher binding capacities.

In addition to growing polymer brushes from inorganic substrate for protein separation, this thesis also tried to explore the possibility of growing polymer chains from protein molecules. From an engineering standpoint, conjugation of a synthetic polymer to a protein is exciting because it allows the merging of the properties of both synthetic polymers and naturally occurring proteins, which could lead to the emergence of a new family of biohybrid materials. Many kinds of proteins including bovine serum albumin, ovalbumin, chymotrypsin, streptavidin, and green fluorescent protein have been reported to prepare protein-polymer bioconjugate for applications ranging from drug delivery, biosensing and biocatalysis. In this thesis, amelogenin, the major protein component of enamel matrix, was utilized to prepare bioconjugate via ATRP in an aqueous solution. The experimental results indicated that the bioconjugate could self-assemble into uniform and stable nanoparticles with an extremely narrow size

distribution, and the nanoassembly process could be regulated by simply varying the pH and temperature. The bioconjugates may serve as a promising platform for advanced drug delivery and improved bioseparation purposes.

Abstract

The objective of this thesis is to investigate the synthesis of well-defined polymer nanohybrid materials bearing desirable functionality via surface-initiated atom transfer radical polymerization (SI-ATRP) for potential bioapplications.

SI-ATRP is an excellent controlled radical polymerization (CRP) method for the synthesis of polymer nanohybrid by growing polymer brushes (chains) from an interface, which allows precise control over polymer composition, topology, and functionality. Polymer brushes have proven to be attractive platforms for applications spanning drug delivery, tissue engineering, biosensors as well as bioseparation.

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Abbreviations

AIBN	Azobisisobutyronitrile
AMEL	Amelogenin
APTES	(3-aminopropyl)-triethoxysilane
ARS	Alizarin Red S
BIBB	2-bromoisobutyrylbromide
BSA	Bovine serum albumin
CRP	Controlled/living radical polymerization
DLS	Dynamic light scattering
DMF	N,N-dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GMA	Glycidyl methacrylate
GPC	Gel permeation chromatography
His-LDH	Histidine-tagged lactate dehydrogenase
His-Hb	Histidine-tagged hemoglobin
HRP	Horseradish peroxidase
Me_6TREN	tris(2-dimethylaminoethyl)amine
NIPAm	N-isopropylacrylamide
OVA	Ovalbumin
PMDETA	N, N, N′, N″, N″-pentamethyldiethylenetriamine
RAFT	Reversible addition-fragmentation chain transfer polymerization
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEA	Triethylamine
TEM	Transmission electron microscopy
TEOS	Tetraethylorthosilicate
TGA	Thermal gravimetric analysis

List of papers

Paper I. **Lingdong Jiang**, Héctor Bagán, Tripta Kamra, Tongchang Zhou and Lei Ye, *Nanohybrid Polymer Brushes on Silica for Bioseparation.* J. Mater. Chem. B, 2016, 4(19), 3247-3256.

Paper II. **Lingdong Jiang**, Maria E. Messing and Lei Ye. *Temperature and pH Dual-Responsive Core-Brush Nanocomposite for Enrichment of Glycoproteins*. ACS Appl. Mater. Interfaces, 2017, 9(10), 8985–8995.

Paper III. **Lingdong Jiang** and Lei Ye. *Temperature Responsive Polymer Brushes for Affinity Separation of Histidine-tagged Recombinant Proteins.* (Submitted to ACS Appl. Mater. Interfaces)

Paper IV. **Lingdong Jiang**, Johan Bonde and Lei Ye. *Temperature and pH Controlled Self-assembly of a Protein-Polymer Biohybrid.* Macromol. Chem. Phys., 2018, 1700597.

Paper not included in this thesis

Paper I. Huiting Ma, **Lingdong Jiang**, Solmaz Hajizadeh, Haiyue Gong, Bin Lu and Lei Ye. *Nanoparticle Supported Polymer Brushes for Temperature Regulated Glycoprotein Separation.* J. Mater. Chem. B, 2018, 6, 3770-3781.

Paper II. Qianjin Li, **Lingdong Jiang**, Tripta Kamra and Lei Ye. *Synthesis of Fluorescent Molecularly Imprinted Nanoparticles for Turn-on Fluorescence Assay Using One-Pot Synthetic Method and a Preliminary Microfluidic Approach.* Polymer, 2018, 138, 352-358.

Paper III. Héctor Bagán, Kamra Tripta, **Lingdong Jiang** and Lei Ye. *Thermoresponsive Polymer Brushes on Organic Microspheres for Biomolecular Separation and Immobilization.* Macromol. Chem. Phys., 2017, 218, 1600432.

My contribution to the papers

Paper I.

I performed the majority of the experiments and most of the experimental data analysis. I contributed to the writing of the manuscript.

Paper II.

I designed the experiments with the help of co-authors, performed most of the experiments and most of the experimental analysis, and drafted the manuscript.

Paper III.

I designed the experiments independently, performed most of the experiments and all the experimental data analysis, and drafted the manuscript.

Paper IV.

I designed the experiments with the help of co-authors, performed all the experiments except the GPC measurement and all the experimental data analysis, and drafted the manuscript.

Chapter 1 Introduction

1.1 Polymer nanohybrid

Nanohybrid materials have gained considerable attention as a new paradigm of materials with the development of nanotechnology. Nanohybrid materials can be broadly defined as synthetic materials that incorporate inorganic, organic, or even bioactive components in a single material. The unlimited possible combinations of different components with distinct properties provide great opportunities to generate multifunctional platforms for highly sophisticated and promising bioapplications in fields as diverse as coatings and adhesives, electronics, biosensing, biomedicine, bioseparation, environment, and countless others [1-8].

Synthesis of organic/inorganic hybrid materials is among the most rapidly developing fields of materials science, and the development has largely been facilitated by controlled radical polymerization (CRP). Properties of this type of hybrid materials can synergistically combine the best of inorganic and organic constituents. Atom transfer radical polymerization (ATRP) is one of the most popular CRP methods and has been an indispensable approach for the controlled synthesis of polymer hybrid with desired composition, complex molecular architecture, and diverse functionalities.

Polymer brushes grown from surfaces can have excellent self-lubricating and tribological properties, which have very good antifouling and antimicrobial properties [9]. Additionally, surface properties such as biocompatibility, hydrophobicity/hydrophilicity, adhesion, adsorption, and corrosion resistance can also be tailored. Polymer brushes grown from nanoparticles can improve their dispersibility and colloidal stability, or enhance the mechanical properties while retaining other properties (magnetic, optical, luminescent, etc.).

In recent times, polymer brushes with active binding sites have proved to be appealing for protein immobilization due to the flexible structure and low steric constraint, which allow rapid protein transport to the binding sites. Polymer brushes functionalized solid particles can be applied as the stationary phase of chromatography columns for protein separation [10]. For example, polymer brushes with carboxylic acid groups and epoxide groups can bind proteins by reacting with amino groups via active ester chemistry and nucleophilic ring-

opening reactions, respectively. However, these immobilization methods lack selectivity. One solution to this problem is the functionalization of polymer brushes with affinity ligands that can specifically bind target proteins.

Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) is well known as the click reaction, and has some important features: high reaction yields, benign reaction conditions and tolerance to diverse functional groups [11-12]. Due to the convenient and effective coupling of complementary functional groups, click reactions have been widely utilized for the design, fabrication, and modification of polymer brushes. In this thesis, taking advantage of ATRP and high-efficiency click reaction, polymer brushes with boronate affinity and metal affinity were prepared for the selective binding of target proteins.

Another very rapidly expanding area for polymeric materials prepared by ATRP includes various bioconjugates with biomolecules such as proteins, nucleic acids, and carbohydrates [13-14]. Synthetic polymers can conjugate with proteins to form protein-polymer bioconjugates, which enable to merge the attractive physical and chemical features of polymers with the biological activity of proteins. For example, the modification of protein with polyethylene glycol can improve the solubility, stability, and circulation lifetime of a protein and decrease its immune response. Many therapeutic proteins tend to denature and aggregate with extended storage time and exhibit poor pharmacokinetics when used without modifications. ATRP is compatible with aqueous media and allows to grow polymers directly from proteinaceous macroinitiators [15], offering many advantages including great chemical flexibility and simple purification process based on the distinct molecular weight differences between unreacted monomers and products.

1.2 Aim and scope of the thesis

Macromolecular engineering via controlled radical polymerizations (CRPs) offers dramatic opportunities to prepare novel functional materials for a wide range of applications. This thesis aims to prepare novel polymeric materials for bioapplications via surface-initiated atom transfer radical polymerization (SI-ATRP), focusing on the design, synthesis, characterization and application of well-defined inorganic/organic and biological/organic hybrid materials.

In Paper I, II and III, we developed a modular approach for the preparation of silica supported polymer brushes that were able to be converted into boronate affinity and metal affinity adsorbents by introducing affinity ligands into the polymer brushes via a high-efficiency click reaction.

In Paper IV, we investigated the possibility of growing thermo-responsive polymer chains from a pH-responsive protein (amelogein) via ATRP in an aqueous solution. TEM was utilized to characterize the morphology of the bioconjugate and DLS was utilized to investigate the self-assembly behavior of the bioconjugate under different conditions.

Chapter 2 Polymer brushes

Polymer brushes are dense polymer chains that are packed close enough on substrate surfaces [16-17]. As shown in Figure 1, when the distance between the neighbouring grafting points is smaller than the radius of gyration, due to the steric confinement, polymer chains tend to stretch away and align themselves along the perpendicular direction of the tethered surfaces, leading to a "brush" conformation. In another situation, if the polymer chains are sparsely grafted such that the distance between the neighbouring grafting points is greater than the radius of gyration, polymer chains adopt a relaxed "mushroom" or "pancake" conformation [18].

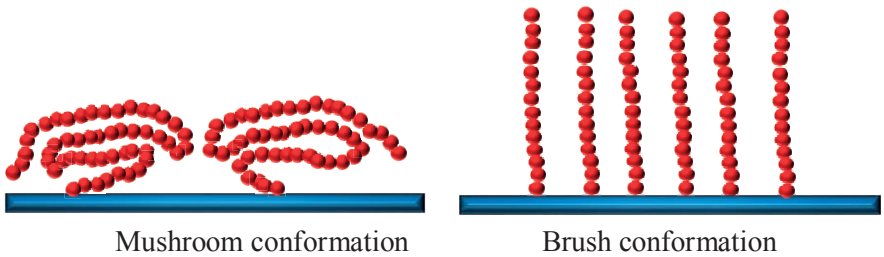

Figure 1. Schematic illustration of the conformation of grafted polymer chains.

2.1 Methods to prepare polymer brushes

There are two main strategies to tether polymer chains to a substrate surface: the "grafting to" and "grafting from" approaches [19-20].

The "grafting to" approach involves the attachment of premade polymer chains to an appropriately functionalized substrate surface [21-24]. The advantage of this approach is that the prefabricated free polymer chains allow for a thorough characterization and the properties can be easily tailored. However, with this approach the achievable grafting density is limited due to the concentration gradient built up by the already-grafted polymer chains. It becomes difficult for the remaining chains to diffuse and reach the reactive sites on the surface.

The "grafting from" approach is a more promising method for the synthesis of

polymer brushes [25-26]. With this approach, small initiator molecules can be immobilized on the surface with a high density, as the addition of monomers to the growing chain ends is not strongly hindered by the already-grafted polymer chains. Therefore, compared with the "grafting to" approach, the "grafting from" approach offers clear benefits in terms of lower steric hindrance and higher conjugation efficiency.

2.2 Atom transfer radical polymerization (ATRP)

As a versatile controlled radical polymerization (CRP), atom transfer radical polymerization (ATRP) [27-28] was independently discovered by Matyjaszewski [29-30] and Sawamoto in 1995 [31]. ATRP allows the controlled synthesis of polymers with predetermined molecular weight, low polydispersity, and complex topology (e.g., rings, bottlebrushes, stars, and combs or networks). The composition of produced polymers can change statistically, periodically, or gradiently. Various functionalities can be precisely incorporated into polymers to provide targeted properties.

