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Responsive Polymers for Biosensing and Protein Delivery

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Abstract: In this feature article, we review some of the most recent advances in the field of materials chemistry for biosensing, disease diagnostics, and drug delivery. Our recent work on the development of responsive polymer-based platforms for biosensing and drug delivery will also be highlighted. This feature article is meant to outline the breadth of the utility of polymer-based materials for select applications, as well as their enormous potential impact on future technologies.

1. Introduction

At the most basic level, a polymer is simply a chain of molecules (or monomers) attached to one another via chemical or physical bonds. This "macromolecule", first discovered as a result of the work of Hermann Staudinger (1953 Nobel Prize in Chemistry) and Wallace Carothers, now has an impact on every part of most of our daily lives. The polymer chain can confer specific properties to materials itself, or can serve as a basic building block that can be crosslinked, again chemically or physically, to yield network polymers with specific properties. One such network polymer is a hydrogel, which is capable of being swollen with water yielding a material that is both soft and structurally robust. Perhaps most importantly, polymeric materials have found their way into numerous biological applications such as: controlled/triggered drug delivery, biosensing, tissue engineering and regenerative medicine.[1-8] Their enormous utility comes from the fact that they are inexpensive, easy to synthesize, offer a wide variety of chemistries and functionalities, and have well known structure-property relationships.

Polymers, both linear and crosslinked networks, can be made to respond physically and/or chemically to the application of a stimulus such as: pH, temperature, magnetic field, and analyte concentration.[9-16] Of these polymers, often called stimuli responsive polymers, poly (*N*-isopropylacrylamide) (pNIPAm) is by far the most extensively studied.[1, 17-35] PNIPAm is fully soluble in water at a temperature < 32 °C, existing as a fully solvated random coil while it transitions to a desolvated globule at T > 32 °C.[36] Likewise, pNIPAm-based polymer networks are responsive to temperature (thermoresponsive), transitioning from swollen to deswollen as the temperature of the water they are exposed to increases to > 32 °C. Finally, hydrogel particles (referred to as micro or nanogels depending on their diameter) can also be synthesized, and

exhibit thermoresponsivity and have found their way into sensors, catalysts, drug delivery platforms, separations and tissue engineering technologies.[35, 37-42]

In this feature article, we review some of the most recent works on responsive polymeric materials for healthcare applications with some highlights to our recent efforts to develop thermoresponsive microgel-based photonic devices for sensing, biosensing, and controlled/triggered drug delivery.[43-51]

2. Applications of Polymeric Materials

2.1 Sensing and Biosensing

A sensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information by transferring specific signals into a form that is readable by an observer or by another instrument to provide a result. A biosensor is a device used for sensing and/or quantifying the concentration of an analyte that is biological in nature. In general, a biosensor should enable quick, and accurate results in a time such that the patient can be treated if needed. In specific cases, the biosensor should be capable of providing a result to patients in resource-limited settings at the point of care (POC).

To develop novel sensing systems, some are turning to responsive polymers. Among the early works featuring responsive microgels for biosensing, Lyon and coworkers developed a novel approach to biosensing using microlenses derived from dual thermo- and pH-responsive pNIPAm-co-acrylic acid microgels.[34, 35] They designed microlenses[35] that would display a change in refractive index and particle diameter upon binding with protein for use in sensing applications. They designed microlenses for two different sensing pathways: a direct binding-induced response and a displacement-induced response. To illustrate each method, the small

vitamin biotin was conjugated to the microgel's acrylic acid (AAc) groups. For the binding-induced method, avidin or anti-biotin was added to the solution around the microlens, resulting in binding of the protein to the microlens surface. Since both avidin (four binding sites) and anti-biotin (two binding sites) are able to bind multiple equivalents of biotin, the protein-binding event increases the surface crosslinking of the microlens and hence changes their refractive index. The displacement-induced method was achieved by designing a reversible antibody–antigen crosslinking construct. In this case, a photoaffinity approach was used to couple a bound antibody to the antigen-laden microlens. When the free biotin disrupts the crosslinks via displacement, the microlens swells and the focal length increases accordingly. This approach offers the benefit of sensor regeneration. Using microgels as microlenses is attractive because of the ability to use many different solution-based bioconjugation methods and the ease of assembly with simple electrostatic adsorption.[52]

Our group fabricated optical devices, which show visual color and multipeak reflectance spectra. The devices are made by painting pNIPAm-based microgels on a Cr/Au coated glass substrate.[53] The excess microgel was washed away and another layer of Cr/Au was deposited on top of the microgel layer, sandwiching the microgel. Hence, the distance between two Au layers depends on the diameter of the microgel. The resultant device is typically referred to as a microgel-based etalon and is shown schematically in Fig. 1a. This device shows visible color and characteristic multipeak reflectance spectra (Fig. 1b) both of which depend on the refractive index of the microgel and the distance between the two Au mirrors based on Equation (1).[54]

$$\lambda m = 2nd \cos \theta \quad \text{.....(1)}$$

where n is the refractive index of the dielectric layer, d is the mirror-mirror distance, θ is the angle of incident light relative to the normal, and m (an integer), is the order of the reflected peak.

