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Preparation and development of block copolypeptide vesicles and hydrogels for biological and medical applications

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Abstract

There have been many recent advances in the controlled polymerization of α-amino acid-N-carboxyanhydride (NCA) monomers into well-defined block copolypeptides. Transition metal initiating systems allow block copolypeptide synthesis with excellent control over number and lengths of block segments, chain length distribution, and chain-end functionality. Using this and other methods, block copolypeptides of controlled dimensions have been prepared and their self-assembly into organized structures studied by many research groups. The ability of well-defined block copolypeptides to assemble into supramolecular copolypeptide vesicles and hydrogels has led to the development of these materials for use in biological and medical applications. These assemblies have been found to possess unique properties that are derived from the amino acid building blocks and ordered conformations of the polypeptide segments. Recent work on the incorporation of active and stimulus responsive functionality in these materials has tremendously increased their potential for use in biological and medical studies.

Introduction

Biological systems produce proteins that possess the ability to self-assemble into complex, yet highly ordered structures.[1] These remarkable materials are polypeptide copolymers that derive their properties from precisely controlled sequences and compositions of their constituent amino acid monomers, as well as domains of ordered conformation within the proteins. There is much recent interest in developing synthetic analogs of these natural polymers to create polypeptide materials for applications in medicine and medical diagnostics, including as vehicles for delivery of therapeutics and scaffolds for tissue

regeneration.[2] To be successful in these applications, it is important that these materials can self-assemble into organized structures with tunable physical properties, as well as incorporate functionality to allow them to interact with biological systems. Polypeptides have many advantages over conventional synthetic polymers for such applications since they use the same building blocks as proteins and are able to adopt stable ordered chain conformations.[3]

Synthetic polypeptides are not new materials: homopolypeptides have been available for many decades and, early on have mainly seen limited use for protein conformation analysis.[4] However, new methods in chemical synthesis have made possible the preparation of increasingly complex copolypeptide sequences of defined chain lengths that possess extraordinary capabilities for assembly into useful structures.[5] Block copolypeptides are well suited for applications where structural and functional domains need to be at length scales ranging from nanometers to microns. Amphiphilic diblock copolypeptides, when dispersed in water, have been shown to be able to form a variety of structures including micelles, vesicles and hydrogels.[6] The regular secondary structures obtainable with the polypeptide segments provide opportunities for dictating self-assembled structure unobtainable with typical block copolymers or small-molecule surfactants.

Upon examining the different methods for polypeptide synthesis, the limitations of these techniques for preparation of well-defined copolymers readily becomes apparent. Conventional solid-phase peptide synthesis is neither useful nor practical for direct preparation of large polypeptides (> 100 residues) due to unavoidable deletions and truncations that result from incomplete deprotection and coupling steps. The most economical and expedient process for synthesis of long polypeptide chains is the polymerization of α-amino acid-N-carboxyanhydrides (NCAs) (Fig. 1).[7] This method involves the simplest reagents, and high molecular weight polymers can be prepared in both good yield and large quantity with no detectable racemization at the chiral centers. The considerable variety of NCAs that have been synthesized (> 200) allows exceptional diversity in the types of polypeptides that can be prepared.[7]

Since the late 1940s, NCA polymerizations have been the most common technique used for large scale preparation of high molecular weight polypeptides.[8] However, these materials have primarily been homopolymers, random copolymers, or graft copolymers that lack the sequence specificity and monodispersity of natural proteins. Until recently, the level of control in NCA polymerizations has not been able to rival that attained in other synthetic polymerizations (e.g. vinyl addition polymerizations) where sophisticated polymer architectures have been prepared (e.g. block copolymers).[9] Attempts to prepare block copolypeptides and hybrid block copolymers using NCAs have traditionally resulted in polymers whose compositions did not match monomer feed compositions and that contained significant homopolymer contaminants.[10] Block copolymers could only be obtained in pure form by extensive fractionation steps, which significantly lowered the yield and efficiency of this method. The limitation of NCA polymerizations has been the presence of side reactions (chain termination and chain transfer) that restrict control over molecular weight, give broad molecular weight distributions, and prohibit formation of well-defined block copolymers.[11] Recent advances enabling the elimination of these side reactions has been a major breakthrough for the polypeptide materials field. In this review, methodology using transition metal complexes for the living polymerization of NCAs into block copolypeptides will be presented. The use of this and related methodologies for development of two different

classes of synthetic block copolypeptide assemblies, vesicles and hydrogels, toward applications in medicine will also be discussed.

