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Review

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Protein−Polymer Delivery: Chemistry from the Cold Chain to the Clinic

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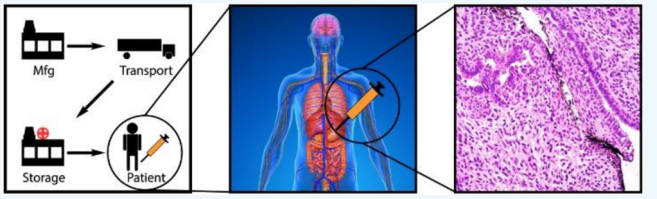
ABSTRACT: Drug delivery is commonly thought of as the performance of a drug *in vivo*. Rather, the process of drug delivery can comprise of the journey of the drug from manufacturer to clinic, clinic to patient, and patient to disease. Each step of the journey includes hurdles that must be overcome for the therapeutic to be successful. Recent developments in proteinaceous therapeutics have made the successful completion of this journey even more important because of the relatively fragile nature of proteins in a drug delivery context. Polymers have been demonstrated to be an effective complement to proteinaceous therapeutics throughout this journey owing to their flexibility in design and function. During transit from manufacturer to clinic, the proteinaceous drug is threatened by denaturation at elevated temperatures. Polymers can help improve the thermal stability of the drug at ambient shipping conditions, thereby reducing the need for an expensive cold chain to preserve its bioactivity. Upon arrival at the clinic, the drug must be reconstituted into a suitable formulation that can be introduced into the patient. Unfortunately, traditional drug formulations relying on oral administration are generally not suitable for proteinaceous drugs owing to the hostile environment of the stomach. Other traditional methods of drug administration—like hypodermic injections—frequently suffer from low patient compliance. Polymers have been explored to design drug formulations suitable for alternative methods of administration. Upon entry into the body, proteinaceous drugs are at risk for identification, destruction, and excretion by the immune system. Polymers can help drugs reprogram immune system response and, in some cases, elicit a synergistic immune response. The next phase of research on protein−polymer-based therapeutics encourages a holistic effort to design systems that can survive each stage of the drug delivery journey.



INTRODUCTION

Proteinaceous therapeutics have begun to surpass conventional small drug pharmaceuticals as industry profit leaders and are widely considered to be the next platform for next-generation custom-therapeutics.[1] Unlike small molecule therapies, however, proteinaceous therapeutics are (i) typically less thermally and solvolytically stable,[2] (ii) their formulation is more complicated as oral delivery is rarely possible,[3] and (iii) as foreign nanoscale proteins, they have an innate capacity to trigger the adaptive immune system,[4−6] which may or may not be desirable. While these three focus areas have emerged as disparate research topics, they share many intellectual overlaps that make their consideration as a whole important. Specifically, synthetic polymers have been used to alter the chemical, physical, or physiological properties of proteinaceous materials via different mechanisms. In many cases, the polymers are either directly attached via bioconjugation or formulated around the specific properties of the protein. Consequently, polymeric materials—married to their proteinaceous therapeutics—have been created to stabilize their cargo for improved shipping, formulation, targeting, and immune evasion. This Review does not intend to cover any single area in great detail—though we direct the reader to comprehensive reviews—rather, we aim to highlight the importance of "drug delivery" as a longitudinal concept, rather than one that is focused on *in vivo* performance. We also aim to highlight these areas as sufficiently interconnected to encourage more synergistic efforts to emerge as a way to improve performance of proteinaceous drugs from the time they are manufactured to the time they exert their therapeutic effect.

We have broken this Review down to three connected foci, as highlighted in Scheme 1. Our first focus is on the macroscale: protein and vaccine protection from creation in a lab or factory until administration into a patient. On the macroscale, drug delivery is focused on getting drugs to their patients with minimal loss of drug effect (Scheme 1A). This entails the proteins surviving several transport and storage steps and ultimately successful preparation or reconstitution at the point of administration into the patient. The existing infrastructures in the developing world are frequently inadequate for these drugs to survive transit to the hard-to-reach places of the globe. Failures in the drug transport infrastructure lead to great cost and potentially ineffective medication.[7] We highlight here several techniques of polymers

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Scheme 1. Illustration of Drug Delivery from A) Macroscale to B) Microscale to C) Nanoscale

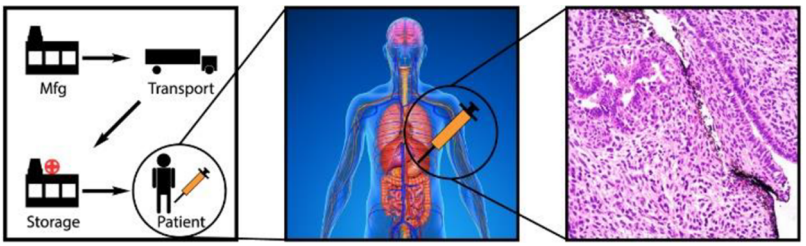

Table 1. Thermostability in the Macroscale

category	ref	biomolecule	polymer system	thermostability
solvent removal	21	LaSota vaccine	trehalose, PVP[a]	40 °C, 21 days
	22	AdHu5,[b] VSV[c]	mannitol/dextran, trehalose, Eudragit	improved T_g
	23	cholera vaccine	Eudragit, Carbopol, alginate	25 °C, 1 year 40 °C, 6 months
immobilization	24	G-CSF[d]	poly(caprolactone)	60 °C, 30 min
	25	goat anti-rabbit IgG, anti-cTnI[e]	silk fibroin	40 °C, 1 week
	26	β-galactosidase, alkaline phosphatase, T4 DNA ligase	photodegradable hydrogel	60 °C, 4 weeks
	27	TMV[f]	ZIF-8	boiling water
	28	NGAL,[g] CA-125	ZIF-8	60 °C, 4 weeks
bioconjugation	29	amelogenin	PNIPAM[h]	40 °C
	30	β-glucosidase	PNIPAM[h]	70 °C
	31	GFP[i]	POEGMA[j]	90 °C
	32	catalase	PAA[k]	50 °C

[a]Poly(vinylpyrrolidone). [b]Adenovirus human serotype 5. [c]Vesicular stomatitis virus. [d]Granulocyte colony-stimulating factor. [e]Anticardiac troponin I antibody. [f]Tobacco mosaic virus. [g]Neutrophil gelatinase-associated lipocalin. [h]Poly(*N*-isopropylacrylamide). [i]Green fluorescent protein. [j]Poly(oligo(ethylene glycol) methyl ether methacrylate). [k]Poly(acrylic acid).

being used to overcome the limitations of the current system. These are primarily centered on improving thermal stability over time by means of solvent removal, protein immobilization, and bioconjugation.

Our second focus is on the microscale: the formulation of the drugs designed for bioavailability, prolonged release, and next-generation methods of administration (Scheme 1B). Because proteins are easily degraded in the gut, they are rarely formulated for oral delivery. Owing to this, time-release formulations that have been so successful in oral therapies must be rethought as the alternative is either continuous IV administration—an impractical approach for many people—or repeated injection. Polymers have shown to be quite effective here as well. We highlight several modes of enhancement for administration into the body based on specific polymer formulations.

Our third focus is on the nanoscale: making sure that the drugs deliver their therapeutic effect unhindered. On the nanoscale, drug delivery is focused on making the therapeutic effect happen without the body's innate defenses interfering (Scheme 1C). The emergence of nonhuman proteinaceous nanoparticles or engineered protein therapies raises the possibility of unwanted immune activation.[8] We highlight here several usages of polymers to help "reprogram" these defenses to activate, inhibit, or augment immune responses.

FROM FACTORY TO CLINIC: DRUG DELIVERY ON THE MACROSCALE

Drug delivery ultimately begins upon manufacture in a laboratory or factory. There is a significant journey between that point and administration into a patient. Proteinaceous drugs and vaccines are, by nature, less stable at elevated temperatures.[2] The bioactivity of vaccines and therapeutic biomolecules is maintained by their shape, which comes from the way the proteinaceous material is folded. As such, changes in temperature outside the stable region can lead to unfolding and subsequent reduction in bioactivity as well as loss of vaccine effectiveness.[9] Therapeutic proteins such as insulin,[10] therapeutic antibodies,[11] growth hormones,[12] erythropoietin,[13] and virus capsids used as vaccines[14] require stabilization to survive outside the thermal window, and high-throughput screening[15] must be carried out to identify vaccine candidates. Researchers have been investigating this issue for over 90 years.[16] Polymers have been shown to be promising components in ambient temperature methods for delivering drugs from point of creation to point of administration. There are various strategies pursued by researchers to tackle protein stability using polymers. One of the reasons for temperature-based unfolding is the effect of solvent molecules in transferring thermal energy from the bulk solution to the protein peptide units, disrupting hydrogen bonding, and imparting enough energy to overcome the energy barrier keeping the protein conformation in a local minimum energy state along the energy landscape.[2,9]

Cold Chain Drug Delivery. The World Health Organization (WHO) recommends that vaccines be stored from 2−8 °C.[17] Currently, in order to keep vaccines within the stable temperature window, a "cold chain" infrastructure is required. This is a system of continuous refrigeration from manufacture to storage to transportation to the clinic to administration into the patient. This includes interim warehouse storage, intermediate hospitals or clinical centers, even the patient's home, and all the transportation steps in between. This sort of infrastructure is also used in food preservation, storage, and distribution.[18] Such a cold chain system requires a large number of refrigerated vehicles, including cars, trucks, aircraft,