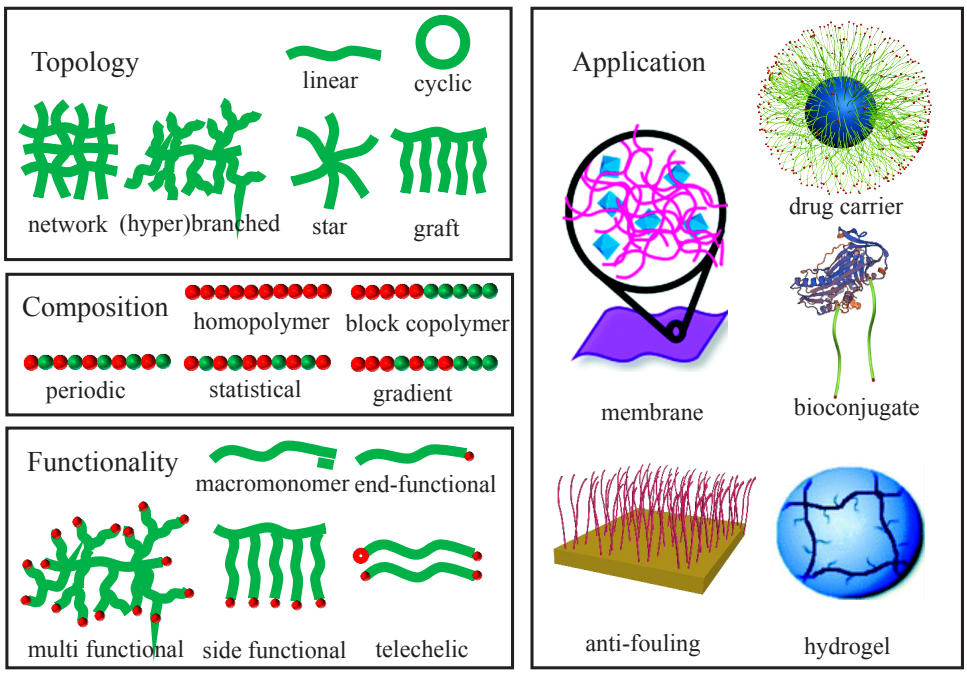

Figure 2. Controlled macromolecular topology, composition, functionality and potential applications of polymer materials prepared by ATRP. Adapted with permission from [18].

An ATRP system is composed of monomers, initiators and catalysts that consist of a transition metal and a suitable ligand. The fact that most of the standard ATRP catalyst and initiators are commercially available also makes ATRP an attractive controlled polymerization technique. Having the capacity to stop and restart the polymerization is also advantageous for preparing complex architectures.

ATRP also presents disadvantages. For example, the reaction requires stringent inert conditions, and the purity of reagents is extremely important. However, the advantages far outweigh the disadvantages. The ability to precisely control the molecular weight and sequence of polymer with a narrow polydispersity is highly desirable.

2.2.1 Monomers

ATRP is compatible with many vinyl monomers including (meth)acrylates, styrenes and (meth)acrylamides, which contain an electron withdrawing substituent adjacent to the vinyl group. Figure 3 provides some examples of monomers that can be polymerized via ATRP.

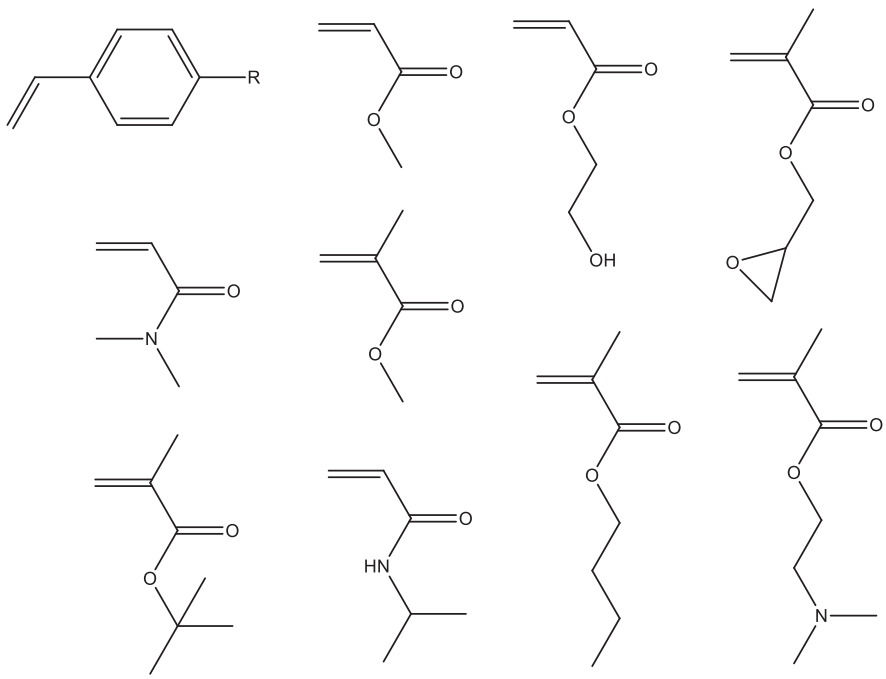

Figure 3. Examples of monomers that can be polymerized via ATRP. Adapted with permission from [32].

2.2.2 Initiators

ATRP initiator is typically an alkyl halide (RX) [33]. Tertiary alkyl halides have been shown to be better initiators than secondary ones, which are better than primary ones. One simple rule to follow is that the R-group in the alkyl halide should be similar in structure to that of the monomer. For example, (1-bromoethyl)benzene is usually used for polymerizing styrene [34], ethyl 2-bromoisobutyrate, and ethyl 2-bromopropionate for meth(acrylates) [35-36] and 2-bromopropionitrile for acrylonitiles[37]. Figure 4 provides some examples of initiators for ATRP.

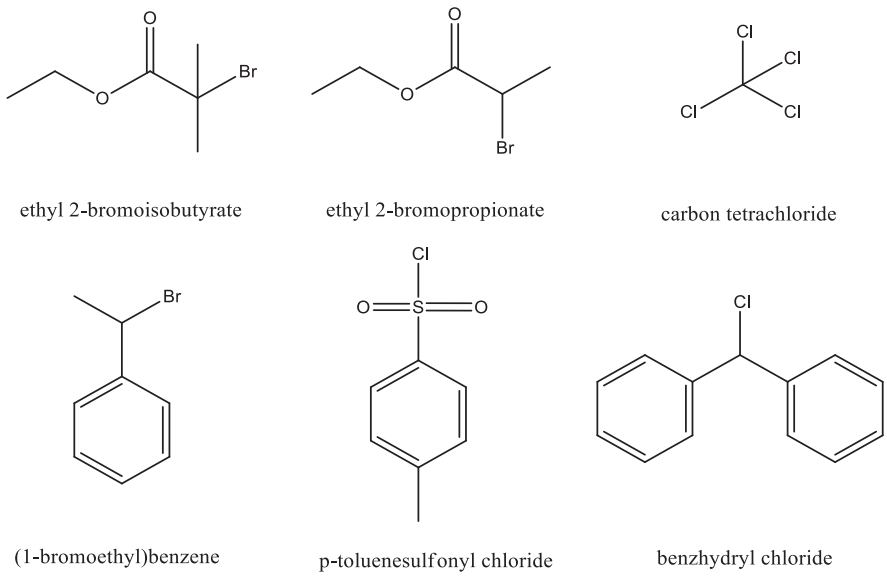

Figure 4. Examples of initiators for ATRP. Adapted with permission from [32].

2.2.3 Transition metals

The transition metals used in the ATRP reaction should be able to readily have access to two oxidation states separated by one electron and have a reasonable affinity towards a halogen while having a low affinity for other atoms such as hydrogen atoms and alkyl radicals. The initial work by Sawamoto et al. utilized a ruthenium (II)-based catalyst [31], whereas Matyjaszewski et al. utilized copper (I) as the superior metal for catalysts due to its versatility and low cost [29-30, 38]. Other less common metals such as iron [39], nickel [40], molybdenum [41], rhenium [42], and palladium [43] have also been used for polymerizing various monomers.

2.2.4 Ligand

The function of ligands is to solubilize the metal in the reaction media and affect the redox chemistry of the final metal complex. Various ligands have been applied to form metal complexes with different transition metals. Copper is usually ligated with nitrogen-based ligands. As shown in Figure 5, bidentate 2,2'-bipyridine (Bipy) and 4,4'-di-5-nonyl-2,2'-bipyridine (dNbpy) [44-46], tridentate pentamethyldiethylenetriamine (PMDETA) [47], tetradentate 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA), tris(2-pyridylmethyl)amine (TPMA) ligands, and tris(2-(dimethylamino)ethyl)amine (Me_6TREN) [48-50] have successfully been applied in copper-based ATRP. The ATRP catalytic activity of copper (I) complexes decrease in the order Me_6TREN > TPMA> PMDETA > dNbpy >HMTETA > Bipy [51].

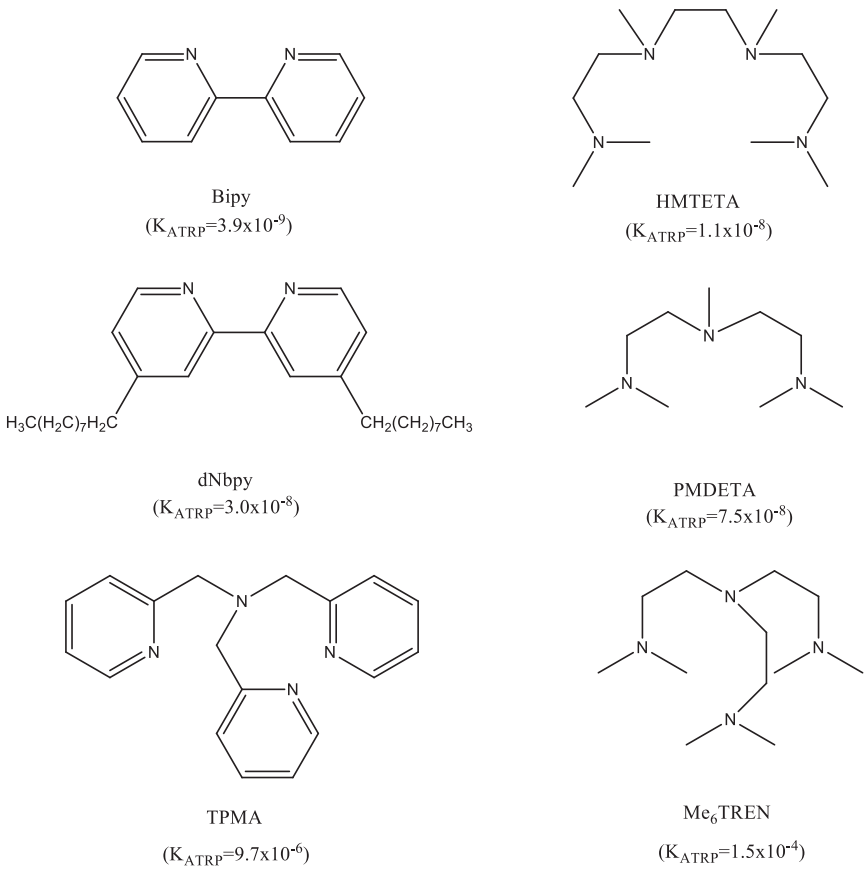

Figure 5. Examples of nitrogen-based ligands and their effect on value of K_{ATRP} for the reaction between ethyl 2-bromoisobutyrate and Cu(I) complexes in acetonitrile at 22 °C. Adapted with permission from [51].