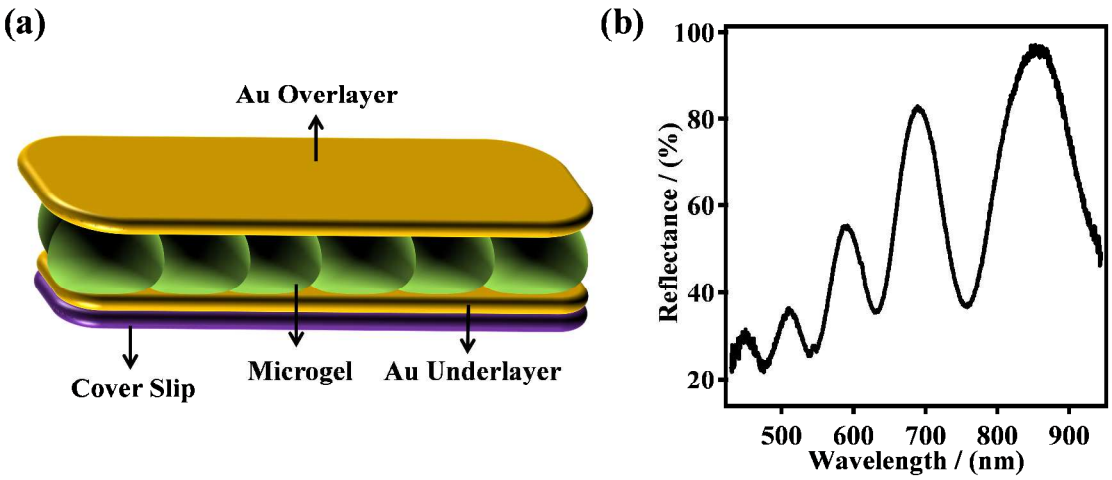

Fig. 1. (a) The basic structure of a microgel-based etalon. The Au overlayer in the figure is drawn as a planar layer, but is actually conformal to the microgel layer. Each Au layer was supported by 2 nm Cr as an adhesion layer. (b) A representative reflectance spectrum from a poly (*N*-isopropylacrylamide)-co-acrylic acid, (pNIPAm-co-AAc) microgel-based etalon. Reproduced with permission from ref. 43.

We used these devices to sense the concentration streptavidin in solution,[43, 46] which was derived from our previous work on polyelectrolyte penetration into microgel-based etalons. Specifically, we determined that pNIPAm-co-AAc microgel-based etalons at high pH (i.e., above the pK_a of AAc is ~ 4.25) could deswell when exposed to poly (diallyldimethylammonium

chloride)(pDADMAc), which is a positively charged linear polymer (polycation).[45] This is a result of the pDADMAc penetrating the outer Au layer of the etalon, resulting in electrostatic interaction induced intra and intermicrogel crosslinking and collapse. This collapse led to an observable shift in the peaks of the reflectance spectrum as predicted from Equation (1). We found that the extent of the shift in peak position depended on the molecular weight (MW) and concentration of polycations. For all experiments, the sides of the etalon were sealed with epoxy to ensure that the polycation only entered the etalon through the Au overlayer.

Therefore, we exploited the above phenomenon to sense streptavidin using a related polycation poly (allylamine hydrochloride) (PAH). To accomplish this, PAH, which is charged at pH < ~9.0, was modified with biotin (PAH-biotin). We showed that the PAH-biotin could also penetrate the etalon and crosslink the microgel layer leading to a spectral shift. Likewise, we found that the extent of the reflectance peak shift depended on the amount of PAH-biotin added to the etalon until the etalon is "saturated". For sensing, we exposed aqueous solution of PAH-biotin to specific amounts of streptavidin; the concentration of PAH-biotin was always high enough to leave excess PAH-biotin in solution after all the PAH-biotin:streptavidin complexes have formed. Then, biotin modified magnetic particles were added to the solution, which bound only to the PAH-biotin:streptavidin complexes. An external magnet was used to remove the magnetic particles bound with PAH-biotin:streptavidin from the solution. The solution containing the excess, unbound PAH-biotin was subsequently separated and added to the pNIPAm-co-AAc etalon stabilized in pH 7.2 (microgels negatively charged) solution maintained at 25 °C. When the PAH-biotin was added to the etalon, it resulted in a blue shift of the etalon's reflectance peaks (the complete protocol is shown in Fig. 2). We found that the extent of the blue shift depends on the amount of streptavidin initially added to the PAH-biotin. Here, a low

concentration of streptavidin initially present in solution yields a large amount of excess, unbound PAH-biotin after the reaction. When the excess PAH-biotin is added to the etalon, we get large shift in spectrum's reflectance peaks. Alternatively, a high concentration of streptavidin initially present in solution will yield a small amount of excess, unbound PAH-biotin that is added to the etalon, which gives a small etalon response. It is important to point out again that we were able to get increasingly large spectral responses with decreasing analyte concentration. This is opposite to what is normally expected from standard sensors.