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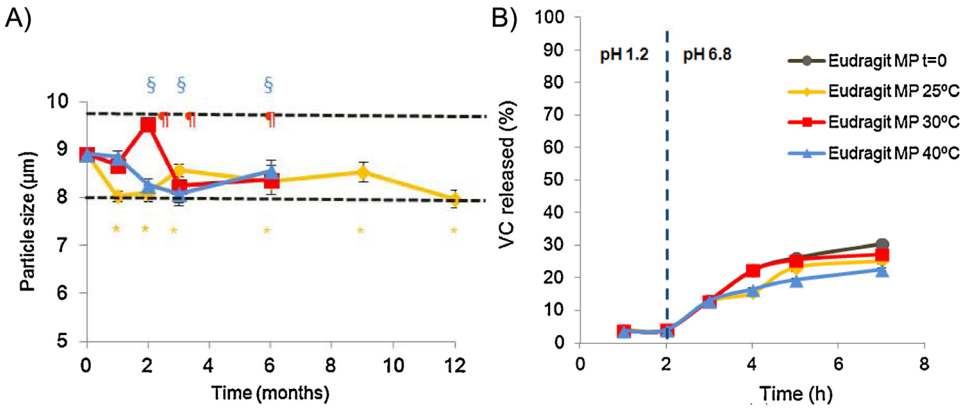

Figure 1. A) Particle size during storage of Eudragit microparticles and B) gastroresistance assay of Eudragit microparticles. Adapted from ref 23, with permission from Elsevier.

boats, trains, and even heat-protective packaging. This requires immense costs to set up, maintain, regulate, and administer in order to remain effective, including significant governmental input. As such, geographically remote places, developing nations, impoverished regions, areas struck by disaster, warzones, and locations lacking government generally have a diminished capability, if any, to support such a system. Even in regions where the cold chain is already established, significant loss of vaccine occurs owing to malfunctions or breakdowns in the refrigeration systems.[19] Total cold chain costs are highly dependent on maintaining temperature in individual components; small temperature perturbations have a large impact on the overall costs.[20]

Several methods have been described recently that improve protein and vaccine stability under shipping and storage temperature conditions (Table 1). These improvements allow less overhead in maintaining refrigeration—the temperatures do not have to be lowered as much—or bypassing the need for a cold chain entirely, allowing traditional ambient-temperature transportation and storage methods. While this does not directly address the problems of vaccine distribution in hard-to-access areas, it allows reallocation of resources away from cold chain management toward addressing those needs. We will focus on three areas: solvent removal, protein immobilization, and bioconjugation.

Solvent Removal. One method to preserve or enhance thermal stability is to remove the solvent from the formulation. This can be accomplished by lyophilization (freeze-drying), spray-drying, or vacuum foam drying to create solid products suitable for shipping without liquid handling or refrigeration. These formulations require stabilizers while drying; often, saccharides such as sucrose or trehalose[33−35] have been used to great effect. Polymers have also been used to stabilize the formulation during solvent removal. Pisal et al.[21] used poly(ethylene glycol) (PEG) and poly(vinylpyrrolidone) (PVP) as a stabilizer of the LaSota virus while vacuum foam drying to increase the virus titer after incubation at 40 °C for 21 days. They found that PEG and PVP as stabilizers also improved the foam characteristics, allowing for a better formulation suitable for shipping. Eudragit, an anionic copolymer of poly(methacrylic acid-*co*-methyl methacrylate), was used by LeClair et al.[22] and Pastor et al.[23] in spray-dried oral vaccine formulations to avoid degradation in the stomach. LeClair maintained thermostability of human adenovirus type 5 and vesicular stomatitis virus stabilized by saccharides and coated in gastroresistant polymer. Pastor protected cholera vaccine for six months at 40 °C and one year at 25 °C, as determined by particle size (Figure 1A) and vaccine release (Figure 1B). While the primary focus of these two studies is preventing premature release and subsequent protein degradation in the stomach, they both also show improved thermostability and potential for cold-chain-free transport. These formulations highlight a synergy of drug delivery on macro- and microscales, as the polymer used to confer increased thermostability also allows the vaccine to survive the low pH stomach conditions, permitting oral administration and extending the vaccine's journey.

Protein Immobilization. In addition to solvent removal, immobilization is an important method of protein protection. Encapsulation in various materials has shown to be effective in creating a barrier between the protein and the external environment, providing increased stability against unfolding or solvent effects. This immobilization can be done by entangling the protein within polymer chains, cross-linking polymer chains around the protein to form a gel, or holding the protein in place within rigid crystalline materials, such as metal−organic frameworks (MOFs).

Immobilization by polymer chain entanglement[36] or within hydrogels[37] allows the protein to be held in place and resist conformational changes. The polymers can be designed with functional groups along the backbone to allow postpolymerization reactions for installing side chains or cross-linking. These side chain modifications may include common stabilizers like trehalose, as used by Pelegri-O'Day et al.[24] to improve the stability of granulocyte colony stimulating factor against shipping temperatures of 60 °C (Figure 2). They used degradable poly(caprolactone) (pCL) as the backbone, modified with thiol−ene chemistry to develop a library of side chains. The use of trehalose here is no surprise, given its success as a stabilizer on its own. In addition to synthetic polymers, the immobilizing polymer could even be a protein itself. Silk fibroin has been used[38] to coat or encapsulate biologics and improve stability. Wang et al.[25] coated plasmonic biochips on gold nanorods with silk fibroin film to preserve antibody activity at 40 °C for 1 week (Figure 3). The thermostability of the silk is not in question because it acts as a sacrificial layer, immobilizing the antibodies with hydrogen bonds and hydrophobic interactions, and is easily removable with a simple water rinse to restore antibody binding capability. Such reconstitution of bioactivity is an important facet of the drug delivery journey; the drugs need to be successfully administered into the body to be of use. For

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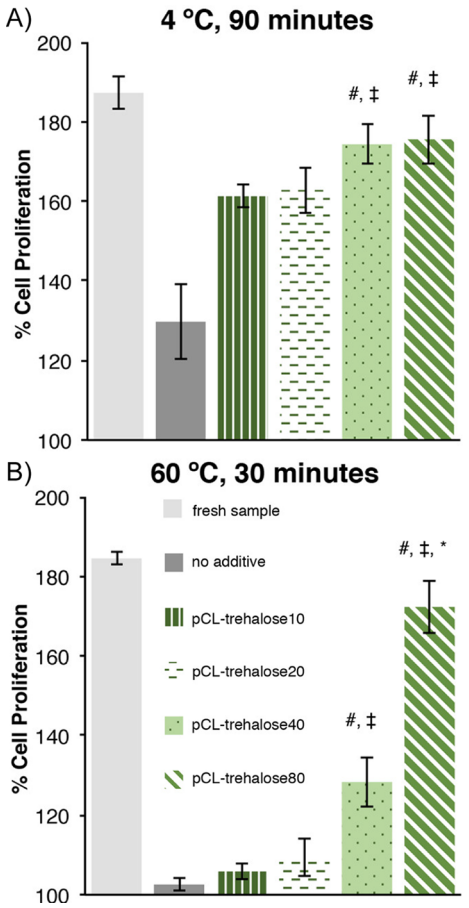

Figure 2. Improvement of thermal stability by immobilization in pCL with trehalose side chains of different lengths at A) 4 °C for 90 min and B) 60 °C for 30 min. Adapted with permission from ref 24. Copyright 2017 American Chemical Society.

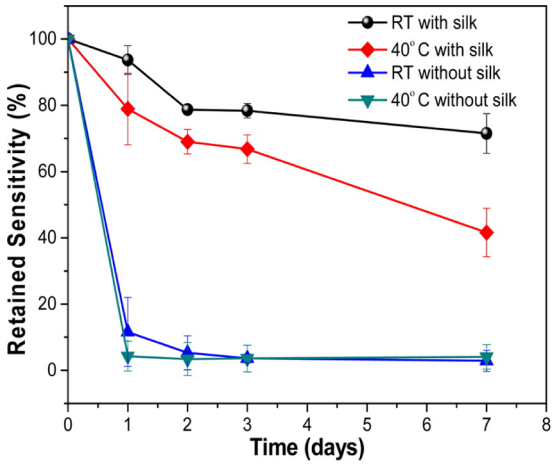

Figure 3. Improved thermal stability through silk film immobilization of gold nanorod plasmonic biochips conjugated with rabbit IgG. Adapted with permission from ref 25. Copyright 2016 American Chemical Society.

example, Sridhar et al. encapsulated[26] various enzymes in a photodegradable hydrogel that preserved bioactivity at 60 °C for up to 4 weeks. This gel keeps the protein stable during transport, and reconstitution upon arrival for patient administration is performed with UV light to trigger degradation of the gel and recovery of the drugs.

In contrast to flexible polymer chains, another method is to use MOFs as immobilizers to provide a rigid structural support.[39] These metal ion clusters and organic linker struts form coordination polymers that hold the protein in place and prevent changes in global conformation, thereby improving stability. These rigid crystalline porous materials have been shown to stabilize biomolecules against temperatures as high as 180 °C.[40] Here, we highlight a few studies utilizing zeolitic imidazolate framework 8 (ZIF-8), a MOF able to form in aqueous conditions. Our own group has shown[27] that ZIF-8 protection can be applied to tobacco mosaic virus (TMV) particles with a tunable shell thickness. The ZIF-8-encapsulated TMV particles show stability against harsh solvents and boiling water. We also show[41] that morphological configurations are dependent on precursor concentrations. Singamaneni's lab[28] encapsulated CA-125, a serum/plasma biomarker for ovarian cancer, in ZIF-8 crystals by drop-drying on paper with the ZIF-8 precursors (Figure 4A), tested the thermal stability (Figure 4B), and mailed it from Missouri to California and back for later extraction and analysis (Figure 4C). This study demonstrates a practical ability to ignore the cold chain and ship sensitive biological samples without refrigeration through the use of ZIF-8 encapsulation.