2.2.5 Kinetics and mechanism

ATRP relies on the dynamic equilibrium (characterized by $K_{ATRP} = k_a/k_d$) between a large majority of dormant (halogen-capped) chains and a very low concentration of active free radicals (Figure 6) [52], which includes several processes. Initiation involves the generation of radicals and the addition of those radicals to a monomer unit, yielding the first propagating species. Propagation is the repeated addition of the polymeric radical species to the monomer molecules. Each propagation reaction results in a polymer chain that is one repeat unit longer and is also a radical. Termination of ATRP leads to the build-up of deactivator, which reduces the concentration of radicals until a steady state is reached. This "self-adjustment" process during the initial stages of the polymerization is called "persistent radical effect" [53].

In a steady state, a transition metal (Z) complex (the activator, e.g., Cu(I)X/L) abstracts a halogen atom (X) from an alkyl halide initiator (R-X) to generate a metal (Z+1) complex (the deactivator, e.g., $Cu(II)X_2/L$) and an initiator radical ($R^{\bullet}$) that can subsequently react with a monomer to start a propagating chain. This chain can then be deactivated by the metal (Z+1) complex transferring the abstracted halogen back to the polymeric radical $P_n^{\bullet}$ (n is the degree of polymerization, DP), forming a dormant chain (P_n-X) and reforming the initial metal (Z) species that can then re-initiate and so on.

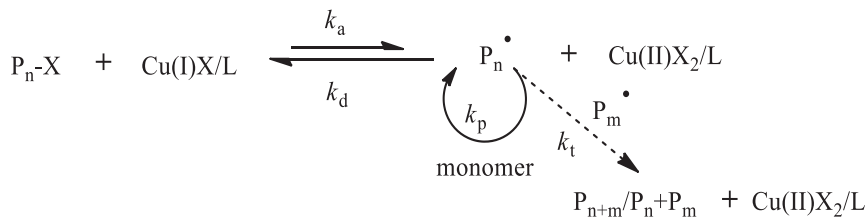

Figure 6. Mechanism of copper-mediated ATRP where k_a, k_d, k_p and k_t are the rate constants for activation, deactivation, propagation and termination respectively.

The degree of polymerization of the produced polymer is determined by the initial concentration ratio of monomer to initiator, $[M]_0/[RX]_0$, and the monomer conversion. The molecular weight distribution ($Đ = M_w/M_n$, where, M_w is the weight-average MW and M_n is the number-average MW) of formed polymers is calculated according to equation 1 [54].

$$Đ = 1 + \frac{1}{DP} + \left(\frac{k_p[RX]_0}{k_d[Cu(II)]_0}\right)\left(\frac{2}{Y} - 1\right) + \left(\frac{k_t k_a[C]_0}{4k_p k_d[Cu(II)]_0}\right)Y, \quad [Cu(II)]_0 \neq 0 \qquad (1)$$

Where, DP is the degree of polymerization; k_p, k_a and k_d are the rate constants of propagation, activation, and deactivation, respectively; $[RX]_0$ is the initiator concentration; $[Cu(II)]_0$ is the deactivator concentration; $[C]_0$ is the catalyst concentration; Y is the monomer conversion.

Control in ATRP depends not only on K_{ATRP} but also on the rate constants of activation and deactivation. Activation and deactivation occur throughout the polymerization process. They determine how many monomer units are added during each intermittent activation step and directly affect the dispersity of the obtained polymers. In order to achieve good control in the ATRP process, (1) initiation of chains must be fast and almost at the same time; (2) termination events must be minimal; (3) exchange between dormant and active species must be fast relative to propagation; (4) the equilibrium between active and dormant species must be greatly shifted towards the side of the dormant species (i.e. $k_a \ll k_d$) in order to ensure that only a small number of monomer units are added to the propagating radicals during each period of activity.

The rate law for ATRP can be described by equation (2) when neglecting the termination step and using a fast equilibrium approximation:

$$R_P = k_P[P_n\cdot][M] = k_P \frac{k_a}{k_d}[P_nX][M]\frac{[Cu(I)]}{[Cu(II)]} \qquad (2)$$

Where, R_p is the rate of polymerization; k_p is the rate constant of propagation; $[P_n^{\bullet}]$ is the concentration of active growing chain radicals; [M] is the monomer concentration; k_a and k_d are the rate constants of activation and deactivation, respectively; $[P_nX]$ is the concentration of dormant species; [Cu(I)] and [Cu(II)] are the concentrations of Cu(I) and Cu(II) catalyst, respectively.

Each monomer has its own unique propagation rate constant and atom transfer equilibrium constant for its active and dormant species. It can be known from equation (2) that the rate of ATRP in solution depends on the components in the ATRP system. It is proportional to the [M], $[P_nX]$, and [Cu(I)] and inversely proportional to [Cu(II)].

2.3 Surface-initiated ATRP

Surface-initiated ATRP has been exploited for the controlled synthesis of polymer brushes from various types of substrates such as silica, iron oxide, gold, and quantum dots [55-58]. It is conducted in the same way as solution ATRP except that the ATRP initiators are immobilized on surfaces that can be concave, convex, or flat. The initiator functionalized substrate is immersed in a solution of

monomer, catalyst, and ligand to initiate polymer growth from the surface.

As the concentration of the surface-bound initiator is very low, which has some effect on surface-initiated ATRP. In solution ATRP, the concentration of the initiator is relatively high, and the termination in the early stage can produce enough deactivators to ensure that the growing chain radicals are efficiently deactivated to the dormant state, giving a controlled polymerization. For surface-initiated ATRP, the concentration of the surface-bound initiator is too low for this mechanism to operate efficiently.

Two main strategies have been developed to solve this problem. One strategy is the addition of free initiators to the ATRP solution [59], which facilitates the formation of deactivators through dormant chain activation and termination reactions in solution. This makes the deactivation of chain radicals on the surface more efficient and leads to better-controlled polymer brush. The free polymer generated in the solution can be characterized by gel permeation chromatography (GPC), giving an indirect measurement of the molecular weight and polydispersity of polymer grafted on the surface. However, this strategy has a disadvantage: the achievable thickness of the polymer on surface is limited, as most of the monomers are consumed by the polymerization initiated in solution.

The other strategy is the addition of deactivator salts to the ATRP solution at the beginning of the reaction. Matyjaszewski et al. first reported the use of deactivator salts ($CuBr_2$) in SI-ATRP to grow polystyrene brushes from initiator functionalized silicon wafer [60]. These added deactivator salts provide a very efficient deactivation of the growing chain radicals on the surface, giving a good control over the polymerization process.

2.4 ATRP in an aqueous system

ATRP has been successfully carried out in a broad range of solvents, including common organic solvents, supercritical CO_2 [61], ionic liquids [62], poly (ethylene glycol) [63], and water [64]. The solvents can interact with the catalytically activator and deactivator, which can have dramatic consequences on the polymerization rate (owing to the effects on the value of K_{ATRP}) and the polymerization control.

Water is the ideal medium for biologically-oriented applications. The need for sustainable and environmentally friendly chemical processes is the driving force behind the development of ATRP in an aqueous media. Jones et al. successfully initiated the polymerization of methyl methacrylate, glycidyl methacrylate, and hydroxyethyl methacrylate from gold surfaces in a water/methanol solvent

mixture with CuBr/Bipy as the catalyst system at room temperature [65].

There are some challenges in conducting ATRP in an aqueous media (Figure 7). One challenge in aqueous systems is the high ATRP equilibrium constant. The polymerization rates increase significantly due to the high dielectric constant of water, which gives high radical concentrations and many "dead" chains, thus leading to a poor control over molecular weight and molecular weight distributions [66]. Additionally, there is partial dissociation of $Cu(II)X_2/L$ deactivator to a free halide anion and $[Cu(II)X/L]^+$ species, which is not able to transfer a halide to a propagating polymer chain [67]. Moreover, certain ligands cause the Cu(I) species to be disproportionated [68]. Furthermore, the carbon–halogen bond can hydrolyze, leading to the loss of chain-end functionality [69].

All of these processes have an effect on the ATRP equilibrium, often resulting in a fast polymerization rate, altered activator and deactivator concentrations, and "dead" chains.

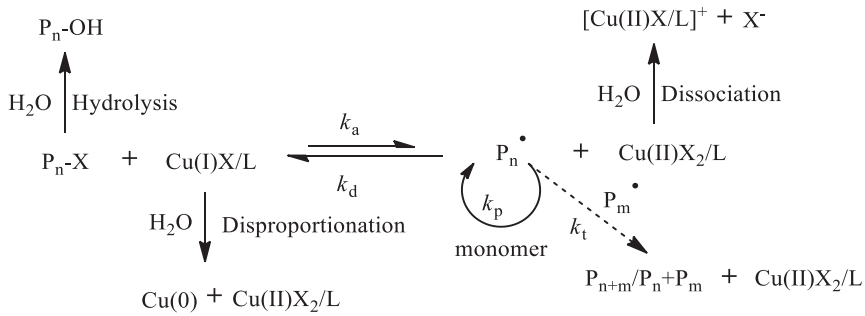

Figure 7. Mechanism of ATRP with potential side reaction and equilibria in aqueous media.

2.5 Improved atom transfer radical polymerization

Conventional ATRP is carried out using a relatively large amount of transition metal catalysts, typically in the range of 0.1-1 mol % versus monomer, in order to compensate for the unavoidable radical termination via biradical coupling/disproportionation [70]. High concentration of catalysts can be disadvantages for electronic and biologically orientated applications, and limit the widespread use of this technique in industrial scale due to the added cost of purification, potential toxicity, and discoloration of products.

In recent times, many approaches have been developed to reduce the amount of catalysts to a few parts per million (ppm), which employ external chemical or physical stimuli to regenerate the active species (Figure 8). These improved

approaches include initiators for continuous activator regeneration (ICAR) ATRP, activators regenerated by electron transfer (ARGET) ATRP, supplemental activators and reducing agents (SARA) ATRP, electrochemical ATRP (eATRP), and photoinduced ATRP. The dramatically reduced level of catalyst used in these approaches could significantly simplify postpolymerization purification of the final products.

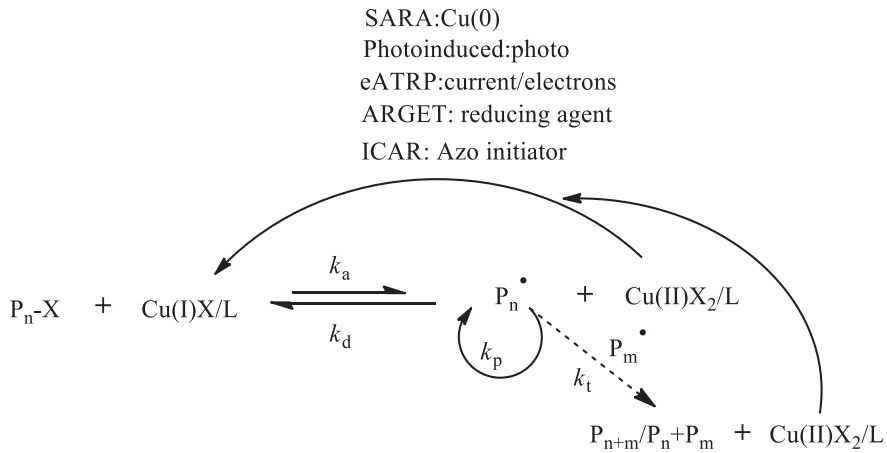

Figure 8. Approaches of activator regenerated used in ATRP.

2.5.1 ICAR ATRP

Initiators for continuous activator regeneration (ICAR) ATRP uses a small amount of conventional radical initiator (e.g., AIBN) to regenerate activators. Compared to conventional ATRP, the continuous regeneration of activator from deactivator facilitated by the radical initiator allows for much lower catalysts to be used [49].