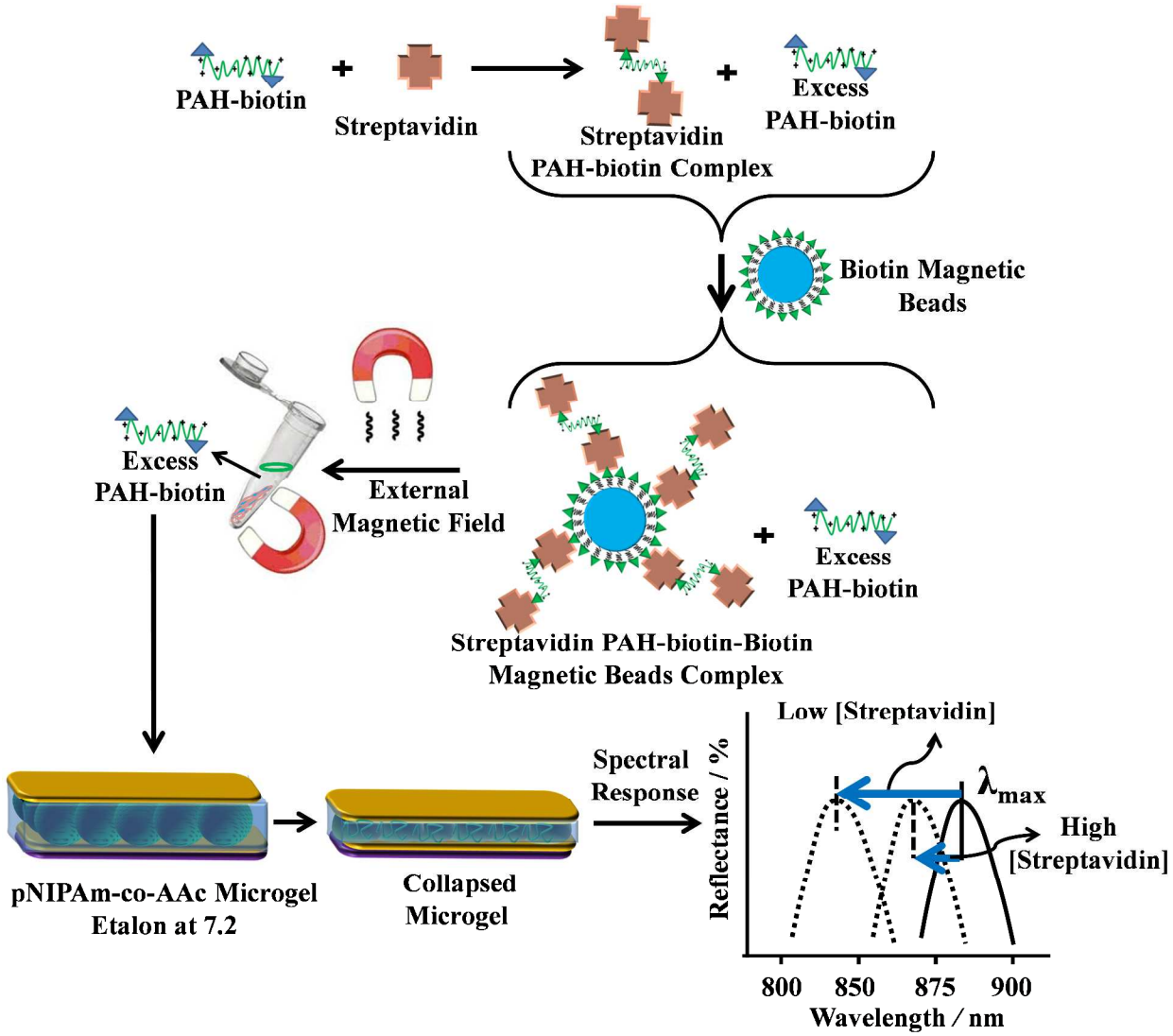

Fig. 2. The proposed sensing mechanism. Streptavidin (the analyte) is added to an excess amount of biotin-modified poly (allylamine hydrochloride) (PAH). The PAH-biotin:streptavidin complex is then removed from solution using biotin modified magnetic particles, leaving behind free, unbound PAH. The unbound PAH is subsequently added to a pNIPAm-co-AAc microgel-based etalon immersed in aqueous solution at a pH that renders both the microgel layer and the PAH charged. As a result, the etalon's spectral peaks shift in proportion to the amount of PAH-biotin that was added. This, in turn can be related back to the original amount of streptavidin added to the PAH-biotin. Reproduced with permission from ref. 43.

A slightly different approach to detecting analytes in solution is to engineer polymer-based materials to undergo crosslinking/decrosslinking in their presence. These processes lead to a subsequent deswelling/swelling response. Maeda et al. detected saccharide–protein interactions using a lectin responsive polymer gel as a signal transducing material bridging between the target and the gate insulator in field effect transistor.[55] This lectin-sensitive polymer gel changes volume in response to the formation of molecular interactions between carbohydrate and the lectin concanavalin A and this polymer gel has the ability to transduce volume changes into electrical signals for the field effect transistor. Kuroki et al. presented for the first time a biomolecule-recognition gating system for avidin. A high-density polyethylene membrane was used as a gating membrane that immobilizes the stimuli responsive copolymer of pNIPAm, and biotin-PEG2-acrylamide onto the pore surface. The pore state (open/closed) of this gating membrane depends on the formation of specific biorecognition-mediated crosslinking in the pores.[56] They used pNIPAm as the stimuli responsive polymer and biotin as a biomolecule-specific receptor for avidin. The pore states can be distinguished by a volume phase change of biotin grafted polymers.

Zhao et al. fabricated DNA-responsive hydrogel photonic beads by polymerizing a DNA monomer containing pre-gel solution in a silica microparticle-based colloidal crystal. [57] Following polymerization, the resultant hydrogel was broken up via vigorous stirring and the resulting hydrogel "particles" were exposed to hydrofluoric acid to dissolve away the silica bead, leaving behind hydrogel particles with an inverse opal structure. In the presence of DNA with a sequence complimentary to the DNA initially incorporated into the hydrogel structure in the pre-gel solution, the photonic particles collapsed, resulting in a blue shift in the Bragg diffraction peak from the particles. Furthermore, quantum dots were introduced into the photonic particles for multiplexed DNA detection. Kivlehan et al. prepared and fabricated surface-attached PEG-diacrylate hydrogel, by a photopolymerization process.[58] They were able to control the process by controlling the light source used. Amino modified oligonucleotides were attached to the gel during photopolymerization, which allow for the diffusion of fluorescently labeled target DNA sequences into the hydrogel matrix to hybridize to probe oligos. The fluorescence after the hybridization was used as the transduction mechanism. This system exhibited a detection limit of 3.9 nM.