These immobilization methods improve thermostability by physically holding the protein higher order structure in place while not being covalently attached. This allows the proteins to be potentially released in their native state, should the drug be designed with that in mind.

Bioconjugation. Another method of protecting proteins is bioconjugation of polymers directly to the protein. Attachment of the polymer directly allows the polymer to stay with the protein longer through its journey, instead of separating upon reconstitution or administration. This may be desired if the polymer is also intended to interact with the immune system in addition to protecting the protein through transport. This provides another synergy between different scales of the drug delivery journey. Covalent attachment also keeps the polymer with the protein even if the solution is diluted.

Polymer conjugation techniques can be generally categorized into three groups: "graft to", "graft from", and "graft through".[42] "Graft to" conjugation is where the polymerization occurs first, and then the full polymer chain is attached to the protein (Figure 5A). This method allows the polymerization to occur separately in conditions harmful to the protein but generally has lower conjugation yields, because the now larger polymer chains are more sterically hindered from conjugation sites. Also, it is more problematic to purify the conjugates because unconjugated polymer chains are closer in size to the protein. In contrast, "graft from" methods involve conjugation of small molecule initiators directly to the protein followed by *in situ* polymerization growing polymer chains outward from the protein (Figure 5B). This method allows for higher density of the smaller initiator molecules on the protein, leading to higher polymer conjugation yields. Purification is also easier, as free monomers and other small molecules present are easily removed via dialysis or size exclusion media. There is also another method, "graft through", that involves copolymerization of biomacromolecules that act as monomers themselves with polymerization that cross-links multiple proteins (Figure 5C). Controlled radical polymerizations such as atom transfer radical polymerization (ATRP) and reversible addition–fragmentation chain transfer (RAFT) are frequently used in bioconjugations to control chain growth and introduce new

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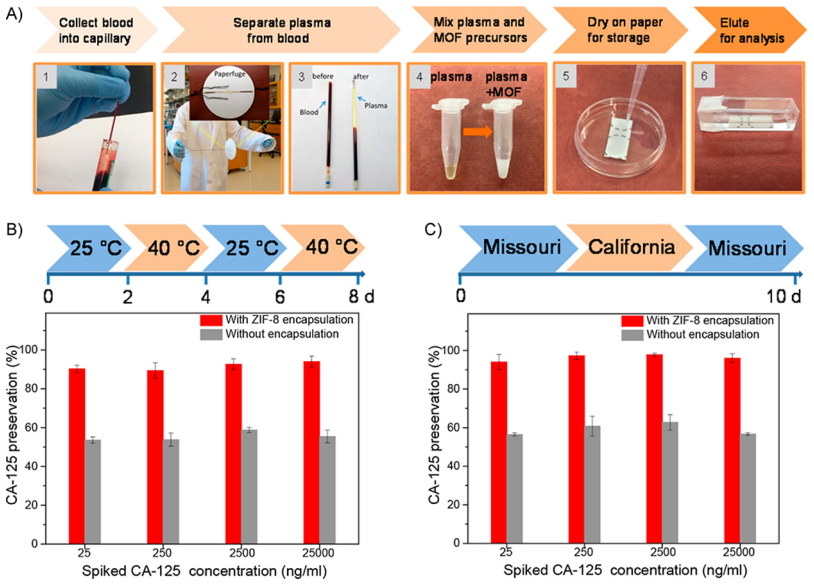

Figure 4. A) Scheme of encapsulation of CA-125 on paper for mailing. B) Thermal and C) shipping stability with ZIF-8 encapsulation. Reprinted with permission from ref 28. Copyright 2018 American Chemical Society.

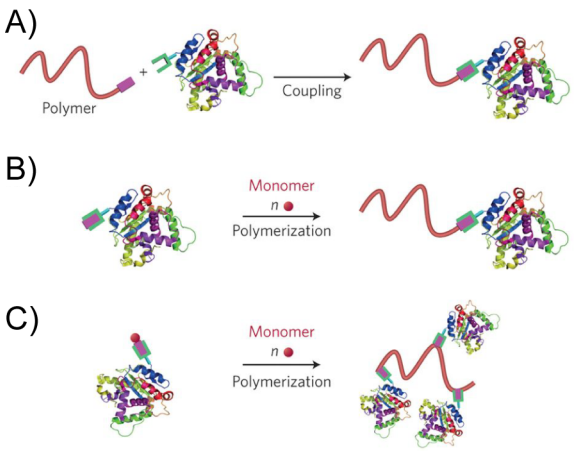

Figure 5. Polymerization methods of bioconjugation: A) "graft to", B) "graft from", and C) "graft through". Reprinted with permission from Springer Nature: Nature Materials, ref 42. Copyright 2015.

functional groups.[43] Examples of ring-opening metathesis polymerization (ROMP) have also been appearing in the literature for these applications.[44]

The choice of polymer and method of attachment depend on the desired property it will add to the protein. For example, poly(*N*-isopropylacrylamide) (PNIPAM) is a thermoresponsive polymer that drastically changes solubility in water as a function of temperature, allowing for a stimuli-responsive bioconjugate. PNIPAM was used by Jiang et al.[29] grafted from amelognin with ATRP, enhancing the thermal self-assembly properties of the protein to create potential drug carrier nanospheres. PNIPAM was also used by Mukherjee et al.,[30] grafting from β-glucosidase using RAFT polymerization, retaining up to 70% of its enzymatic activity at 70 °C compared to native enzyme. In both of these studies, the hydrodynamic diameter of the bioconjugates drastically increased with temperatures greater than the PNIPAM lower critical solution temperature of 33 °C, highlighting the thermoresponsive nature the PNIPAM polymer confers to the conjugates.

The polymer itself does not have to be thermoresponsive, however. Poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) is used to enhance circulation and retention times in the body, and poly(acrylic acid) (PAA) is used as a polyelectrolyte, but both can still confer thermostability to the protein. For example, Hu et al.[31] used circularized green fluorescent protein (GFP), which was linked via the C- and N-termini, and grafted POEGMA from it using ATRP. After thermal denaturation, the conjugated GFP recovered its fluorescence after just a few minutes compared to no recovery for linear GFP or linear GFP-POEGMA (Figure 6). The

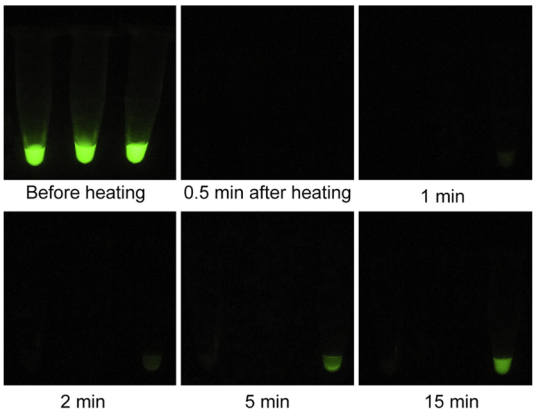

Figure 6. A) Visual recovery of GFP fluorescence after denaturing at 90 °C of *l*-GFP (left tube), *l*-GFP-POEGMA (middle tube), and *c*-GFP-POEGMA (right tube) and B) fluorescence recovery percent. Adapted from ref 31, with permission from Elsevier.

bioconjugations hold the protein structure together enough that refolding of the fluorophore is possible. The POEGMA also enhanced tumor retention, a synergy between the macro- and nanoscales. Riccardi et al.[32] used EDC coupling to graft PAA to catalase. The bioconjugates showed retention of enzymatic activity and thermal stability up to 80 min at 50 °C, in addition to lowered rates of trypsin and chymotrypsin digestion and increased activity rate in the presence of

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Table 2. Delivery Systems on the Microscale

category	ref	biomolecule	polymer	formulation
injections	26	β-galactosidase, alkaline phosphatase, T4 DNA ligase	photodegradable hydrogel	reconstitution
	49	Qβ VLP[a]	PEG[b]	bioconjugation
	50−52	insulin, LyzSH[c], GOX[d], HRP[e]	trehalose glycopolymer	bioconjugation
oral	37	BSA[f]	alginate	spray drying
	53	insulin	PEG,[b] ethyl cellulose	tablet
	22	AdHu5,[g] VSV[h]	mannitol/dextran, trehalose, Eudragit	spray-drying
	23	cholera vaccine	Eudragit, Carbopol, alginate	spray-drying
alternative	54	influenza	PVA[i]	microneedles
	55	insulin	PVA[i] trehalose	microneedles
	56	Qβ VLP[a]	PLGA[j]	implants

[a]Virus-like particle. [b]Poly(ethylene glycol). [c]Thiolated lysozyme. [d]Glucose oxidase. [e]Horseradish peroxidase. [f]Bovine serum albumin. [g]Adenovirus human serotype 5. [h]Vesicular stomatitis virus. [i]Poly(vinyl alcohol). [j]Poly(lactic-*co*-glycolic acid).

inhibitors compared to native enzyme. It is worth noting that often a systematic approach is required to test various formulations to see which polymers enhance and which polymers hinder the desired effects.[45−47]

Looking Ahead. All of these methods can lead to enhanced thermal stability of proteins that can be used to protect potential drugs and carriers throughout the long journey from factory to clinic. As cold-chain bypass methods have begun to proliferate, the Controlled Temperature Chain Working Group of the WHO recently expanded[48] their guidelines for temperature storage for certain vaccines stable up to 40 °C. Increased vaccine, protein, and biomolecule stability at ambient or above ambient temperatures can drastically reduce costs and greatly improve public health. These reduced costs will provide a large incentive to adopt these techniques and secure government and private sector support. There is room for further improvements, however.