Lamson et al. prepared polyacrylonitrile (PAN) via ICAR ATRP [71]. The authors investigated the effect of a few parameters on the polymerization process including ligand and initiator type, the amount of catalyst and the targeted degree of polymerization. It was reported that 50 ppm of $CuBr_2$/TPMA as the catalyst and 2-bromopropionitrile (BPN) as the initiator resulted in PAN with the narrowest molecular weight distribution ($M_w/M_n = 1.11–1.21$). Even lowering the catalyst to 10 ppm, excellent control ($M_w/M_n < 1.30$) was maintained with 56% conversion in 10 h.

With this approach, Konkolewicz et al. successfully prepared polymers of oligo(ethylene oxide) methyl ether acrylate in aqueous media by using 20-100 ppm of CuBr/TPMA catalyst in the presence of excess bromide anions [72]. The authors found that the key to controlling the polymerization was the addition of

a bromide salt. The bromide ion was able to promote the formation of the deactivators, leading to the low dispersity of the products. Therefore, ICAR ATRP is promising for controlled polymerization in aqueous media. However, the addition of free-radical initiator limits its applicability to the synthesis of high-order macromolecular architectures. Scaling-up ICAR ATRP may be challenging due to the large amounts of free radical initiator needed, which may quickly decompose and lead to fast and exothermic polymerization if the temperature is not controlled precisely.

2.5.2 ARGET ATRP

Activators regenerated by electron transfer (ARGET) ATRP was first developed by Matyjaszewski's group [73-74]. This provided a continuous controlled polymerization with a low amount of copper catalyst (typically 10-250 ppm versus monomer). The Cu(I) complexes were constantly regenerated in situ from oxidatively stable Cu(II) species by the action of reducing agents such as tin(II) 2-ethylhexanoate (Sn-$(EH)_2$) [75] and ascorbic acid [76]. To select the proper reducing agent, its reactivity toward all reaction components need to be considered. Reducing agents that yield relatively strong acids upon oxidation may protonate the ligand, leading to catalyst "poisoning" and loss of control.

In ARGET ATRP, the reaction is allowed to start with the stable Cu(II) species owing to the presence of reducing agents. This avoids the usage of Cu(I) species in the laboratory and can reduce the weighing errors, as Cu(I) salts can be oxidized to Cu(II) during its storage.

In an ARGET system, although much lower amount of catalysts are used compared to conventional ATRP, this does not lead to a greatly reduced polymerization rate. As shown in equation 2, the polymerization rate does not depend upon the absolute value of [Cu(I)] but upon the ratio of [Cu(I)] to [Cu(II)].

In order to compensate for the competitive complexation of the low amount of catalysts with the monomer, strong and excess ligands (usually 3 to 10 times molar excess) are required in an ARGET system. Matyjaszewski's group often used tetradentate ligands such as Me_6TREN and TPMA in their ARGET ATRP systems [73-74].

As to the amount of reducing agent, the optimum quantity depends on the reactivity of the reducing agent and its solubility in the system. Excessive reducing agent would result in a fast and uncontrolled polymerization, while too little would lead to a slow polymerization and low conversions. In systems reported by Matyjaszewski's group, a fair control was achieved when the ratio of reducing agent to copper was chosen to be 10:1 [77-78].

2.5.3 SARA ATRP

Supplemental activator and reducing agent (SARA) ATRP utilizes zero-valent metal to reduce the deactivator to the activator [79-80]. Most reports of SARA ATRP utilized Cu(0) in the form of copper powder or copper wire as the SARA agent, which simplified the reaction setup, and allowed easy handling of the reaction process. In these systems, Cu(I) was continuously generated through the reduction of the oxidatively stable Cu(II) by Cu(0).

Inorganic sulfites such as sodium dithionite ($Na_2S_2O_4$), sodium metabisulfite ($Na_2S_2O_5$), and sodium bisulfite ($NaHSO_3$) can also be utilized as SARA agents. Polymerization of methyl acrylate in DMSO at ambient temperature was demonstrated using various sulfites in combination with a $CuBr_2$/Me_6TREN catalyst [81]. Aqueous SARA ATRP was carried out for the preparation of poly[oligo(ethylene oxide) methyl ether acrylate] with less than 30 ppm of the copper catalyst using TMPA instead of Me_6TREN ligand in the presence of an excess of halide salts (e.g., NaCl) [82]. Inorganic sulfites (e.g., $Na_2S_2O_4$) were continuously fed into the reaction mixture. The mechanistic studies proved that these salts can activate alkyl halides directly and regenerate the activator complex. The polymerization could be directly regulated by starting or stopping the continuous feeding of the SARA agent.

2.5.4 eATRP

Electrochemical ATRP (eATRP) reversibly generates Cu(I) species from Cu(II) through the application of an electric field [83]. In eATRP, activator regeneration is accomplished by the electrons supplied by the cathode. Varying the applied potential can effectively control the rate of polymerization.

Matyjaszewski et al. utilized this technique in the polymerization of oligo(ethylene glycol)methyl ether methacrylate in aqueous media [84]. Reactions were conducted with a Cu/TPMA catalyst in water with various electrolytes. As with the polymerization of methyl acrylate reported in organic media, increasing the applied potential was found to increase the rate of the polymerization; however, in aqueous media, it was found that a high rate had negative consequences on the degree of control over the polymerization. The low level of control at higher applied potential is attributed to increased bimolecular termination reactions at higher radical concentrations.

Additionally, aqueous eATRP has been used to control the polymerization of acrylamides, which is significant because of the inability of many other ATRP techniques to effectively control the polymerization of this class of monomer [85].

One disadvantage of this procedure is the use of platinum electrodes, which is

currently an obstacle to larger scale synthesis due to its cost. In 2016, Isse and co-workers demonstrated that eATRP could also be conducted with non-noble metals such as NiCr and stainless steel [86].

2.5.5 Photoinduced ATRP

Photoinduced ATRP has attracted considerable attention due to its wide availability, environmental benignity, and the possibility of controlling the reaction by simply switching the light on and off [87]. Copper-mediated photo-ATRP relies on a free amine ligand in solution that is able to reduce Cu(II) species to Cu(I) when in a photoexcited state.

Photoinduced ATRP in aqueous media was first reported in 2015 and was promoted by visible light and a $Cu(II)X_2$/TPMA catalyst [88]. The key to controlling the reaction was found to be the addition of an additional halide salt, which offsets the dissociation of Cu(II) species in aqueous media. Temporal control was demonstrated by cycling the reaction between illumination and darkness. Catalyst concentrations were reduced significantly while maintaining control over the polymerization by the addition of sodium bromide. Control over polymerization was demonstrated at copper concentrations as low as 26 ppm.

2.6 Architecture of polymer brushes

Polymer brush prepared via ATRP is terminated with a “living” halogen atom that can be used for further chain extension to construct complex architectures when the monomer supply is replenished. Various polymers with controlled composition, topology, and functionality can be achieved by the ATRP technique.

2.6.1 Random copolymer brushes

Random copolymer brushes are prepared by copolymerizing different monomers in one step reaction mainly to tune the properties (e.g., hydrophilicity and stimuli-responsiveness) of polymer brushes [89]. Random copolymers exhibit interesting and unique properties of each individual homopolymer. Due to differences in monomer reactivity, the ratio of the monomers incorporated into the copolymer brush is not necessarily identical to the monomer feed.

Klok et al. prepared RGD peptide-modified poly(2-hydroxyethyl methacrylate-co-2-(methacryloyloxy)ethyl phosphate) brushes via SI-ATRP and post-polymerization modification with peptide [90]. The RGD peptides were able to

mediate osteoblast adhesion, and the phosphate groups could mimic the function of natural bone extracellular matrix phosphorylated proteins and stabilize the bone mineral phase.

In Paper I, taking advantage of SI-ATRP, we successfully prepared a thermo-responsive random copolymer brush composed of pNIPAm and pGMA from initiator functionalized silica (Figure 9). pNIPAm is a thermo-responsive polymer that has a characteristic lower critical solution temperature (LCST) in water at around 32 °C [91]. Above LCST, the polymer undergoes a phase transition from hydrophilic state to hydrophobic state. pGMA is widely employed for the preparation of functional polymer materials due to its epoxy group that can undergo ring-opening reactions and allows the introduction of various functionalities into polymers [92].

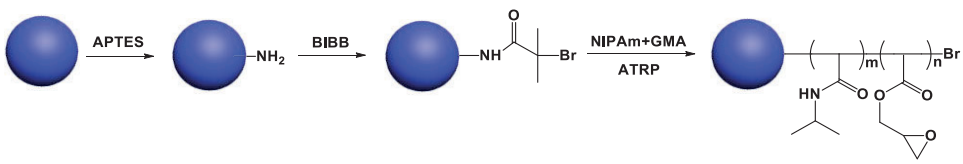

Figure 9. Synthesis of P(NIPAm-co-GMA) random copolymer brushes from ATRP initiator functionalized silica nanoparticles via ATRP.

2.6.2 Block copolymer brushes

Block copolymer brushes consist of two or more homopolymer segments linked together. There are two important requirements for the synthesis of well-defined block copolymers: efficient initiation and preservation of chain end functionality, i.e., "livingness". SI-ATRP has been extensively applied to prepare block copolymer brushes via sequential block growth [93].

Bruening et al. managed to prepare triblock copolymer brushes composed of poly(methyl methacrylate)-*b*-poly(dimethylamino ethylmethacrylate)-*b*-poly(methyl methacrylate) and poly(methyl acrylate)-*b*-poly(methyl methacrylate)-*b*-poly(hydroxyethyl methacrylate) brushes at room temperature via the ATRP technique [94]. The chain extension confirmed the presence of survivor halogen atom at the end of the polymer brushes.

It was reported that the nature of the monomer would influence the success of preparing block copolymer. Genzer et al. reported that multiblock copolymer brushes of poly(methyl methacrylate) and poly(2-hydroxyethyl methacrylate) were able to be readily prepared, but the synthesis of poly(methyl methacrylate)-b-poly(dimethylaminoethyl methacrylate) brushes turned out to be much more difficult [95].

In Paper II and III, we investigated the possibility of preparing block copolymer brushes composed of pNIPAm and pGMA segments with different sequences from ATRP initiator functionalized silica nanoparticles (Figure 10). We discovered that the on-particle synthesis of pNIPAm-b-pGMA brush was much more efficient than pGMA-b-pNIPAm brush from the initiator functionalized silica surfaces.

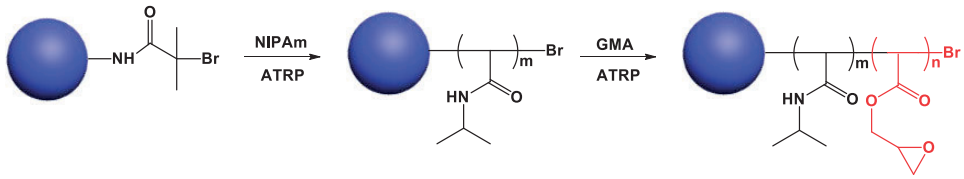

Figure 10. Synthesis of block copolymer brushes of pNIPAm and pGMA from ATRP initiator functionalized silica nanoparticle via consecutive SI-ATRP.

In Paper II, the morphologies and sizes of Si@initiator, Si@pNIPAm, and Si@pNIPAm-b-pGMA particles were characterized by SEM and TEM. As seen in Figure 11 (images a and b), the Si@initiator nanoparticles were spherical and had similar diameters (~200 nm). After grafting pNIPAm brushes from the Si@initiator surface, the Si@pNIPAm particles exhibited a core−shell structure where the silica core and the surrounding polymer shell (~5 nm) could be distinguished in the TEM image (Figure 11, image d). After further grafting the pGMA block, the thickness of the polymer shell in the obtained Si@pNIPAm-b-pGMA particles increased to ~30 nm (Figure 11, images e and f).