Micro and nanoparticles-based approaches to sensing and biosensing have also found traction over a number of years. Myung et al.[59] employed seventh-generation (G7) poly(amidoamine), PAMAM dendrimers and the anti-epithelial cell adhesion molecule (anti-EpCAM) known as the most commonly used circulating tumor cells capturing agents. G7 PAMAM dendrimers were carboxylated and conjugated with anti-EpCAM. The binding avidity of the G7-aEpCAM conjugates was measured by surface plasmon resonance spectroscopy (SPR), along with the enhanced binding stability of the tumor cells on the dendrimer-

functionalized surfaces. This proves that the dendrimer-mediated multivalent binding effect can be exploited in cell capture on engineered surfaces.

Thermoresponsive poly(*N*-isopropylacrylamide-acrylamide-allylamine), (PNIPAAm-AAm-AH) coated magnetic nanoparticles (PMNPs) were developed[60] and conjugated with prostate cancer-specific cell permeable oligo-arginine peptide R11 for active targeting and imaging of prostate cancer. The stable nanoparticles have an average diameter of 100 nm and surface charge of -27.0 mV. The polymer had a lower critical solution temperature of 40 °C. *In vivo* bio-distribution and tumor-specific targeting studies confirmed that peptide coated R11-PMNPs accumulated specifically in tumor regions. In another report,[61] a bioaffinity matrix of viruses integrated into poly(3,4-ethylenedioxythiophene) (PEDOT) films for prostate-specific membrane antigen (PSMA), a prostate cancer biomarker was described. This matrix shows high sensitivity to PSMA due to the synergistic action by the two different ligands to PSMA on the same phage particle. One ligand was genetically encoded, and the secondary recognition ligand was chemically synthesized to wrap around the phage. The dual ligands result in a bidentate binder with high-copy (production of DNA per mL of Lysogeny broth), dense ligand display for enhanced PSMA detection through a chelate-based avidity effect. These films provide a 100 pM limit of detection for PSMA in synthetic urine without requiring enzymatic or other amplification.

Finally, lysozyme specific protein-imprinted spherical nanogel particles were prepared via aqueous free radical precipitation polymerization with the aid of a surfactant, sodium dodecyl sulfate (SDS).[62] Using lysozyme as the protein template and N-isopropylacrylamide as the major monomer, they found that the diameter of nanogels could be controlled from a few hundred down to ~ 50 nanometers by adjusting the SDS concentration during polymerization. It was

revealed that the lysozyme-imprinted nanogels possessed higher rebinding capacity, more rapid rebinding kinetics, and much higher specificity toward lysozyme than non-imprinted counterparts. Importantly, both the rebinding and release characteristics of lysozyme-imprinted nanogels showed dramatic temperature-dependence, with clear on–off transition around 33 °C which is close to the volume phase transition temperature of the thermoresponsive polymer poly (*N*-isopropylacrylamide). This protein imprinted nanogel platform is promising for a broad spectrum of biomedical applications, such as controlled drug delivery, protein separation, biosensing, and therapeutics.

2.2 Drug delivery

Traditionally, administration of drugs is via the oral route, although injections are common if the efficacy of the particular drug can be affected by stomach digestion. Since these mechanisms for drug administration lead to drug distribution to the whole body, there are many challenges associated with natural resistance mechanisms. While this is the case, some recent drug formulations are able to target specific regions of the body, and be undetected by the immune system. Furthermore, there is a considerable amount of research going into the development of systems that not only target specific regions of the body, but also can be triggered to release their cargo only when needed (triggered drug delivery). One way to accomplish this is to use stimuli responsive polymers. They have also been combined with a variety of bioactive molecules by physical mixing, chemical conjugation and/or complexation to facilitate the targeted/triggered release. These bioactive molecules include small molecules, protein and peptides, and nucleic acids. These efforts have been the subject of many reviews.[63-65]

Here, we describe some of the latest examples of responsive polymeric materials developed for drug delivery during the last few years.

One such material used widely for drug delivery is hydrogels. Hydrogels are advantageous due to their porous structure and solvent swellability (among other things).[66, 67] Their porosity permits loading of drugs into the gel matrix and subsequent drug release at a rate dependent on the diffusion coefficient of the small molecule or macromolecule through the gel network. In addition, many hydrogels can alter the degree of swelling in response to changes in their environment as mentioned earlier.[9, 12, 15, 16]

In addition to macroscopic (bulk) hydrogels, microgels and nanogels (hydrogel particles) with diameters ranging from tens of nanometers to several microns can also be used.[68-71] Similar to bulk gels, microgels can also be made biocompatible, however, due to their small size, they exhibit many advantages over bulk gels when used as biomaterials. One major advantage is that the rate of the microgel response to external stimuli is much faster than bulk gels. Secondly, colloidal microgel particles allow for minimally invasive administration when used as drug carriers. In addition, colloidal microgel particles can be used as building blocks for the fabrication of biomedical devices with improved and/or new functionalities[72-74].