More research is required regarding reconstitution of vaccine solutions for delivery after formulation for cold-chain free transport. Some of the researchers highlighted here have begun to do so, but most have focused primarily on maintaining stability through transport and storage, an important hurdle to overcome first. As improvements in stability continue, development could be done furthering our fundamental understanding of general protein stabilization methods that could be applicable to a broad range of proteins, instead of protein- or vaccine-specific formulations. Such development would allow more general logistic protocols to be adopted by administrating agencies, further lowering costs.

FROM CLINIC TO PATIENT: DRUG DELIVERY ON THE MICROSCALE

The protected biomolecule has safely arrived at the clinic and now has to be introduced into the patient. Normally with small molecules, the formulation can be taken easily at home as a pill to provide continuous release over several hours.[57] Orally available drugs have high patient compliance[58] compared to injectable protein therapeutics because patients can take pills in the comfort of their homes while avoiding visits to the clinic and painful needle injections.[59,60] Additionally, injectable proteinaceous therapeutics are commonly administered in multiple doses; for example, insulin injections must be taken before every meal, which is a tedious problem that can be avoided with improved therapeutics.[61,62] Unfortunately, biomolecules are not often orally available because the proteolytic and acidic conditions in the upper digestive tract denature most proteins.[63,64] Thus, a clinician- and patient-friendly development for proteinaceous therapeutics would include formulations that offer improved stability, painless injections, and delayed-release delivery. We have discussed methods of bioconjugation and noncovalent entanglement as ways to protect proteins with polymers. At this point, the proteins here are already prepared, and our focus now shifts to explaining how they are reconstituted before delivery into the body (Table 2). We will highlight current developments in injection, oral, and alternative methods that offer prolonged delivery and patient compliance.

Hypodermic Injections. The primary routes of protein administration have been injections, but for this method to continue being used as the main choice, improvements in formulations must be investigated. Specifically, the steps taken in the formulation of protein−polymer combinations must be considered before the delivery into the body, as proteins can easily aggregate and denature.[65] We will not focus on polymers that offer a triggered stimuli-release as this has been extensively reviewed.[42,66−69] Our goal here is to discuss the reconstitution of proteins for injections and provide examples of improvements being investigated for injections. For instance, a prominent candidate discussed above for bypassing the cold chain was a photoresponsive hydrogel investigated by Sridhar et al., who demonstrated[26] improved thermal stability up to 60 °C. The various enzymes encapsulated in the hydrogel then could be reconstituted easily by UV light exposure and administered via intravenous injection into a patient (Figure 7). However, in addition to needing a purification step, this method still requires multiple injections to provide the desired therapeutic effect. PEG is a very common polymer used to increase the thermal stability of proteins for the cold chain and *in vivo*. For formulations, PEG can improve the colloidal stability of proteins that have been conjugated heavily with molecules that are hydrophobic. For example, Chen et al. synthetically modified[49,71] Qβ—a virus-like particle (VLP) used as a nanocarrier—with doxorubicin, causing it to precipitate out of solution. Fortunately, they were able to overcome this colloidal instability by orthogonally bioconjugating PEG across surface-exposed disulfides on the VLP. This both improved the thermal and colloidal stability of the biocomposite. In a second example, Maynard and co-workers have synthesized[52] different trehalose glycopolymers that were attached to proteins by RAFT polymerization. This attachment improved thermal stability up to 90 °C—an affect attributed to the alcohol group on trehalose stabilizing the protein by replacing the water molecules that evaporate.[51] In following studies they tested their glycopolymer *in vivo*,[50] with site

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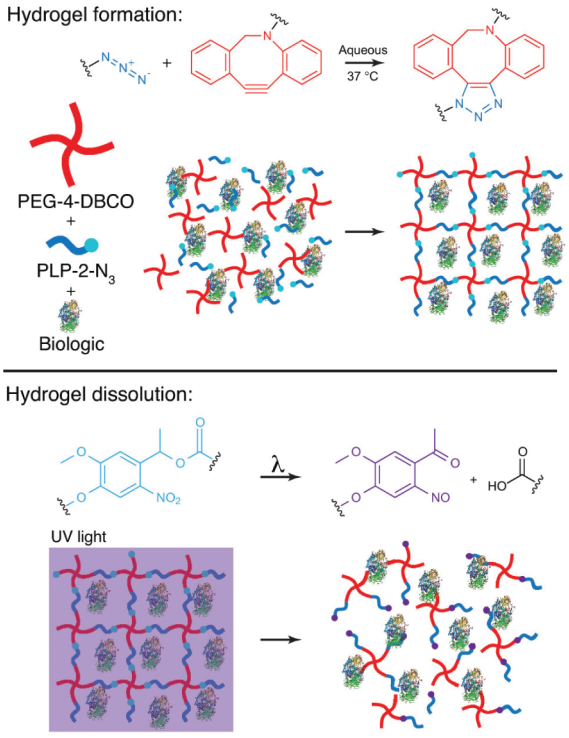

Figure 7. An example of reconstitution of a hydrogel after shining with ultraviolet light that can then be injected intravenously. Adapted with permission from ref 26. Copyright 2018 American Chemical Society.

specific attachment,[72] and for hydrogel formation[73,74] with the goal of improving thermal stability of the proteins. Their investigations with insulin have shown improved circulation half-life of the protein−polymer conjugate compared to the native protein while maintaining bioactivity despite being directly conjugated to the protein surface. Like PEG, trehalose is an example of a biocompatible polymer that can be used from manufacture to *in vivo*. Recently, Liow et al. formulated[70] a thermogel copolymer, poly(PEG/PPG/TPE urethane) (EPT), consisting of a hydrophilic PEG segment and a hydrophobic poly(propylene glycol) (PPG) segment that releases biomolecules in the body up to 14 days after injection (Figure 8). They used doxorubicin as a proof of concept and envision this as a technique to slowly release proteins over time—a direction needed for hypodermic injections that should be tested *in vivo* with a therapeutic protein.

Oral Delivery. An encouraging but technically challenging path that avoids hypodermic administration altogether is oral delivery.[75−77] Although, as stated earlier, this method has been difficult to formulate for proteins because of the physiological, physical, and chemical barriers that need to be overcome before finally flourishing as a method of delivery. Common methods for oral delivery include attaching proteins to cationic polymers[78−80] or polysaccharides[81] that protect them from the acidic environment. Polymers offer protective zones around the proteins to shield them from the denaturing environment of the gut and can be attached to permeation enhancers.[82−84] For example, the Whitehead lab ran[85] an MTT assay on phenyl piperazine derivatives—cell membrane permeation enhancers—with Caco-2 epithelial cells to show low cytotoxicity. After this discovery, they used ATRP to attach POEGMA to BSA followed by a sequential reaction to graft phenyl piperazine acrylamide.[86] In this study, they conducted an MTT assay to show that the polymer with piperazine had a lower cytotoxicity than free piperazine in Caco-2 epithelial cells. Additionally, the protein−polymer conjugate showed an

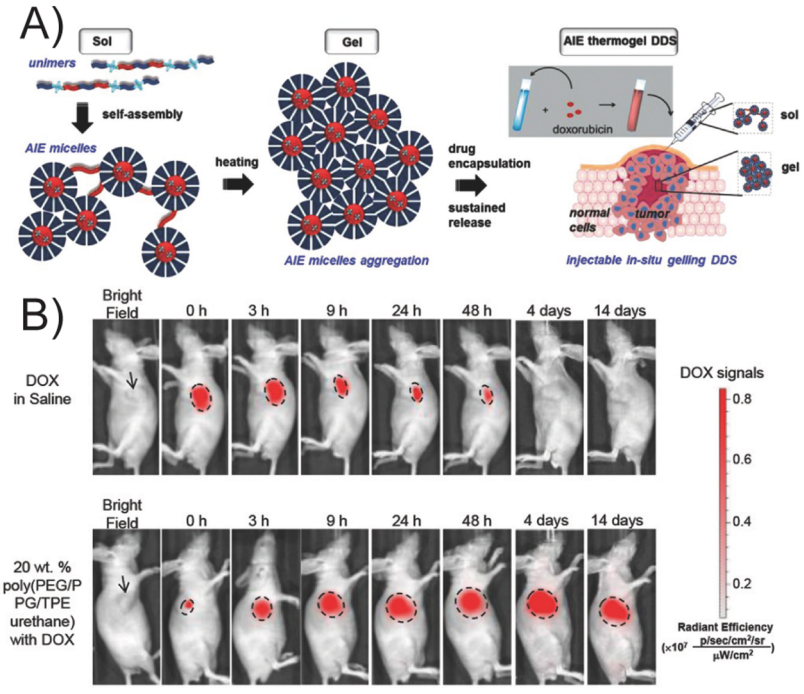

Figure 8. A) The mechanism of micelle particles that aggregate in the body. B) Top image is Dox, and the bottom image is Dox encapsulated inside the AIE micelle. Both samples were injected into mice and monitored for release over time. Adapted with permission from ref 70. Copyright 2017 John Wiley and Sons.