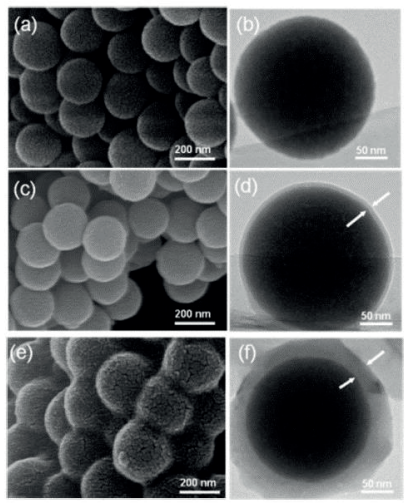

Figure 11. SEM images of (a) Si@initiator, (c) Si@pNIPAm, and (e) Si@pNIPAm-b-pGMA. TEM images of (b) Si@initiator, (d) Si@pNIPAm, and (f) Si@pNIPAm-b-pGMA.

2.6.3 Complex polymer brushes

ATRP can be exploited to prepare architecturally more complex brushes such as hyper-branched, comb-shaped, and star-shaped polymer brushes.

Hyper-branched polymer brushes can be prepared by polymerizing inimers, i.e., compounds that act as initiators and monomers [96]. Mori et al. described the preparation of hyper-branched polymer brushes through copolymerizing 2-(2-bromopropionyloxy) ethyl acrylate and 2-(2-bromoisobutyryloxy)ethyl methacrylate, respectively [97-98]. Incorporation of a degradable linker between the ATRP initiating site and vinyl group allows the production of degradable branched polymers [99].

Comb-shaped polymer brushes can be prepared by first growing a homopolymer brush, followed by functionalization of the side chains with initiating groups for the growing polymer arms [100-101]. Neoh et al. immobilized ATRP initiator (2-chloropropionic acid) to pGMA brushes, followed by the growing of pNIPAm brushes from the initiators, which were distributed along the pGMA brushes, leading to comb-shaped polymer brushes for accelerated temperature-dependent cell detachment [100].

Multifunctional initiators attached to a central core can yield star polymers with multiple linear polymers emanating from a central moiety. Liu et al. reported the fabrication of amphiphilic multiarm star block copolymer for cancer targeted drug delivery and magnetic resonance imaging (MRI) contrast enhancement (Figure 12) [102]. In aqueous solution, the star block copolymer exists as structurally stable unimolecular micelles possessing a hyperbranched polyester (*Boltorn H40*) as the core, a hydrophobic poly(ε-caprolactone) (PCL) inner layer, and a hydrophilic outer corona of poly(oligo(ethylene glycol) monomethyl ether methacrylate) (POEGMA). The outer corona was covalently labeled with Gd and folic acid (FA) for synergistic targeted drug delivery and MRI.

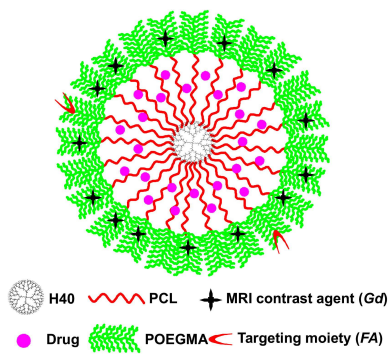

Figure 12. Schematic illustration of multifunctional unimolecular micelles based on amphiphilic multiarm star block copolymers, *H40*-PCL-*b*-P(OEGMA-*Gd*-*FA*). Adapted with permission from [102].

2.7 Functionalities of polymer brushes

The functional groups of polymer brushes can be placed at the α-end (tail), in the backbone (repeat units), or at the ω-end (growing head). Initiators with azide, alkene [103], or alkyne [104] functionality are attractive, as they allow access to more functional polymers with the development of the very efficient azide–alkyne, thiol-ene, and thiol-yne click chemistry. Macroinitiators containing degradable functional groups [105] can incorporate functionality in the center of the chain. Initiators with N-hydroxysuccinimide and disulfide groups can react with exposed amino and thiol groups on proteins, respectively [106-107]. A biotin-containing ATRP initiator can be used to prepare a polymer that can selectively interact with avidin [108].

A wide range of functional monomers can be polymerized yielding polymers with pendant functional groups. For example, monomers with epoxide groups are extensively employed for preparing functional polymers [109].

Polymers prepared by ATRP are actually macromolecular alkyl halides, and the halogen atom at the ω-end can be further transformed to various functionalities by standard organic chemistry procedures. For example, the nucleophilic substitution of halides with sodium azide is very efficient, yielding azido end-functionalized polymers that can be further modified via click reaction with alkynes [110].

Chapter 3. Polymer brushes for protein separation

Bioseparation is the process of separating biomacromolecules from biological samples based on molecular size, electrostatic attraction, or affinity interaction.

Polymer brushes have proved to be very attractive for protein binding due to its soft and flexible structure that can provide rapid protein transport to binding sites. The three dimensional structure with significant internal volumes facilitates protein binding.

Polymer brushes with carboxylic acid groups such as poly(acrylic acid) (PAA) and poly(carboxybetaine methacrylate) (PCBMA) can be used to bind proteins via active ester chemistry. These carboxylic acid brushes can be activated with N-hydroxysuccinimide (NHS) using a carbodiimide reagent to mediate the reaction between carboxylic acid groups and amino groups [111-112]. With this approach, Cullen et al. successfully immobilized RNase A onto PAA brush. The bound enzyme was found to show a high enzyme activity [113]. Zhang et al. managed to immobilize antihuman chorionic gonadotropin (anti-hCG) antibody onto PCBMA brushes [114]. Surface plasmon resonance (SPR) measurements demonstrated that the antibody-modified polymer brush was able to specifically bind hCG while resisting the nonspecific adsorption of other proteins.

A drawback of immobilizing proteins via active ester chemistry is the susceptibility of the hydrolysis of the active esters. Upon exposure to an aqueous solution, NHS ester hydrolysis competes with protein immobilization, thus limiting the binding capacities [115].

Polymer brushes with epoxide groups are attractive candidates for protein binding, as they can react irreversibly with various nucleophilic groups in proteins such as amino and hydroxyl groups. However, due to its hydrophobicity nature, pGMA brush is not suitable for the separation of biological samples. This problem can be resolved by copolymerizing GMA with appropriate hydrophilic comonomers. For example, Huang et al. successfully prepared a water-dispersible magnetic microsphere by copolymerizing GMA and 2,3-dihydroxypropyl methacrylate via SI-ATRP for the immobilization of penicillin G acylase and BSA [116-117].

3.1 Glycoprotein separation

Glycoproteins are proteins containing carbohydrates (Figure 13) that are attached to the proteins in a post-translational glycosylation process. Glycosylation of a protein is one of the most important and ubiquitous processes existing in various biological activities including molecular recognition, protein folding, cell division and differentiation, signal transduction, and immune response [118]. It has been known that altered and aberrant glycosylated proteins are correlated with the occurrence of many types of cancer [119].

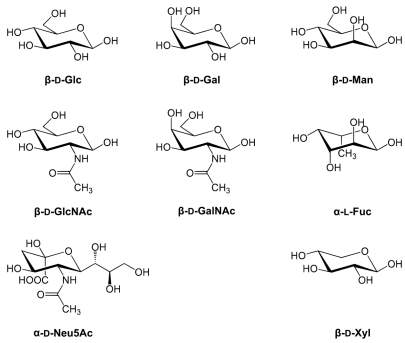

Figure 13. The principal sugars found in human glycoproteins.

There are two major types of glycosylation: N-linked glycosylation, which involves the attachment of glycans to asparagine residues and O-linked glycosylation, which involves the attachment of glycans to serine or threonine residues (Figure 14). Systematic investigation of glycoproteins can facilitate the discovery of new diagnostic biomarkers and provide important information for therapeutic treatment [120]. However, due to the relative low abundance of glycoproteins in complex biological samples, it is still a challenging task to enrich glycoproteins in complex samples.

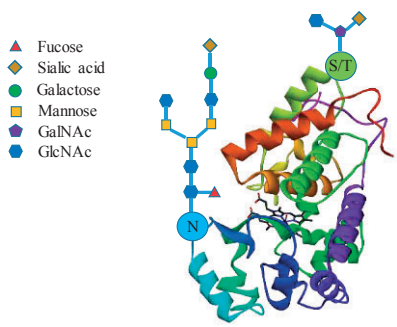

Figure 14. Illustration of N-linked and O-linked glycoprotein.

Currently, the techniques utilized for glycoprotein separation includes hydrazide chemistry, lectin affinity, and boronate affinity-based enrichment.

3.1.1 Hydrazide chemistry

Hydrazide chemistry based enrichment involves the reaction between hydrazide functionalized materials and aldehyde groups that are converted from secondary hydroxyl groups of glycans upon peroxidation (Figure 15) [121-122].

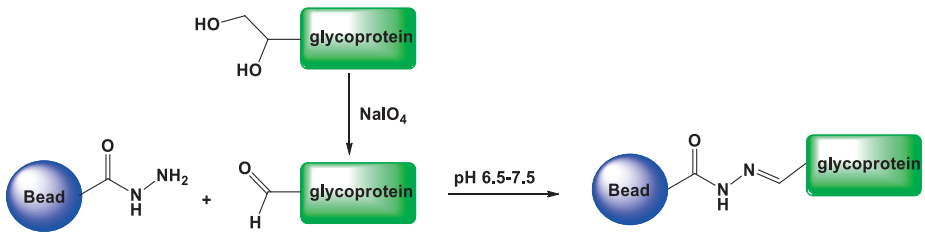

Figure 15. Isolation of glycoproteins on hydrazide-modified beads.

To release the bound glycoproteins from the adsorbents, another chemical reaction or additional enzyme is needed because the hydrazone bond is nearly irreversible. PNGase F is the commonly used enzyme in an elution step. Chen et al. combined multienzyme digestion and hydrazide chemistry for the human liver N-glycoproteome analysis with an identification of 939 N-glycosylation sites and 523 N-glycoproteins [122].

Zou et al. developed a hydroxylamine assisted PNGase F deglycosylation strategy to release glycopeptides efficiently [123]. Hydroxylamine was used to release the former oxidized intact N-glycopeptides through transamination, and then PNGase F deglycosylation was performed in the free solution, which could avoid the inefficiency of the enzymatic reaction in heterogeneous conditions.

3.1.2 Lectin affinity interaction

Lectins are a unique class of proteins found in plants, bacteria, fungi, and animals that exhibit affinity for carbohydrates. Lectin affinity separation is based on the binding ability of lectins towards oligosaccharide structures on glycoproteins. Lectins offer relatively weak binding ($K_d \sim 10^{-4}$-10^{-7} M) compared to antibodies ($K_d \sim 10^{-9}$ M), but because of their carbohydrate specificities, they are eminently suited for glycoprotein separation. Different lectins display different binding specificities. For example, Concanavalin A (ConA) specifically binds to

mannose-containing glycans, Sambucus nigra (SNA) to sialic acid-containing glycans, and wheat germ agglutinin (WGA) to GlcNAc residues of a glycan structure.

Zhu et al. combined surface plasmon resonance imaging (SPRi) with the lectin microarray for rapid and reliable glycoprotein profiling (Figure 16) [124]. The effectiveness of this system was demonstrated by automatically monitoring the interactions between lectins and the lysates of several mouse stem cell. In total, 40 lectins were spotted on the sensor chip to characterize the stem cell markers associated with pluripotency and non-pluripotency.

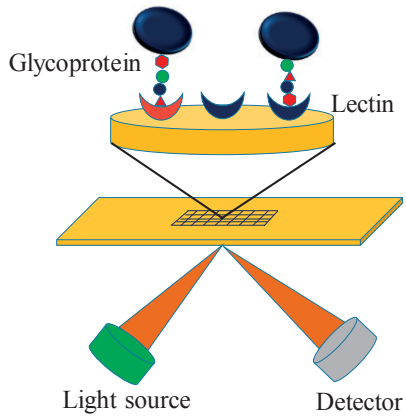

Figure 16. Schematic representation of lectin array on surface plasmon resonance imaging (SPRi). Adapted with permission from [124].