As a drug carrier, pNIPAm microgels combine the advantages of both hydrogels and nanoparticles. PNIPAm microgel particles have a sponge-like structure with interstitial spaces filled with solvent. In some cases, drug molecules can be loaded by equilibrium partitioning between the solution and microgel phases. Electrostatic interaction, hydrophobic interaction, and H-bonding may play an important role for the drug loading process. Our group developed a

novel microgel based sandwich structure as a new platform for drug delivery.[51] A device composed of a poly(N-isopropylacrylamide)-co-acrylic acid (pNIPAm-co-AAc) microgel layer sandwiched between two thin Au layers (all on a glass support) was used as a novel platform for controlled and triggered small molecule delivery. Tris(4-(dimethylamino)phenyl)methylium chloride (Crystal Violet, CV), which is positively charged, was loaded into the microgel layer of the device and released in a pH dependent fashion, at a rate that could be controlled by the thickness of the Au layer coating the microgel. The loading process and release data is shown in Fig. 3. Furthermore, the model drug could be released in an "on-off" fashion, by systematically varying the solution pH.

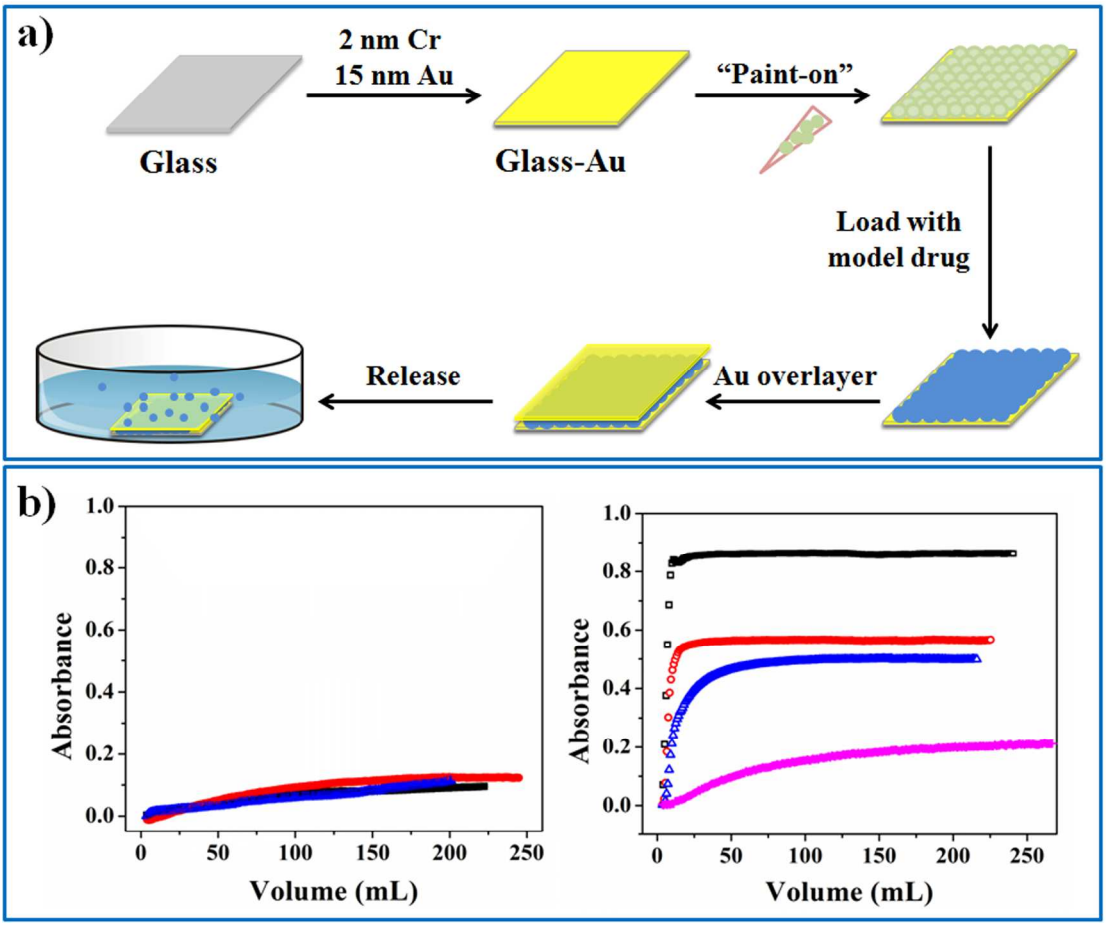

Fig. 3. (a) Schemetic illustration of fabrication of microgel based drug release device; and (b) drug release profiles at (left) pH 6.5 and (right) pH 3.0. Reproduced with permission from ref. 51.

Proteins are important engines of life that perform essential functions inside cells, such as enzyme catalysis, signal transduction, gene regulation, and maintain a fine balance between cell survival and programmed death. Drugs based on proteins are being developed over the last decades. As a consequence, delivery of functional proteins has significant therapeutic implications in biological applications, including disease therapies, vaccination, tissue engineering and diagnostics. Most peptides and proteins are potent and become drugs of choice for specific physiological conditions. Despite rapid progress in the large-scale manufacture of therapeutic proteins, the convenient and effective delivery of these drugs to the body remains a major challenge. Proteins can be physically incorporated in a hydrogel matrix, and their release can be controlled via several ways, such as diffusion, swelling, erosion/degradation, or a combination of these mechanisms.