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improved permeation across a Caco-2 monolayer membrane compared to the unmodified protein *in vitro*. Another aspect for oral delivery that needs to be improved is drug release over time, which can be tuned by altering the length of the attached polymer[46] and by increasing the amount of cross-linking in a network. Hariyadi et al. used[37] ionic cross-linking to develop an alginate hydrogel that encapsulated bovine serum albumin (BSA). Alginate is a polysaccharide that easily dissolves in water and can be mixed with BSA to form a model freeze-dried oral vaccine with a process known as aerosolization (Figure 9).

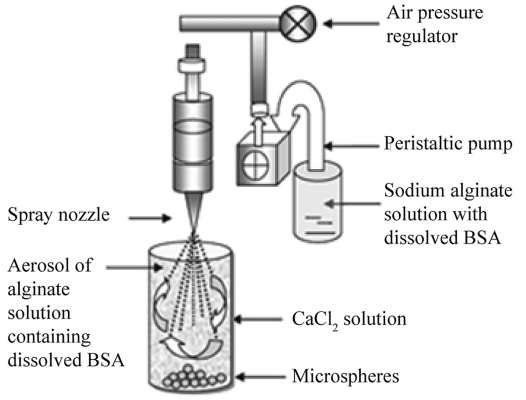

Figure 9. Machinery to produce alginate microspheres. Adapted from ref 37, with permission from Elsevier.

The vaccine was studied *in vitro* where it displayed minimal protein release in a low pH environment that mimicked the conditions of the stomach. Upon changing the environmental conditions to a pH of 7.4, which simulates the small intestines, the protein was slowly released over the course of 8 h. This phenomenon at neutral pH is attributed to the protonation of the sugars and displacement of calcium ions from the hydrogel network.[87] The Sun lab has developed[53] a method that delays release of insulin by incorporating the protein in a mesoporous phosphonate tablet and then dip-coating it in a solution of ethyl cellulose, PEG 400, and anhydrous ethanol containing diethyl phthalate (Figure 10A). The tablet was dipped two to four times to demonstrate that the release over time was dependent on the coating (Figure 10B). This is a promising study toward delayed release, which still needs to be investigated further *in vivo*. While all of these methods require multiple dosages owing to their quick release and low loading capacity,[88] they offer a patient-friendly method of self-administration.

Alternative Administration. Alternative methods have been extensively researched to incorporate small lipophilic drugs into polymers. A more in-depth explanation on specific techniques of incorporation, such as electrospinning, microneedles, and nanocarriers, can be found in several reviews.[66,89−92] A relatively new idea is to blend proteins with polymers to develop implants and microneedles—a painless injection that improves patient compliance. However, this necessitates the polymers to be biodegradable and biocompatible, and the conditions required to process the polymers must not denature the mixed proteins. Vrdoljak et al. blended[54] the influenza vaccine with trehalose to show improvements in thermal stability and, more importantly, developed a method to stabilize the vaccine in a microneedle without denaturing it. The method involves the dropcasting of trehalose, poly(vinyl

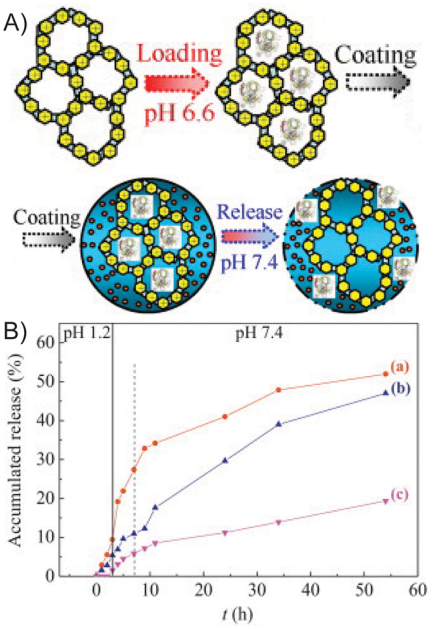

Figure 10. A) The loading of insulin into mesoporous framework and B) the simulated release over time. Adapted from ref 53, with permission from Elsevier.

alcohol) (PVA), and a vaccine mixture into a polydimethylsiloxane (PDMS) mold that concentrates the proteins into the needle tips via centrifugation (Figure 11A). PVA provides the vaccine stability upon drying up to 1 year at 40 °C and helps retain its bioactivity (Figure 11B). The microneedle patch was administered to mice for 18 h, and the vaccine elicited an immunogenic response for the duration of the study. A few months later, the Liu lab demonstrated[55] a multilayered microneedle formulation of PVA and silk fibroin that was mechanically strong enough to penetrate the abdominal skin of mice up to 150 μm. PVA alone does not offer enough mechanical strength to puncture the *stratum corneum*, and silk fibroin alone can puncture but tends to break easily. The composed mixture offered controlled release for up to 3 h. A hurdle for a vast amount of these proteins has been the poor thermal stability that limits the polymers involved in formulations. Lee et al. cleverly combined[56] lyophilization, which has been shown to improve thermal stability, with a melt process technique. Qβ was lyophilized and mixed with crushed poly(lactic-*co*-glycolic acid) (PLGA) that was inserted into a heated 95 °C syringe-die (Figure 12A), as a proof of concept for cancer immunotherapy implants. PLGA will degrade in the body over time and release the lyophilized Qβ; the observed antibody response proved that the temperature used to melt PLGA did not denature the Qβ capsid (Figure 12B−C). Further studies need to be conducted to increase the number of polymers used in this area that aim toward delayed release, painless injections, and protein-friendly drug delivery.

Looking Ahead. The examples of formulations presented here have all been aimed toward patient compliance while retaining the efficacy of the drug. These tools for drug delivery are still expanding, and the aim will be to look for methods that offer long-term release, administration at home, and virtually painless transdermal delivery. New and exciting opportunities are presenting themselves for combinations of proteins and polymers with molds, implants, and 3D printing.[93,94] Unfortunately, a great deal more research needs to be

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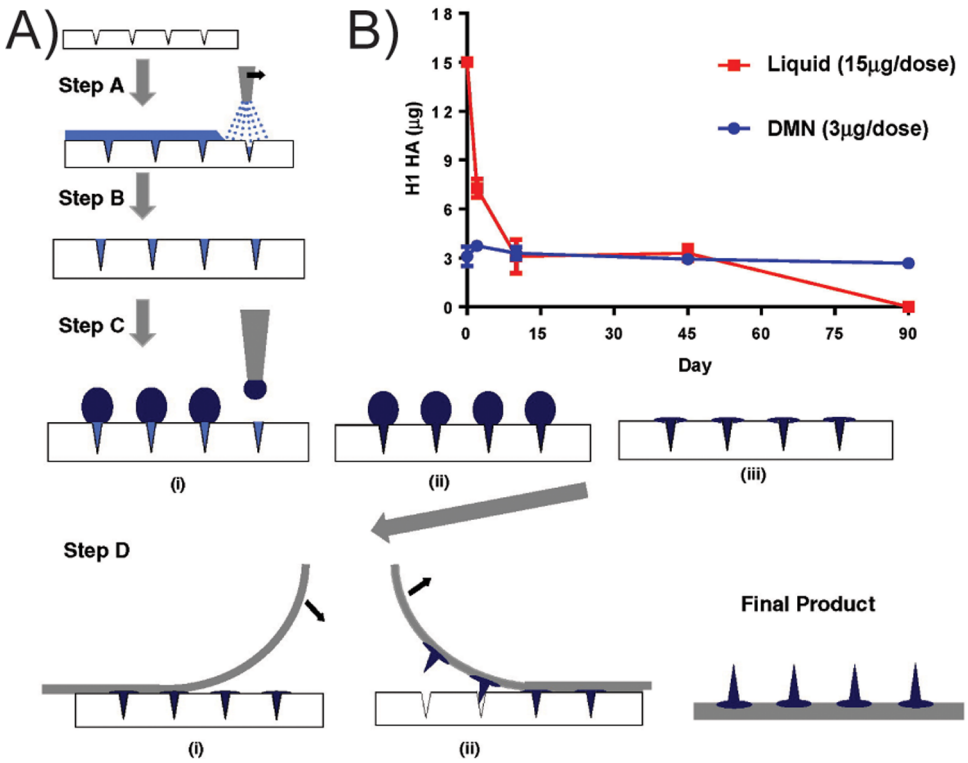

Figure 11. A) Drop casted formulation that centrifuges the polymer into the mold. B) H1 HA strain viability was examined in dissolving microneedle versus liquid formulation in storage at 40 °C up to 3 months and for microneedle up to a year. Adapted from ref 54, with permission from Elsevier.