Lectin affinity separation is highly selective and thus provides target proteins with a high purity while the biological activity and natural glycosylation pattern is conserved due to the mild separation conditions. The drawback of this technique is that lectins are associated with high cost and poor stability, which limit their widespread applications.

3.1.3 Boronate affinity interaction

In recent years, many types of boronate affinity materials including macroporous monoliths [125], molecularly imprinted polymers [126], and magnetic materials [127] have aroused enormous interest for the enrichment of glycoproteins.

Boronic acids can react with cis-diol-containing biomolecules in mildly basic solution by forming boronate ester bonds. The bound biomolecules can be eluted in acidic solution via the dissociation of boronate ester bonds (Figure 17).

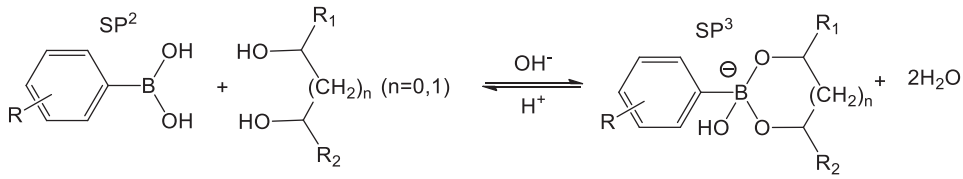

Figure 17. Schematic of the interaction between boronic acids and cis-diol-containing compounds.

Liu et al. reported a boronate affinity-based surface imprinting approach for the efficient imprinting of glycoproteins [126]. A glycoprotein template was first anchored onto a boronic acid functionalized substrate, which was then deposited with a thickness-controllable imprinting coating generated by self-copolymerization of dopamine and m-aminophenylboronic acid (APBA). The approach has significant advantages, including high specificity, high imprinting efficiency, and widely applicable substrates. The author also reported a dendrimeric boronic acid-functionalized magnetic nanoparticle for the separation of glycoproteins [128]. Highly branched dendrimers allowed the introduction of a high density of boronic acid ligands.

In this thesis, by combining SI-ATRP and high-efficiency click reaction, we successfully introduced a high density of boronic acid ligands into polymer brushes material for selectively isolating glycoproteins (Figure 18).

In Paper I, we prepared random copolymer brushes by copolymerizing NIPAm and GMA monomers from initiator functionalized silica nanoparticles via the ATRP technique, followed by opening the epoxide ring of GMA with propargylamine to produce multiple alkyne groups, which were then utlized to conjugate with azide-functionalized boronic acid through the CuAAC click reaction.

In Paper II, we prepared block copolymer brushes by growing pNIPAm and pGMA segments sequentially from initiator functionalized silica nanoparticles via the ATRP technique, followed by opening the epoxide ring of GMA with NaN_3 and subsequent modification with alkyne-functionalized boronic acid via CuAAC click reaction to yield the desired nanohybrid.

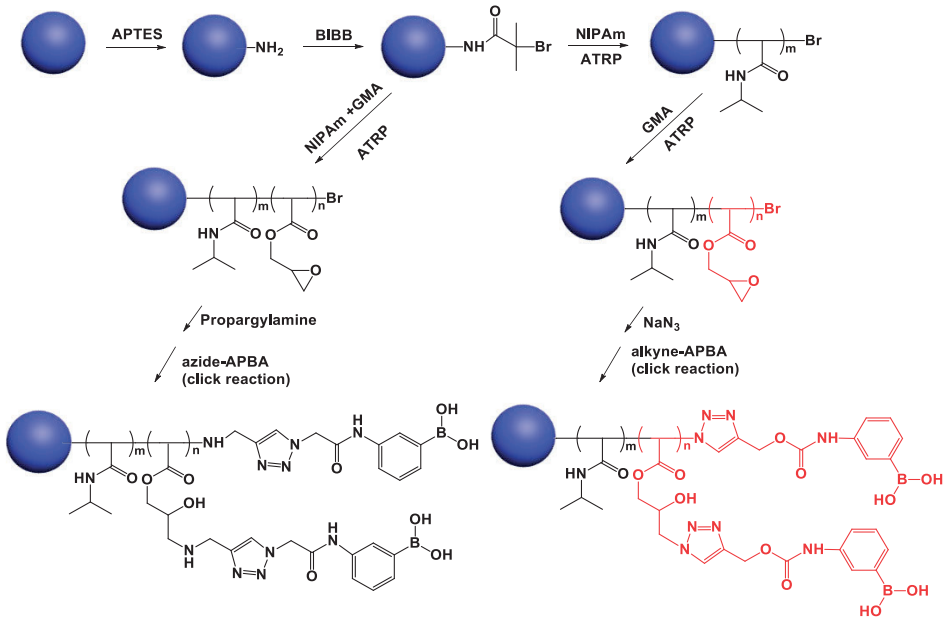

Figure 18. Preparation of boronate affinity nanohybrid via the combination of SI-ATRP with CuAAC click reaction.

3.2 His-tagged protein separation

Since it was first reported in 1975, immobilized metal ion affinity chromatography (IMAC) has been extensively applied for protein separation [129]. Subsequent studies reveal that histidine amino acid could strongly coordinate with transition metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} [130].

The development of recombinant protein technology allows a number of histidines to be incorporated into the N- or C-terminus of the protein of interests by genetic engineering. Therefore, the affinity of the protein for the metal ion will be remarkably increased. When a histidine (His)-tagged protein contacts with metal ions that are immobilized on supporting materials via the chelating ligands such as iminodiacetate (IDA) [131] and nitrilotriacetate (NTA) (Figure 19) [132], it will be immbolized and other proteins can, therefore, be removed by washing with an appropriate buffer. The bound His-tagged proteins can be eluted with an excess of competitor ligands such as imidazole. Moreover, the material can be easily regenerated by washing with a stronger chelator such as ethylenediaminetetraacetic acid (EDTA) and subsequently reloading with metal ions.

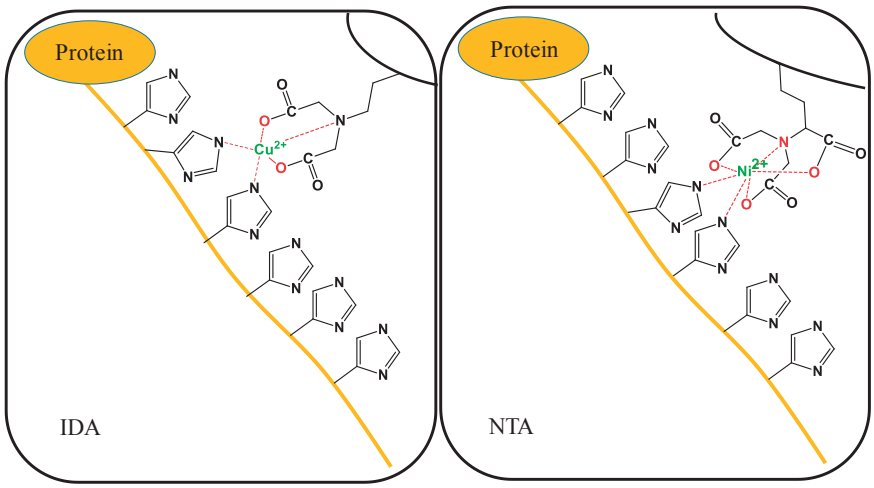

Figure 19. Model of the interaction between a His-tagged protein and the metal ion in IDA and NTA ligands.

3.2.1 His-tagged protein

A His-tag (also known as hexa histidine-tag, 6xHis-tag, His6-tag) is an amino acid motif in proteins that normally consists of six consecutive histidine residues incorporated into the N- or C-terminus of the protein by genetic engineering. The His-tag has been the most widely used affinity tag due to its low immunogenicity, small size and general applicability [133-134].

3.2.2 IMAC adsorbents

Nowadays, various IMAC adsorbents of His-tagged proteins are available in the market, which include MagneHis Ni-Particle (Promega), Ni-NTA magnetic agarose beads (QIAGEN), Dynabeads TALON (Dynal Biotech), and HIS-Select nickel magnetic beads (Sigma). These commercial adsorbents normally have a limited binding capacity (lower than 40 mg g^{-1}) [135]. Thus, developing novel IMAC adsorbents with higher capacity is necessary.

Zhang et al. developed a molecularly imprinted polymer for the purification of His-tagged green fluorescent protein (GFP) (Figure 20). By surface imprinting of the His-tag, the authors obtained IMAC adsorbents with improved selectivity for the His-tagged GFP [136].

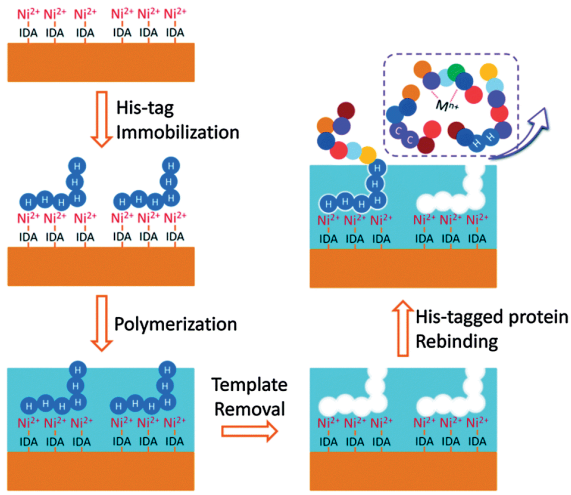

Figure 20. Outline of the preparation process of epitope imprinting enhanced IMAC (EI-IMAC) and the rebinding toward His-tagged protein. Adapted with permission from [136].

Mattiasson et al. prepared soft macroporous gels with a large surface area for the enrichment of His-tagged proteins [137]. However, the slow diffusion of proteins into the imprinting pocket or porous gels often necessitated a longer separation time that may harm sensitive proteins [138-139].

Tan et al. successfully designed and synthesized anionic conjugated polyelectrolytes with IDA pendant groups (PPEIDA), which were exploited to immobilize His-tagged proteins [140]. Both fluorescence anisotropy and fluorescence resonance energy transfer experiments demonstrated the prominent recognition of His-tagged proteins. Compared to the conventional Western blot analysis, the method using PPEIDA–Ni^{2+} significantly reduced the operation time and cost.

In Paper II, taking advantage of the SI-ATRP and the high-efficiency click reaction, we have successfully prepared polymer brushes with boronate affinity for glycoprotein separation. Inspired by these research results, in Paper III, we further explored the feasibility of preparing polymer brushes with a metal affinity for the enrichment of His-tagged proteins using His-tagged hemoglobin (His-Hb) and His-tagged lactate dehydrogenase (His-LDH) as models. As shown in Figure 21, based on the intermediate nanohybrid platform, Si@pNIPAm-b-pGMA, alkyne-functionalized IDA ligands were introduced via high-efficiency CuAAC click reaction. The long and flexible polymer brushes enabled to provide local proximity and facilitate multiple IDA-Cu ligands to bind His-tagged proteins via multipoint attachment.

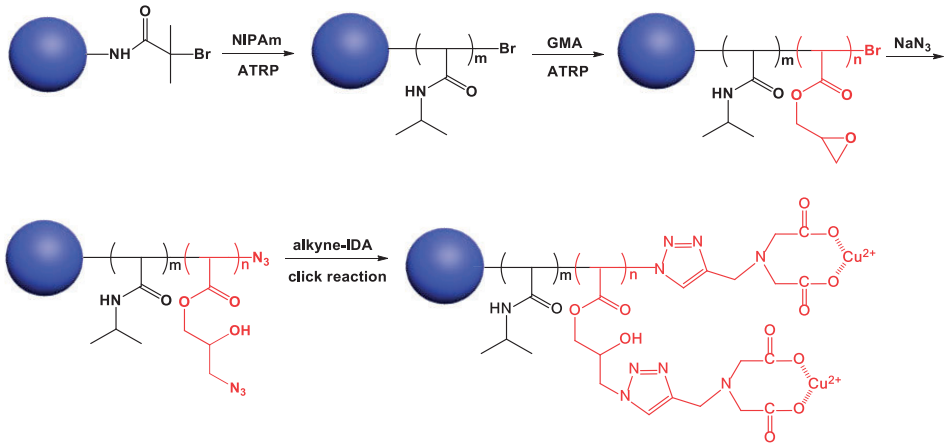

Figure 21. Preparation of IDA-Cu affinity core-brush nanohybrid by combining SI-ATRP with CuAAC click reaction.