There are several issues that need to be addressed when developing and delivering protein-based drugs such as: low permeability of proteins through the skin or gastro intestinal track, short half-life, chemical and enzymatic degradation and immunogenicity.[75, 76] It was found that PEGylation of protein drugs enhances the permeability of the drugs through the cellular track and protects the drug from being deactivated or degraded by enzymatic action. Additionally, the half-life of the protein drugs could be increased by reducing the density of the PEG linker on the protein backbone. In a recent publication, Browning et al. showed[77] that acrylamide-PEG-

isocyanate (AAm-PEG-I) linker can be incorporated onto proteins with PEG diacrylamide (PEGDAA) to enhance the hydrolytic stability and half-life of protein drugs. In another study da Silva Freitas et al.[78] reported site specific PEGylation of recombinant human growth hormone (hGH) by N-terminal PEGylation and microbial transglutaminase (mTGase) mediated enzymatic PEGylation and found that both of the strategies keeps the selectivity, secondary structure and pharmacokinetic profiles of the protein drugs. Finally, site directed PEGylation of fibroblast growth factor 21 (FGF21) known for treatment of type II diabetes was found to show sustained potency and minimum vacuole formation.[79]

Hydrogels allow fine-tuning of protein release by tailoring their crosslinking density via changes in polymer architecture, concentration, molecular weight, and/or chemistry. Other strategies to tailor drug release from hydrogels rely on reversible protein-polymer interaction or encapsulation of the protein in a second delivery system (e.g., micro- or nanoparticles) dispersed in the hydrogel network.

In one example, Lyon and coworkers[69, 80] reported that loosely crosslinked microgels (composed of a random copolymer of N-isopropylacrylamide (NIPAm) and acrylic acid (AAc)) demonstrated a high loading capacity for protein. These kinds of microgels or microgel-based devices can be potentially used as protein drug delivery systems. The investigation also elucidated the effects of gel network structure on macromolecule encapsulation.[80] Using network swelling and ionization as a tunable variable, future delivery vehicles may be designed with specific encapsulation and release properties for biomedical applications as shown in Fig. 4.

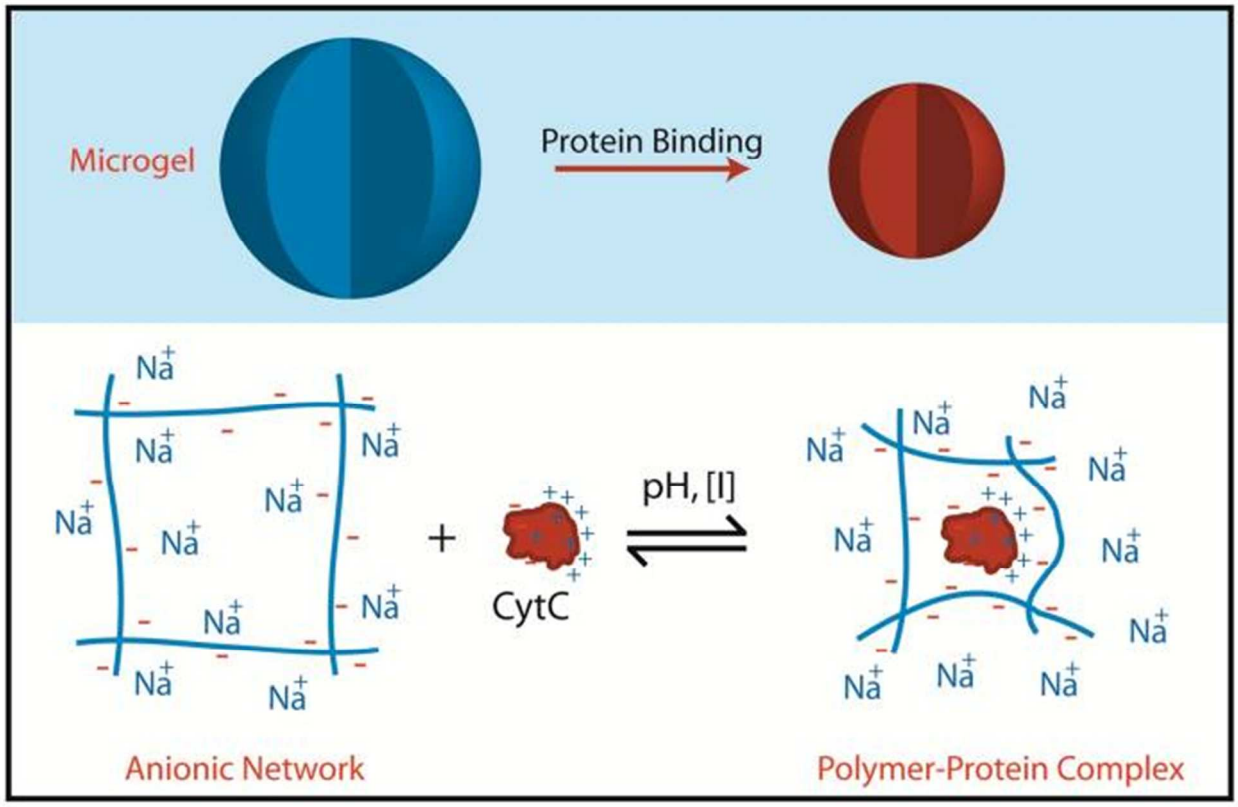

Fig. 4. Proposed interaction of microgels with oppositely charged cytochrome complex (cyt c.) Reproduced with permission from ref. 80

In general, protein delivery from micro/nanogels suffers from a limited release time due to the large surface area of nanogels compared to that of macrogels. For sustained release over longer periods of time, nanogels can be incorporated into macrogels. Polymer-protein conjugates are one of the methods that researchers use to develop new protein or peptides drug delivery systems. In this way, the low delivery efficiency and poor stability against proteases in the cell, which digest the protein, caused by other delivery methods can be limited. Yan and co-workers[81] developed a novel delivery platform based on nanocapsules consisting of a protein core and a

thin permeable polymeric shell that can be engineered to either degrade or remain stable at different pH. Non-degradable capsules show long-term stability, whereas the degradable ones break down their shells, enabling the core protein to be active once inside the cells as shown in Fig. 5. Multiple proteins can be delivered to cells with high efficiency while maintaining low toxicity.