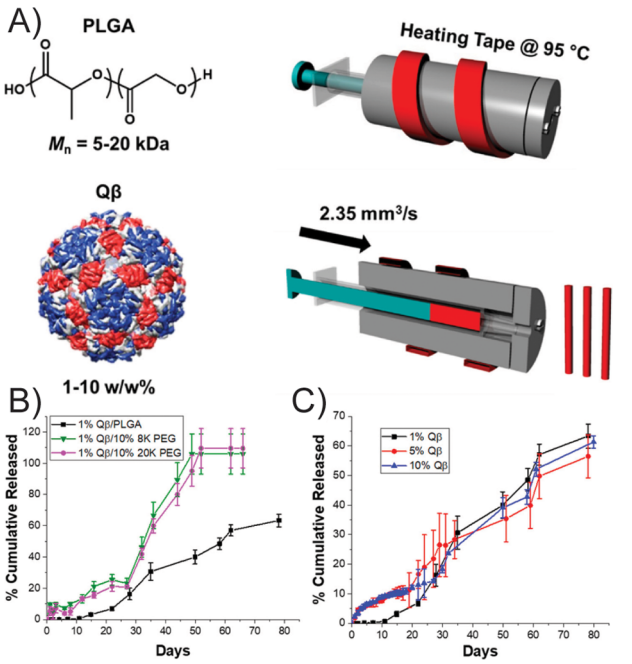

Figure 12. A) A heated syringe used to develop implants for slow release vaccine delivery. B) Qβ release over time with different PEG additives. C) Qβ release over time at different Qβ concentrations. Adapted with permission from ref 56. Copyright 2017 American Chemical Society.

conducted before these alternative methods replace injections via hypodermic needles. A key objective for these formulations is to monitor long-term effects that polymers can have in the epidermis and begin introducing these strategies *in vivo*.[95,96] It would also be beneficial to develop methods for industrial scale production of these formulations, so as to make them more competitive in the market. Some researchers are beginning to take the necessary steps to push this field further into clinical studies,[97,98] but most have focused on developing new formulations. A deeper understanding of the way these polymers can stabilize proteins *in vivo* can lead to better personalized medicine.

FROM PATIENT TO DISEASE: DRUG DELIVERY ON THE NANOSCALE

Once *in vivo*, the delivery of many drugs, especially proteins and nucleic acids, faces several obstacles. Issues regarding bioavailability and circulation lifetimes of drugs, in part addressed above, become critical upon entry of the drug into the circulatory system. In particular, nonhuman proteins and nucleic acids face opsonization and antibody formation, which can complicate subsequent administration of the drug or cause allergic reactions. These reactions from the immune system must be considered when designing therapeutics for a particular condition, and sometimes disorders of the immune system may be the underlying condition. The ability to modulate immune responses to achieve a specific therapeutic outcome without causing unintended consequences for the patient is a major goal of modern medicine and would be extremely beneficial in developing therapeutics for cancers or autoimmune disorders. Recent efforts toward this objective have utilized polymers in various applications owing to their low production cost, facile scalability, and potent tunability. Whether they are modified for direct functionality or used as a carrier for more complex systems, polymers have found a place in applications involving modulation of the immune system

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(Table 3). We discuss immune system evasion, inhibition and activation, and augmentation in the following sections.

Table 3. Targeting on the Nanoscale

category	ref	target(s)	polymer system
immune evasion	111	DCs[a]	PEG,[b] PMAA[c]
	112	uric acid	HSA[d]
immune inhibition and activation	113	DCs[a]	PLGA[e]
	114	PD-L1,[f] tumor cells	pyropheophorbide–lipid conjugate NCPs[g]
	115	MR[h]	PBAE[i]
	116	DCs[a]	pHEMA,[j] PDMS[k]
immune augmentation	117	TAMs[l]	PBAE[i]
dynamic materials and lessons from natural products	118	N/A	PBAE[i]
	119	tumor cells, NK cells	GFP1[m]
	120	acidic microenvironments	Qβ VLP[n]

[a]Denritic cells. [b]Poly(ethylene glycol). [c]Poly(methacrylic acid). [d]Human serum albumin. [e]Poly(lactide-*co*-glycolic acid). [f]Programmed death-ligand 1. [g]Nanoscale coordination polymer. [h]Mannose receptor. [i]Poly(beta-amino ester). [j]Poly(2-hydroxyethyl methacrylate). [k]Poly(dimethylsiloxane). [l]Tumor-associated macrophages. [m]Ginseng fruits polysaccharide. [n]Virus-like particle.

Immune Evasion. There are a number of excellent reviews on targeted drug delivery,[67,99−101] though we wish to briefly shed light on another area—cellular avoidance. We will specifically discuss therapeutics avoiding the immune system and detrimental effects including low circulation half-lives, antibody formation, and allergic reactions. Some of the earliest drug delivery systems developed with immune interactions in mind were based on nanoparticles that were coated with PEG. In-depth discussions on PEGylation are available[67,99−101] but will not be presented in this Review. While the PEGylation of nanoparticles has often achieved its primary goal of shielding nanoparticles from the immune system and increasing the efficacy of drugs, it is not effective on all systems, control over the targeting of specific cells or immune responses is limited, and anti-PEG antibody responses can develop.[102−105] There have been many attempts to not only optimize the shielding of drugs and nanoparticles from the immune system but also increase the functionality of modified nanoparticles.[106−110] These attempts have achieved success in various contexts. Silica-templated polymer hydrogel nanoparticles developed by De Koker et al. combined[111] PEG and poly(methacrylic acid) to form polymer hydrogel nanoparticles that were shown to target lymph node dendritic cells (DCs) and B cells. This targeting resulted in the increased priming of antigen-specific CD8 T cells. PEGylation was shown to have no effect on the uptake of nanoparticles by DCs but was shown to enhance nanoparticle mobility and lymph node drainage. Proteins have also been used to shield other proteins from the immune system and extend the circulation time of therapeutics. By conjugating palmitic acid (Pal), a natural human serum albumin (HSA) ligand, to urate oxidase (Uox), Cho et al. have demonstrated[112] a system in which the shielding of foreign proteins using the body's own proteins can be performed *in situ*. The Uox–Pal conjugate has the potential to bind HSA in the circulatory system and create a protein corona that allows for evasion of the immune system. The nanoparticle systems described above have the potential to enhance the potency of therapeutics by conditioning the immune system's response to specific stimuli, avoiding irrelevant or dangerous responses that reduce the efficacy of therapeutics or harm the patient.

Immune Inhibition and Activation. The idea of specifically targeting and inhibiting or activating immune responses has shown more promise in developing effective therapeutics than attempting to solely evade them. There are many potential immune pathways available for inhibition or

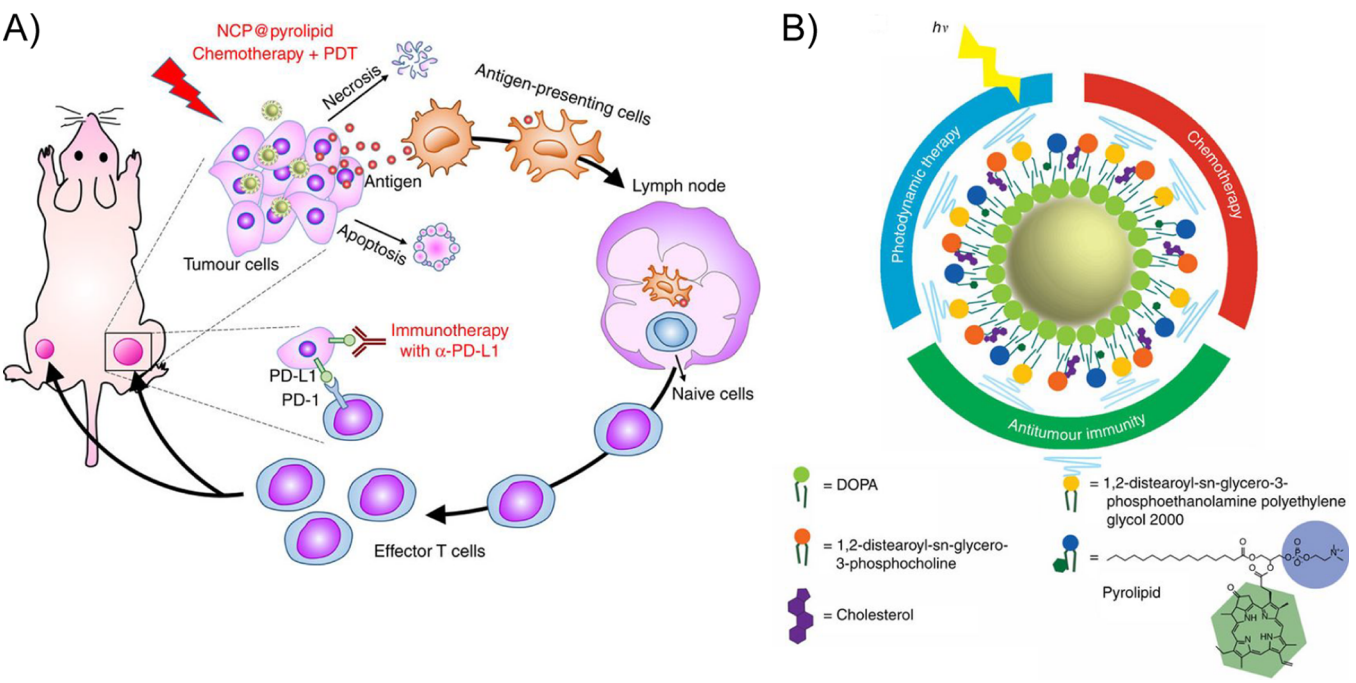

Figure 13. A) Chemotherapy and PDT of NCP@pyrolipid potentiate PD-L1 blockade to induce systemic antitumor immunity. B) Preparation and characterization of NCP@pyrolipid. Adapted with permission from ref 114.