The equilibrium binding isotherms (Figure 22) showed that the nanohybrid with IDA ligands could significantly bind a higher amount of His-Hb (184.4 mg g^{-1}) and His-LDH (93.3 mg g^{-1}) than BSA (19.2 mg g^{-1}). The His-Hb variant used in this study is composed of a single polypeptide chain (MW of 62 kDa), where the His tag is located at the N-terminal of the elongated XTEN sequence linked to the fHbF protein. The easily accessible His-tag in this particular His-Hb may be the main factor to enhance the protein binding to the nanocomposite. The generally high binding capacity of the nanocomposite towards His-tagged proteins can also be attributed to the open 3D architecture of the IDA-Cu ligand-rich polymer brushes. The hydrated polymer brushes are expected to adopt an extended conformation in aqueous solution at room temperature, thereby facilitating the infiltration of His-tagged proteins to the binding sites.

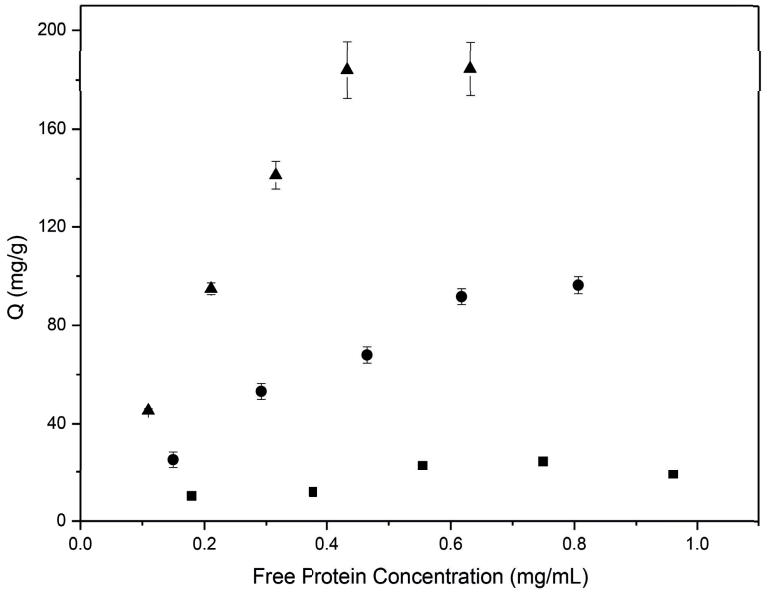

Figure 22. Protein binding isotherm measured at pH 8.0 for His-Hb (▲), His-LDH (●) and BSA (■).

Chapter 4. Polymer-protein bioconjugate

Protein–polymer bioconjugates have attracted considerable interests in many biological related fields. From an engineering standpoint, conjugation of a synthetic polymer to a protein is exciting because it allows the merging of the properties of both the synthetic polymer and the protein [141-142].

Protein-polymer bioconjugates can be prepared by "grafting to" and "grafting from" approaches (Figure 23).

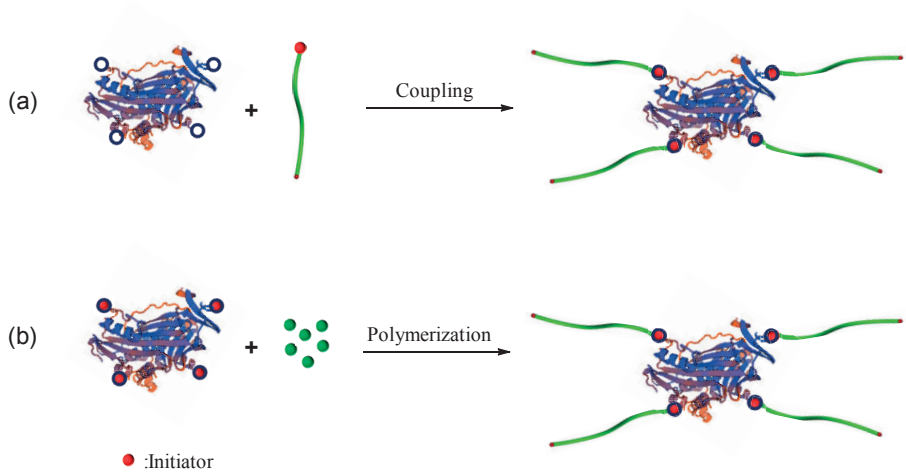

Figure 23. General synthetic strategies to form polymer–protein conjugates.

4.1 "Grafting to" approach

The "grafting to" approach involves the synthesis of a polymer with a functionality capable of bonding to a natural product, followed by a coupling reaction. A few reliable reactions that can take place under mild reaction conditions have been extensively applied to prepare protein-polymer bioconjugate. The most commonly used chemistries include: N-

hydroxysuccinimidyl (NHS) activated ester chemistry (Figure 24a), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) mediated chemistry (Figure 24b), thiol–maleimide Michael addition (Figure 24c), and thiol-disulfide exchange reaction (Figure 24d).

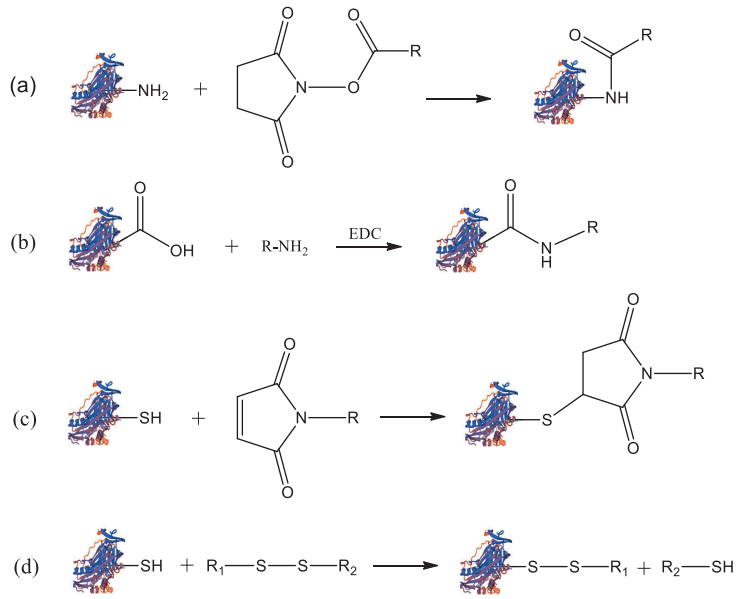

Figure 24. Common bioconjugation techniques for the modification of reactive amino acid side chains. (a) NHS-chemistry; (b) carbodiimide chemistry; (c) cysteine-Maleimide chemistry; (d) thiol-disulfide exchange chemistry.

As mentioned in section 2.1, the advantage of "grafting to" approach is that the polymer can be prepared and fully functionalized under harsh conditions before its conjugation with proteins. However, due to the steric hindrance between two large macromolecules, the "grafting to" approach often involves low coupling efficiency and low conjugation yields.

Besides chemical reactions, specific noncovalent interactions, for example, the affinity interaction between biotin and streptavidin, have also been employed to prepare bioconjugates.

4.2 "Grafting from proteins" via aqueous ATRP

ATRP is compatible with aqueous media, thus allowing the growth of polymer chains *in situ* from protein surfaces under benign conditions. The general process involves the modification of a protein with ATRP initiators, followed by ATRP

of selected monomers. Until now, various kinds of proteins including streptavidin, myoglobin, chymotrypsin, BSA, lysozyme, GFP, trypsin, and HRP have been utilized for the preparation of protein-polymer bioconjugate via the ATRP technique (Figure 25) [142]. This synthetic route offers clear benefits in terms of lower steric hindrance and higher conjugation efficiency. Moreover, the unreacted monomers can be easily removed from the product by dialysis.

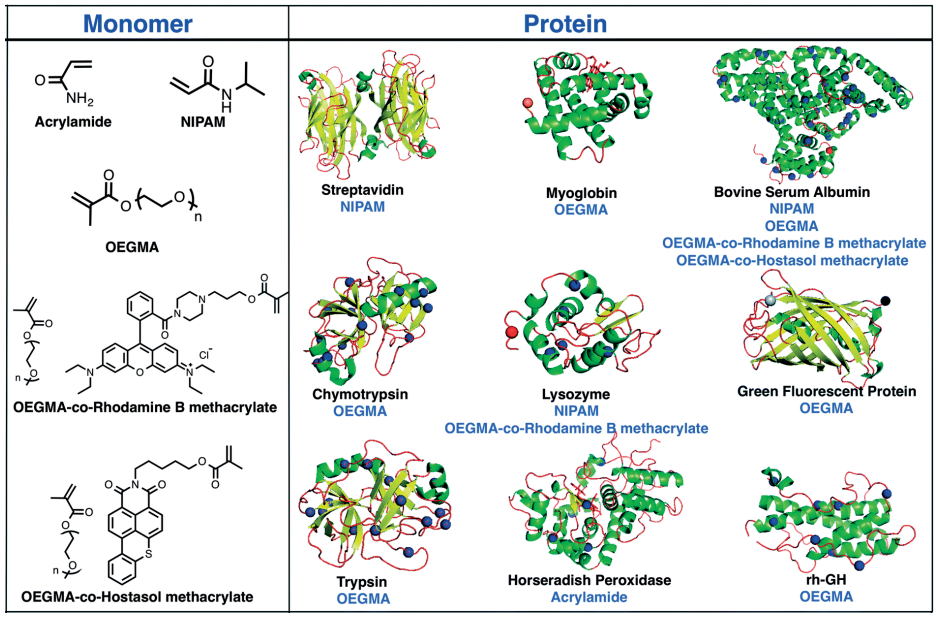

Figure 25. Current protein–polymer conjugates created using ATRP technique. Adapted with permission from [142].

In order to produce homogeneous recombinant protein that contains polymer initiators at defined sites, Mehl et al. designed the amino acid 4-(2'-bromoisobutyramido)phenylalanine as an ATRP initiator and incorporated this initiator to GFP for polymerizing oligo(ethylene oxide) monomethyl ether methacrylate from the selected site in buffered solution(Figure 26) [143].

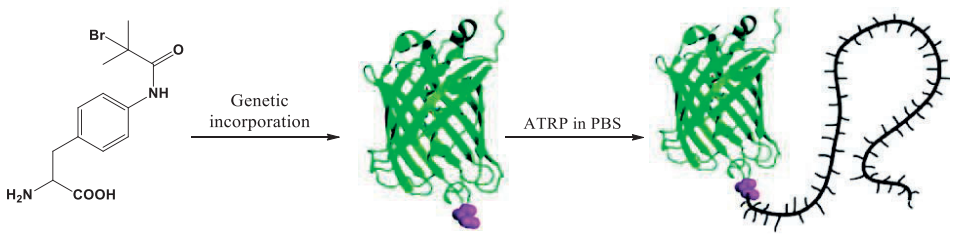

Figure 26. Genetic incorporation of ATRP initiator into proteins for growing polyer brushes. Adapted with permission from [143].

Alexander et al. demonstrated the successful synthesis of "smart" hybrid polymer-protein conjugates by aqueous ATRP from trypsin protein [144]. The structures of resultant hybrids were examined by aqueous phase atomic force microscopy and cryo transmission electron microscopy.