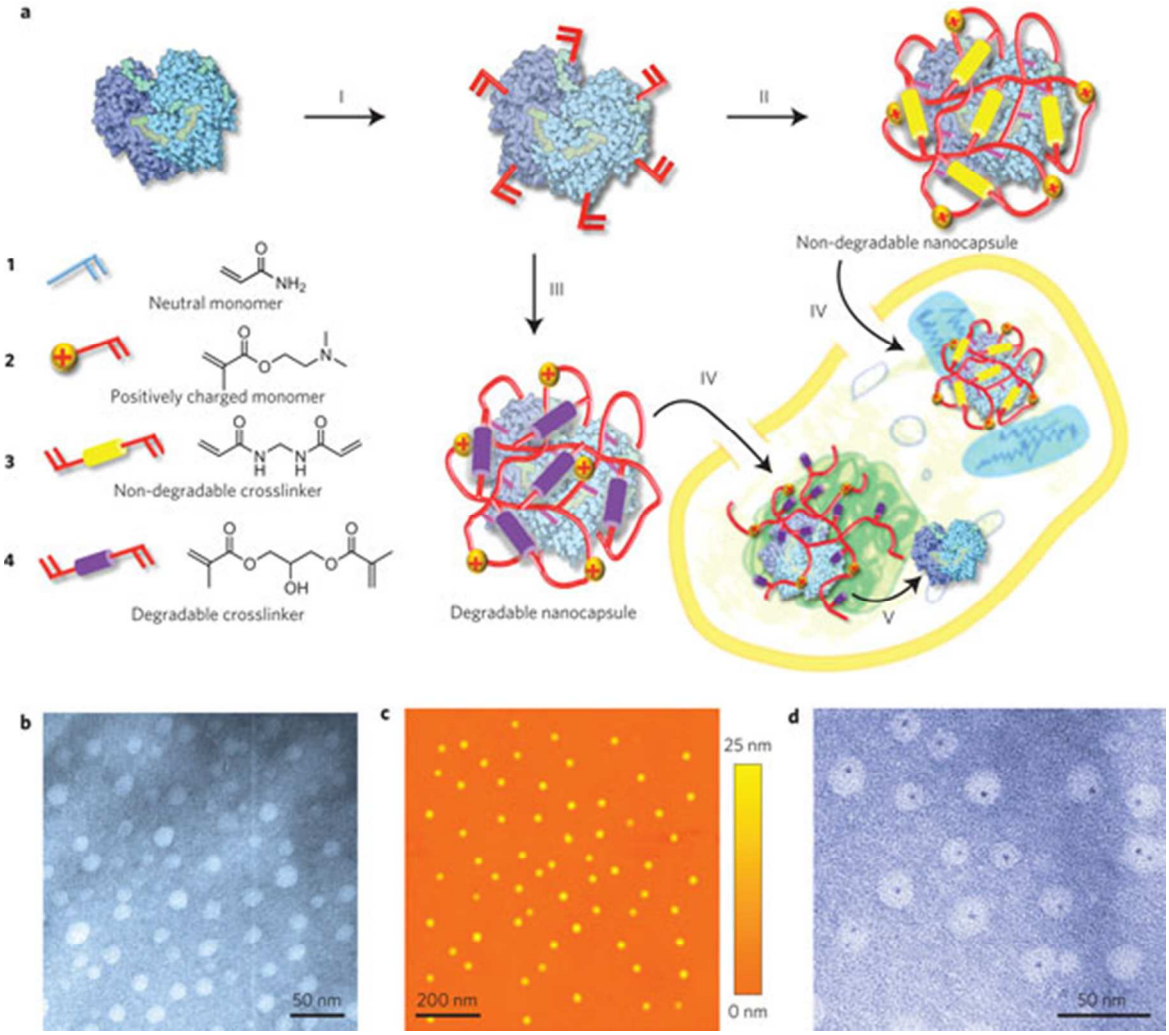

Fig. 5. a) Schematic showing the synthesis and cellular uptake of cationic single-protein nanocapsules with degradable and non-degradable polymeric shells prepared by in situ co-polymerization of acrylamide 1, 2-dimethylaminoethyl methacrylate 2 and non-degradable

crosslinker methylenebisacrylamide 3 or acid-degradable glycerol dimethacrylate 4: I, formation of polymerizable proteins by conjugating polymerizable acryl groups to the protein surface; II, formation of non-degradable nanocapsules from 1, 2 and 3; III, formation of degradable nanocapsules from 1, 2 and 4; IV, cellular uptake of the degradable or non-degradable nanocapsules via endocytosis; V, shells of degradable nanocapsules break down after internalization to release the protein cargoes, allowing them to interact with large molecular substrates. (b) Representative TEM and (c) AFM images of the HRP nanocapsules. (d) TEM image of nanocapsules containing a single 1.4-nm gold-quantum-dot-labelled HRP core confirms the formation of a single-core nanoscale architecture. Reproduced with permission from ref. 81

More recently, Buchar et al.[82] reported on the synthesis of thermoresponsive, degradable, and hydrophilic nanogels for the uptake and delivery of proteins. Core cross-linked micelles (CCL, also termed nanogels) were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using the macro-RAFT agent poly(2-methacryloyloxyethyl phosphorylcholine) (poly(MPC)). Nanogels containing a poly(MPC) shell and an acid degradable, poly(methoxydiethylene glycol methacrylate) (poly(MeODEGM)-co-poly(2-aminoethyl methacrylamide hydrochloride) (poly(AEMA) core were obtained in a one-pot process. They found that these nanogels can uptake protein (e.g., insulin) and release the same protein over extended period of time (24-48 h) at low pH.