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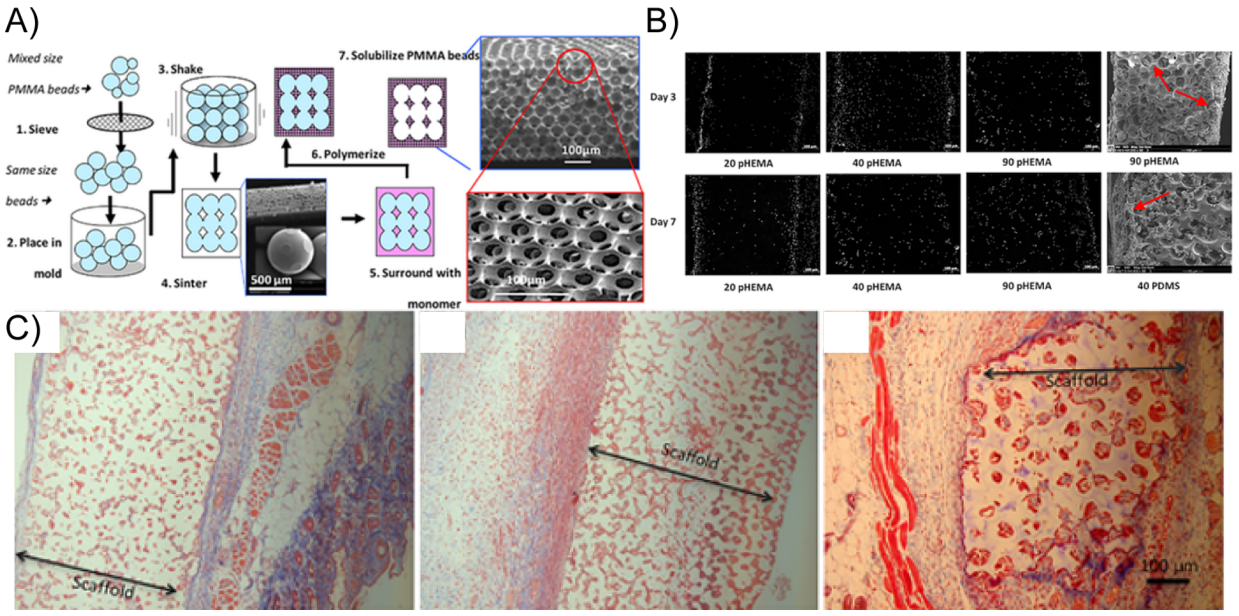

Figure 14. A) Construction of 3DOM porous templated scaffolds. B) Scaffold cell infiltration *in vivo* analysis. C) Masson's trichrome stain of tissue sample after 3 weeks implantation of scaffolds. Adapted with permission from ref 116. Copyright 2018 John Wiley and Sons.

activation, and a thorough discussion of each would be impossible in this context. However, there are some notable examples of immune-targeting mechanisms that will be discussed in this Review. Maldonado et al. have demonstrated[113] the ability to inhibit CD4+ and CD8+ T-cell activation, increase regulatory cells, improve B-cell tolerance, and inhibit antigen-specific hypersensitivity reactions. These results were achieved using self-assembling PLGA tolerogenic nanoparticles containing either protein or peptide antigens and rapamycin. Immune inhibition strategies like the one mentioned in the previous example would be helpful for managing or treating allergies and autoimmune disorders. Immune inhibition strategies like the one mentioned in the previous example would be helpful for managing or treating allergies and autoimmune disorders. Immune inhibition strategies could also be used to reduce undesired antibody formation in response to protein- or nucleic acid-based therapeutics, leading to increased circulation lifetimes, improved bioavailability, and reduced side effects.

Activation of certain immune responses can be useful for situations where traditional therapeutics become ineffective at treating the condition or cause serious side effects at effective dosages. In the context of cancer, chemotherapy has been the most common treatment method for several decades, but drug resistance and extreme side effects limit the extent of its usefulness.

Combination therapies that introduce radiation- or immune-based therapies have proved to be far more effective at increasing the survival rates of patients with cancers that are difficult or impossible to treat with traditional chemotherapy alone. While some of the work described throughout this section may not directly incorporate proteins, they do pertain to the larger theme of immune modulation and demonstrate that immune modulation can be a viable strategy to overcome difficulties faced by polymer-, nucleic acid-, and protein-based therapeutics. The structural motifs used in these studies and the insight into immune modulation could prove useful in designing protein-based therapeutics in the future. He et al. have developed[114] nanoscale coordination polymers (NCPs) that combine the chemotherapy drug oxaliplatin and pyropheophorbide−lipid conjugate (pyrolipid) as a photosensitizer (Figure 13). Administration of the NCP along with a PD-L1 checkpoint blockade kills cancer cells and induces a strong tumor-specific immune response. Recruiting the immune system in this manner lets the patient's body fight their cancer with tools they already have while reducing side effects resulting from high dosages of traditional chemotherapeutics.

The treatment of infections caused by microbial pathogens is another area where the activation of specific immune responses would prove beneficial. Various bacteria have been known to evade the immune system by using polysaccharide or lipid films to mask protein domains that the immune system uses to recognize foreign substances. Many of these bacteria can also become resistant to antibiotics, limiting the treatment options provided by traditional therapeutics. Jones et al. have developed[115] polymer:DNA polyplexes that are capable of targeted gene delivery to modulate humoral immune responses safely and effectively. The polymer:DNA polyplexes utilize mannosylated poly(beta-amino esters) (PBAEs) to target antigen-presenting cells (APCs) through the activation of the mannose receptor (MR). MRs selectively recognize polysaccharides that are abundant on microbes, and the expression of MRs on APCs is a significant feature of the recognition and subsequent immune response against microbial pathogens. The development of immune modulators that target APCs and deliver genes in this manner has the potential to be very useful in the treatment of infections caused by antibiotic resistant bacteria and bacteria that rely on polysaccharides to mask antigens and evade attack by the immune system.

Immune activation strategies do not necessarily have to rely on the delivery of a payload to elicit a response from the immune system. Providing an environment that can recruit immune cells and encourage their migration, maturation, and activation can be a powerful strategy to enhance the response of the immune system against cancer cells or microbial pathogens. Chen et al. have developed[116] polymeric 3D ordered mesoporous (3DOM) scaffolds that promote DC

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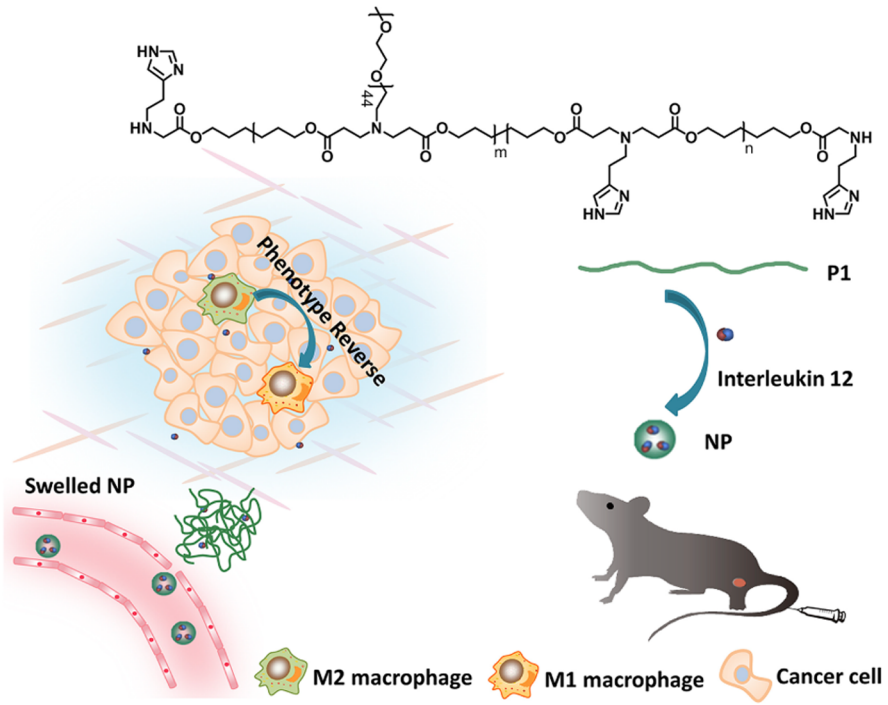

Figure 15. A) Schematic illustration of IL-12⊂P1 nanoparticles that passively target tumors, respond to the microenvironment, and locally reverse macrophage phenotypes. Reprinted from ref 117, with permission from Elsevier.