Liu et al. reported a simple and efficient method for the preparation of uniform HRP bioconjugates via ATRP in aqueous media (Figure 27) [145]. Their study demonstrated that the HRP conjugates essentially retained the catalytic behavior of its native counterpart, but exhibited significantly enhanced stability against high temperature and trypsin digestion. Moreover, the uniform size distribution was designable and tunable through adjusting the monomer loading and reaction time.

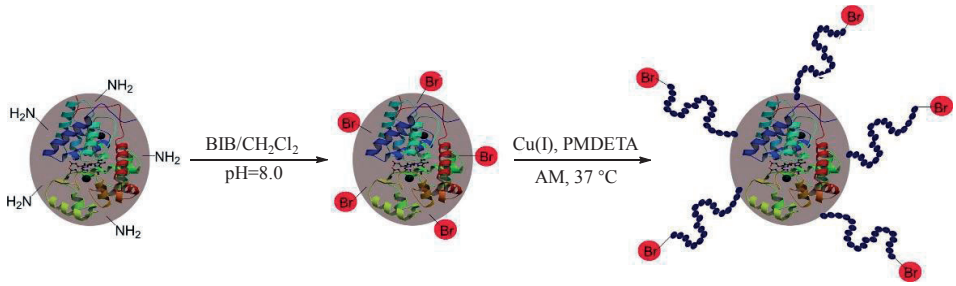

Figure 27. Preparation of conjugates by two-step in situ aqueous AGET ATRP. Adapted with permission from [145].

In Paper IV, amelogenin (AMEL) was employed for the first time to prepare a protein-polymer bioconjugate via the ATRP technique. AMEL (~20 kDa) is the major protein component of the enamel extracellular matrix, playing an important role in enamel formation. Interestingly, it has a natural propensity to self-assemble in weakly alkaline conditions to form globular nanospheres, with a diameter typically around 30 nm [146]. This self-assembly behavior is highly affected by pH, giving AMEL a distinct solubility profile with a low solubility close to neutral pH, and a high solubility in acid (as soluble monomers) and alkaline conditions (as soluble nanospheres) [147].

As depicted in Figure 28, a hydrophilic initiator (2-bromoisobutanoic acid *N*-hydroxysuccinimide (NHS-BiB)) was first immobilized on the surface of the AMEL nanosphere to form the macroinitiator (AMEL initiator), followed by the controlled grafting of PNIPAm chains at room temperature to form AMEL-PNIPAm bioconjugate. The bioconjugate was characterized to determine its particle size, temperature and pH responsiveness, the number of polymer chains, and the molecular weight of the polymer.

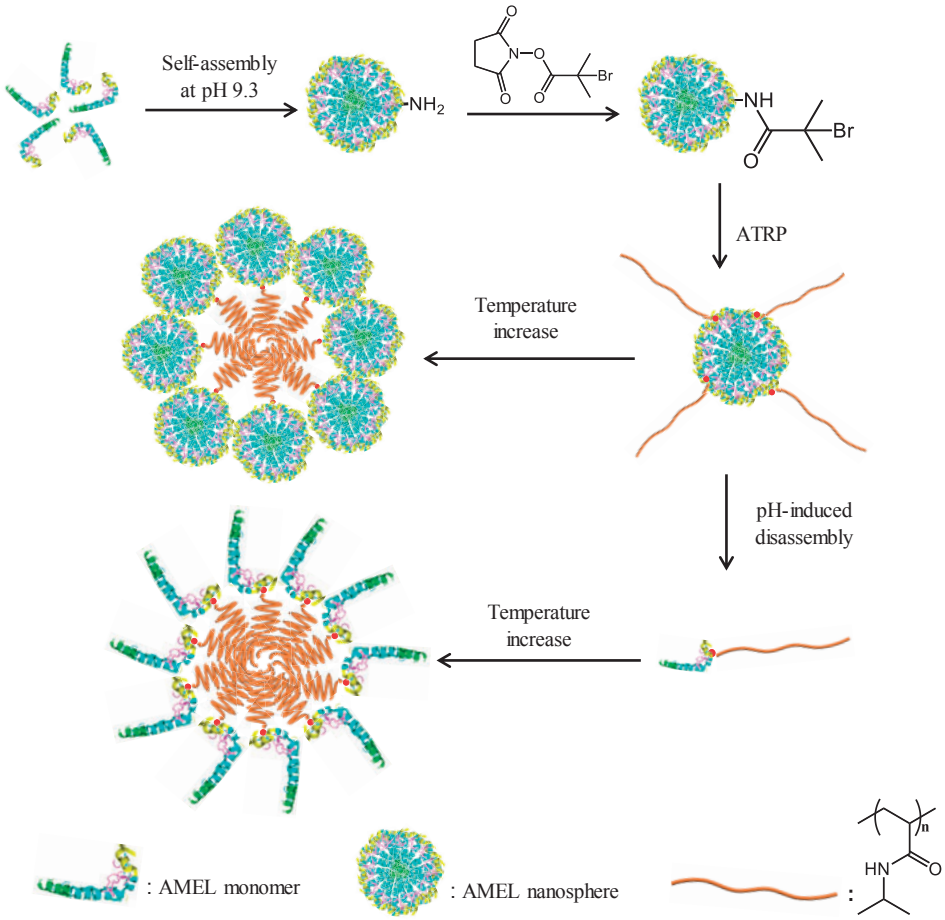

Figure 28. The synthesis of pH and temperature dually responsive AMEL-PNIPAm bioconjugates via "grafting-from" ATRP polymerization.

Dynamic light scattering (DLS) was used to investigate the size of the conjugates below and above the low critical solution temperature (LCST) of PNIPAm. As shown in Figure 29, the size of AMEL-PNIPAm nanosphere was 61.0 nm (PDI = 0.341) at 20 °C (Figure 29a). Upon heating to 40 °C, the particle size increased to 218.7 nm with an extremely narrow size distribution (PDI = 0.089) (Figure 29b). These results indicate the formation of uniform and stable nanoparticles from AMEL-PNIPAm.

The pH-responsiveness of the bioconjugate was also investigated. When lowering the pH of the solution to pH 3.2, the mean particle size was measured to be 16.2 nm (PDI = 0.284) (Figure 29c) at 20 °C, which can be explained by the high solubility and low propensity of AMEL to form self-assembled structures in

acidic solvents. Interestingly, the mean particle size increased to 286.9 nm (PDI = 0.094) upon heating to 40 °C (Figure 29d), indicating the formation of uniform and stable nanoparticles again.

The observed phenomena can be explained by the fact that PNIPAm undergoes rapid chain dehydration and aggregation when the environmental temperature increases to above its LCST (≈ 32 °C), changing from a hydrophilic state to hydrophobic state. PNIPAm conferred its temperature responsiveness to the attached AMEL, leading to an amphiphilic bioconjugate when the environmental temperature was raised above the LCST. The dehydrated PNIPAm chains drove the amphiphilic AMEL-PNIPAm to self-assemble into larger particles composed of PNIPAm-rich core and AMEL-rich domain on the surface.

This pH and temperature dually responsive AMEL-PNIPAm bioconjugate could potentially act as a supramolecular carrier for controlled drug delivery in cancer therapy, for example, by responding to lower pH and temperature variation in tumor tissues.

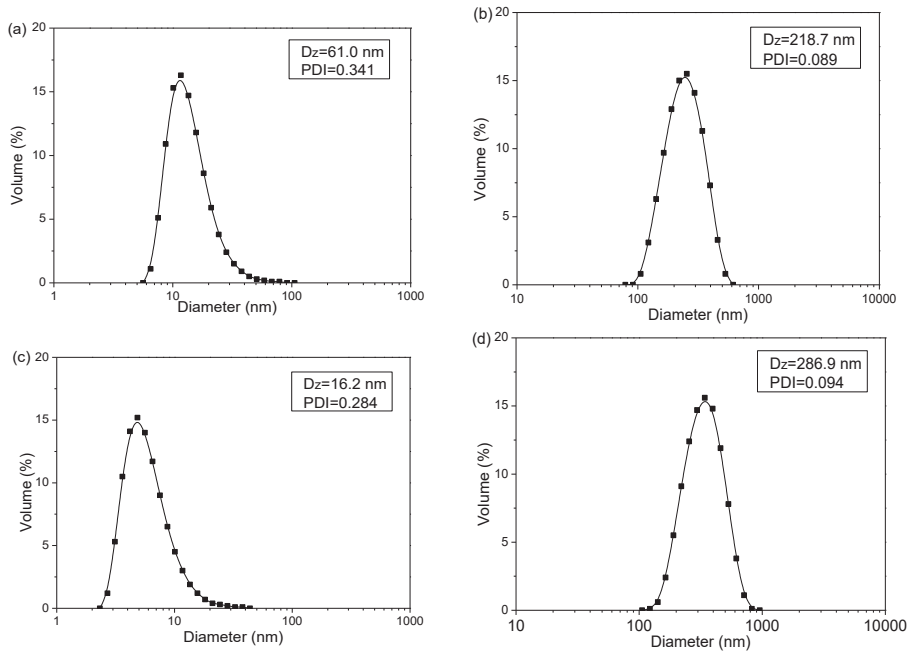

Figure 29. Hydrodynamic particle size of a) AMEL-PNIPAm at pH 9.3 at 20 °C, b) AMEL-PNIPAm at pH 9.3 at 40 °C, c) AMEL-PNIPAm at pH 3.2 at 20 °C, and d) AMEL-PNIPAm at pH 3.2 at 40 °C. Dz stands for Z-average diameter.

5. Conclusions and future outlooks

ATRP provides facile access to produce polymer nanohybrids with precise control over the chemical composition, topology, and functionality under the undemanding reaction conditions. The high-efficiency click reaction enables to introduce a high density of affinity ligands into the polymer brushes via post-polymerization modifications. Polymer brushes prove to be very attractive for protein binding due to their soft and flexible structure that can provide rapid protein transport to binding sites. The swollen polymer brushes could provide sufficient internal space for a high amount of protein binding.

In Paper I and II, intermediate polymer brushes, pNIPAm-co-pGMA, either in the form of a random copolymer or block copolymer, were grown from initiator functionalized silica nanoparticles via the SI-ATRP technique in an organic solvent. The pNIPAm segments endowed the polymer brushes with thermo-responsive property and could increase the hydrophilicity of the brushes. The pGMA segments were utilized to introduce boronate affinity into the brushes via a high-efficiency click reaction. These polymer nanohybrids exhibited great efficiency for glycoprotein separation. More importantly, the thermo-responsive pNIPAm segment could serve as an intelligent "switch" that enabled to regulate the on/off adsorption of glycoproteins through controlling environmental temperature.

In Paper III, based on the block copolymer brushes developed in Paper II, a high density of clickable IDA ligands were introduced to polymer brushes for His-tagged protein separation. The polymer nanohybrid exhibited high binding capacities towards His-tagged protein.

In Paper IV, a novel pH- and temperature-responsive protein-polymer bioconjugate was prepared by grafting pNIPAm chains on amelogenin nanospheres via ATRP in aqueous solution. The bioconjugate could self-assemble into uniform and stable nanoparticles with an extremely narrow size distribution, and the nanoassembly process could be regulated by simply varying the pH and temperature. The protein and the polymer components can be further modified through protein engineering and controlled radical polymerization techniques.

For future perspectives, new ATRP systems that allow fast and precise synthesis

of various polymer hybrids with more complex and precisely controlled architecture in environmentally benign and industrially scalable processes are highly desirable. Smart materials that can specifically self-assemble, self-repair, or interact under external stimuli are particularly interesting. Degradable and therapeutic bioconjugates that can be degraded to well-defined short primary chains and be cleared from the body are also particulary interesting. Nowadays, polymers can be grown from viruses, bacteria, or even living cells via ATRP. We believe that the combined molecular engineering from the bio- and the chemical directions will open great opportunities to fabricate more multifunctional hybrid materials for broad bioapplications including bioseparation, drug/gene delivery, tissue engineering, and biosensing.

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