In another publication, RAFT polymerization was used to generate water soluble poly(ethylene glycol)-*b*-poly(2-(hydroxyethyl)methacrylate-co-acryloyl carbonate) (PEGP(HEMA-co -AC)), which formed disulfide crosslinked nanogels in the presence of cystamine, as shown in Fig. 6.[83] These nanogels were stable under physiologically relevant conditions, but degrade rapidly in presence of 10 mM dithiothreitol (DTT). Furthermore, the

authors found that fluorescein isothiocyanate (FITC) labelled cytochrome C can be loaded into the nanogels with more than 98% loading efficiency and the release is minimal at physiological conditions. Again, in the presence of DTT, the nanogels degraded, which released up to 97% of the loaded protein within 22 hours. These nanogels were found to be nontoxic to HeLa cells up to a tested concentration of 2 mg/mL.

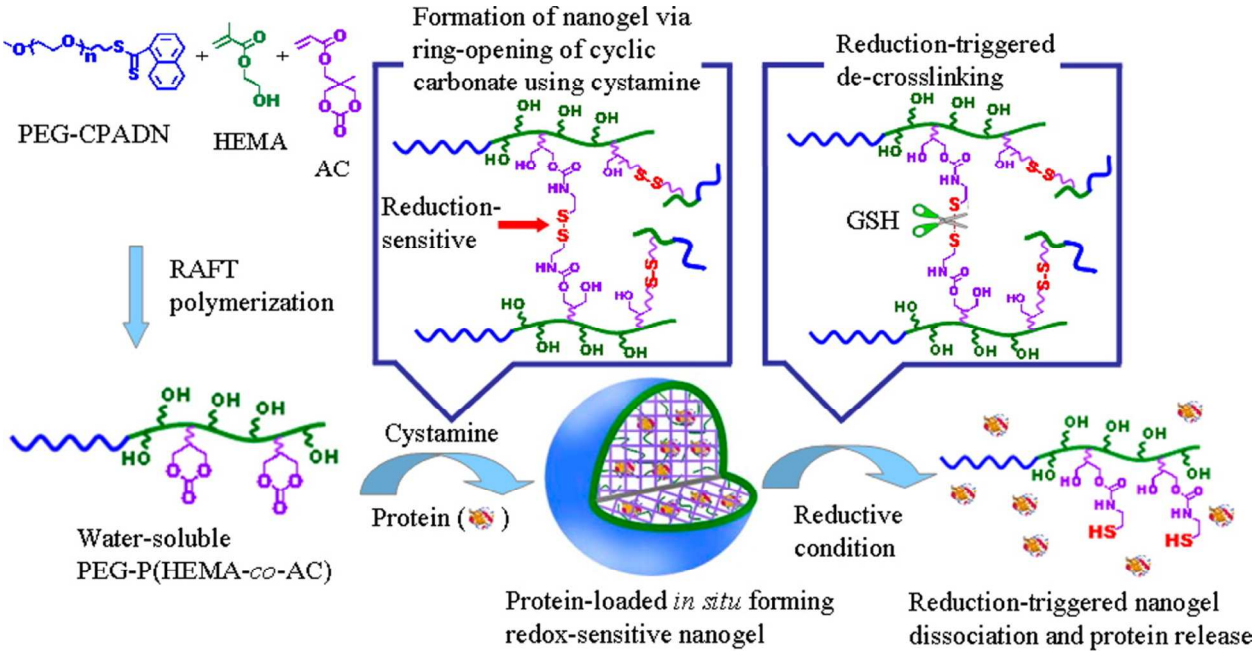

Fig. 6. Illustration of the in situ formation of the reduction-sensitive nanogels, and their degradation in the presence of DTT to release their protein cargo. Reproduced with permission from ref. 83

3. Conclusions

The transformation of polymers from the concept of mere “large molecules” to an indispensable part of life was not so easy. Researchers invested a significant amount of effort over more than a half-century to understand polymers and their structure-property relationships.

Control over their synthesis, self-assembly, and chemistry created new opportunities in responsive materials, gels and composites. These advances are constantly leading to new applications, perhaps most interesting are their utility for maintaining human health. Specifically, and as was highlighted in this review, the usage of polymers for disease diagnostics and drug delivery have led to exciting advances, which will positively affect lives. While this is the case, there are numerous challenges that lie ahead. One such challenge is the continued development of biocompatible and water-soluble polymeric materials for disease diagnostics and drug delivery. For example, much effort is required to design the next generation of biocompatible polymeric materials that degrade into inert fragments. Furthermore, advances in the fields of biopolymers and biomass-derived polymers will certainly lead to new and exciting opportunities for clinicians and researchers alike. While these are just a couple of examples of where progress can be made, there is enough discovery to be made in these select areas that it could last many the remainder of their careers to achieve.

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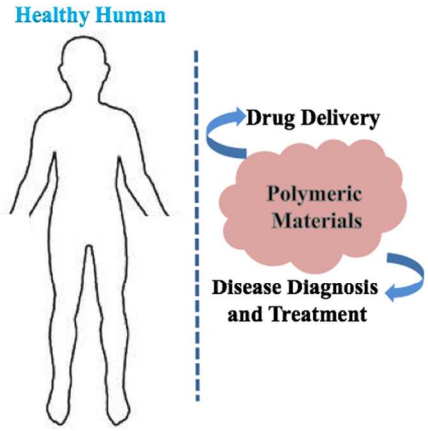

Responsive polymers have found their way into sensors and drug delivery platforms; some examples of biosensing and protein delivery are highlighted here.