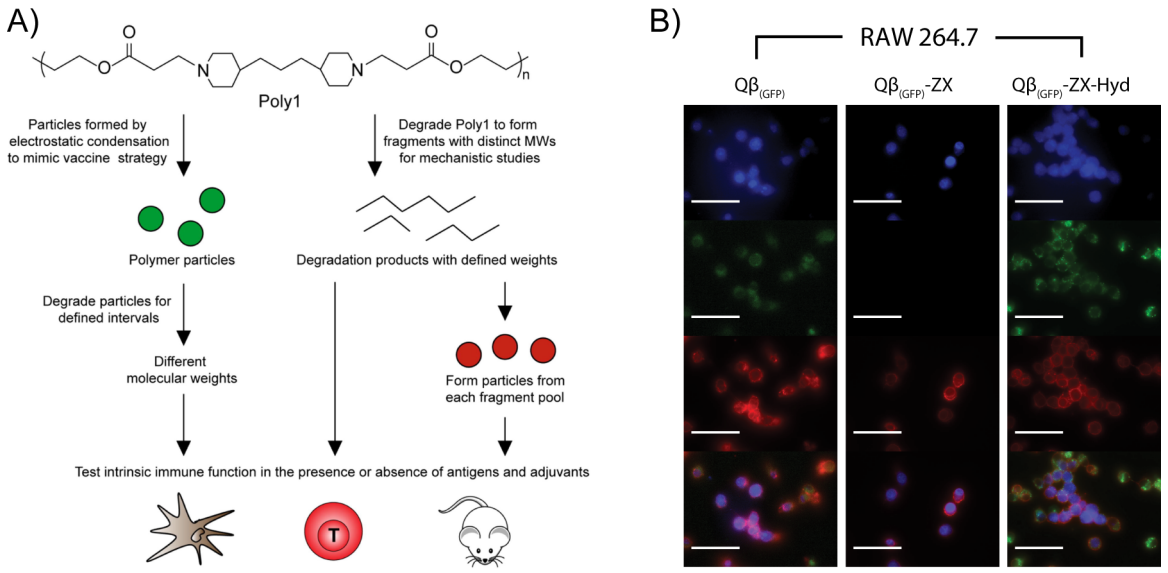

Figure 16. A) Schematic depicting the approach to investigate the intrinsic immunogenicity of Poly1, a degradable, cationic PBAE. Reprinted from ref 118, with permission from Elsevier. B) Epifluorescence microscopy images corresponding to cells incubated with their respective Qβ samples. Adapted with permission from ref 120. Copyright 2018 American Chemical Society.

activation and maturation (Figure 14). The poly(2-hydroxyethyl methacrylate) (pHEMA) and PDMS scaffolds were demonstrated to promote the maturation of JAWSII DCs *in vitro*. pHEMA and PDMS scaffolds were also shown to promote DC maturation and recruitment *in vivo*, when they were implanted subcutaneously in mice. The results reported by the study suggested that the polymer used did not seem to have a large effect on DC maturation or recruitment but that the pore size of the scaffolds did. Smaller pore sizes correlated with higher DC activation both *in vitro* and *in vivo* and DC maturation and scaffold infiltration properties can be modulated by tuning the pore size of the scaffolds. Immune activation strategies based on scaffolds may someday offer the advantage of drug-free therapies that have a low risk of side effects and do not require complex dosage procedures.

Immune Augmentation. In addition to directly targeting the immune system to inhibit or activate responses, therapeutics have been designed to use existing components of the immune system to target hostile cells. This approach of augmenting the immune system takes advantage of tools that the immune system already offers and repackages them in such a way that can target cancer-related cells or pathogens that the immune system would not (normally) respond to. Wang et al. have developed[117] polymeric nanoparticles that have been

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demonstrated to re-educate tumor-associated macrophages (TAMs) from the M2 to the M1 phenotype (Figure 15). The results are achieved using microenvironment-responsive PBAE nanoparticles that carry a payload of the cytokine interleukin-12 (IL-12). IL-12 is known to induce the conversion of tumor-supportive (M2) macrophages to tumor-suppressive (M1) macrophages. However, cytokines like IL-12 are unstable and can induce a wide range of immune responses that result in systemic side effects. The packaging and targeted delivery of IL-12 in PBAE-based nanoparticles can provide a stabilized release profile of IL-12 to tumor microenvironments and provide insights on developing new strategies to treat various forms of cancer. Advantages of such systems include a natural response from the immune system when clearing out the hostile cells and reduced side effects attributed to the potential of traditional small molecule drugs being unnecessary for treatment.

Dynamic Materials and Lessons from Natural Products. Although new materials and strategies for immune modulation are constantly being developed, new lessons and properties are still being learned from older materials. Many studies focus on the initial effects observed upon introduction of a therapeutic, but the components of a therapeutic must eventually be degraded and cleared from the body. Polymers like PBAEs have gained popularity for use in therapeutics due to biocompatible properties that include solubility, functionalizability, and low immunogenicity. Immunogenicity is an important factor for any therapeutic, but there is still much to be known about how the metabolites and degradation products of polymers commonly used in therapeutics interact with the immune system. Andorko et al. have elucidated[118] the intrinsic immunogenic properties of PBAEs as a function of the degree of polymer degradation and polymer form—soluble or particles (Figure 16A). Polymer molecular weight was found to strongly affect the activation of DCs, drive antigen presentation, and enhance T cell proliferation in the presence of antigen. High molecular weight polymers were found to provide short degradation times and maximum immune stimulation. Polymers in nanoparticle form behaved similarly to high molecular weight polymers. Low molecular weight free polymers were found to be immunologically inert. The immunogenic properties of the polymers changed as they degraded due to the changing physicochemical properties and concentration of the polymers. Understanding how these components degrade and their changing properties is important for optimizing a therapeutic for maximum efficiency and safety. Proper optimization of a therapeutic to account for degradation and metabolism can accelerate its time to market.

Similar to how important lessons can be learned from widely established materials, natural products can provide insight into how existing biological systems have evolved to target certain immune pathways. While many natural products are not optimized to treat specific conditions, analyzing their effects on the immune system can lead to the discovery of new structure−activity relationships and the development of therapeutics that take advantage of these.[121] Wang et al. have demonstrated[119] the immune modulating properties of ginseng fruits polysaccharide (GFP1). GFP1 was found to inhibit tumor growth and lung metastasis *in vivo* by activating several immune pathways including the promotion of ConA or LPS-induced spleen lymphocyte proliferation. In another example, Lee et al. showed[120] that surface functionalization VLPs derived from bacteriophage Qβ inhibited the cellular uptake of the VLPs by HeLa cells and RAW 264.7 macrophages (Figure 16B). The inhibition of uptake by macrophages was surprising given that the surface-exposed amines of Qβ VLPs were functionalized with terminal hexanoic acid moieties. These findings suggest that natural products and nontraditional polymers hold potential for the discovery and design of novel ways to modulate the immune system.

Looking Ahead. An increased understanding of the immune system has spurred the development of therapeutics that not only evade immune responses to improve delivery of active components to their targets but also target and take advantage of specific immune responses to aid in the treatment of the patient. It is encouraging that the paradigm of therapy development is moving away from the designs that focus on drugs acting on their target in a vacuum to designs that work together with the immune system to achieve the desired outcome. The immune system should be viewed as a powerful tool that can be harnessed to reach targets and elicit systemic responses that could not be achieved by traditional therapeutics alone. Recent developments in targeted therapeutics, including the above-mentioned examples, have already demonstrated that conditions thought untouchable by the immune system, like certain cancers, can be primed for recognition by the immune system. This priming increases the therapeutic efficiency in terms of the quantity of drugs needed to treat the condition and the severity of side effects induced.

Recent developments in targeted therapeutics have also demonstrated that there is hope in treating previously untreatable autoimmune disorders or allergies. The continued discoveries and optimizations of drug delivery systems that respect the delicate balance of biological systems will hopefully lead to an era when no condition is untreatable.

CONCLUSIONS

This Review considers a holistic view of drug delivery by first considering the delivery from factory to clinic, clinic to patient, and patient to disease. Polymers play a crucial role in each stage in drug delivery by enhancing stability, maintaining bioactivity, and improving targeting of the drug molecule.

In order to deliver drugs and vaccines from factory to clinic, an extensive and expensive cold chain is in place to preserve the integrity of the drugs. Failures at any point in the cold chain can result in destruction of the vaccines. By bypassing the need for a cold chain, the cost and availability of vaccines can be improved. Polymers have been utilized by many research groups to devise strategies to increase the temperature stability of vaccines and proteinaceous drugs. Three prominent methods of stabilization involving polymers have emerged—solvent removal, protein immobilization, and bioconjugation. These methods work to protect the vaccines from the environment and ultimately preserve the structure and function of the proteinaceous drugs. These methods have proven successful under laboratory conditions in preserving vaccines during transportation and storage at elevated temperatures. A next step for researchers in this field is to study the recovery of the drugs at the clinic.

The next consideration is the delivery of the drug from clinic to patient. The preserved drug has survived cold chain transportation and storage and must now be reconstituted for administration. Three common methods are typically used for drug administration—oral, injection, and alternative. Oral drug administration has the highest level of patient compliance but is limited by proteinaceous stability in the acidic

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environment of the stomach. On the other hand, intravenous injections by hypodermic needles have lower patient compliance but can bypass the hostile digestive system. Alternative methods work to devise painless methods of drug delivery that utilize polymers to help stabilize the proteinaceous drug as it enters the body. Methods such as microneedles and implants have found success as they accomplish these goals and also extend the duration of release, which helps to improve patient compliance. Polymers are ideal candidates for modification as they can be tuned to serve a specific purpose.

Once the drug has been introduced into the patient, the interaction of the drug and body must be considered. Polymers have been used in drug delivery systems to evade, activate, inhibit, and augment the immune system. By determining the immune response elicited by drug delivery, the overall therapeutic outcome can be predicted and manipulated. Early on in immune system modulation studies, it was found that polymeric shielding agents were useful in improving the circulation time and efficacy of drugs by evading immune detection. Later it was discovered that eliciting a synergistic immune response can increase the potency of therapeutics. Studies today have moved on to reprogram the immune system for cancer immunology and the treatment of autoimmune diseases. By modulating the immune system through the use of tunable polymers, a higher control over therapeutic outcomes can be achieved.

As demonstrated in this Review, polymers are valuable tools that allow for the successful journey of proteinaceous drugs from the macroscale to the microscale to the nanoscale.

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The manuscript was written through contributions of all authors.

Notes

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