BLOCK COPOLYPEPTIDE SYNTHESIS

The first successful strategy to eliminate side-reactions in NCA polymerizations was the use of transition metal complexes as active species to control addition of NCA monomers to polymer chain-ends. The use of transition metals to control chemical reactions has been proven in organic and polymer synthesis as a means to increase reaction selectivity, efficiency, and rate.[12] Using this approach, a significant advance in controlled NCA polymerization was realized in 1997. Highly effective zerovalent nickel and cobalt initiators (i.e. bpyNi(COD) and $(PMe_3)_4Co$)[13,14] were developed by Deming that promote living polymerization of NCAs via unprecedented, highly active covalent propagating species. Following polymerization, the metal ions can be conveniently removed from the polymers by simple precipitation or dialysis of the samples, yielding polypeptides suitable for biological use.

Mechanistic studies on the initiation process showed that both these metals react identically with NCA monomers to form metallacyclic complexes by oxidative addition across the anhydride bonds of NCAs.[13,14] These oxidative-addition reactions were followed by addition of a second NCA monomer to yield complexes identified as six-membered amido-alkyl metallacycles (Fig. 1). These intermediates were found to further contract to five-membered amido-amidate metallacycles upon reaction with additional NCA monomers. This ring contraction is thought to occur via migration of an amide proton to the metal-bound carbon, which liberates the chain-end from the metal (Fig. 1).[15] The resulting amido-amidate complexes were thus proposed as the active polymerization intermediates. Propagation through the amido-amidate metallacycle was envisioned to occur by initial attack of the nucleophilic amido group on the electrophilic C_5 carbonyl of an NCA monomer. This reaction results in a large metallacycle that can contract by elimination of CO_2. Proton transfer from the free amide to the tethered amidate group further contracts the ring to regenerate the amido-amidate propagating species, while in turn liberating the end of the polymer chain.

In this manner, the metal is able to migrate along the growing polymer chain, while being held by a robust chelate at the active end. The formation of these chelating metallacyclic intermediates appears to be a general requirement for obtaining living NCA polymerizations using transition metal initiators. These cobalt and nickel complexes are able to produce polypeptides with narrow chain length distributions ($M_w/M_n < 1.1$) and controlled molecular weights ($500 < M_n < 500{,}000$).[16] This polymerization system is very general, gives controlled polymerization of a wide range of NCA monomers, and provides propagation rates typically orders of magnitude greater compared to other controlled NCA polymerizations. These polymerizations can be conducted in a variety of solvents (e.g. THF, DMF, EtOAc, dioxane, nitrobenzene, DMAc) and over a broad range of temperatures (e.g. 10 to 100 °C) with no loss of polymerization control. By addition of different NCA monomers, the preparation of block copolypeptides of defined sequence and composition is feasible.[5,17]

Block copolypeptides prepared via transition-metal mediated NCA polymerization are well-defined, with the sequence and composition of block segments controlled by order and quantity of monomer added to initiating species, respectively. These block copolypeptides can be prepared with the same level of control found in anionic and controlled radical

polymerizations of vinyl monomers, which greatly expands the potential of polypeptide materials. The unique chemistry of NCAs allows these monomers to be polymerized in any order, which is a challenge in most vinyl copolymerizations, and the robust chain-ends allow the preparation of copolypeptides with multiple block domains. The robust nature of transition metal initiation was shown by the linear, stepwise synthesis of triblock and pentablock copolypeptides (Fig. 1).[18,19] The self-assembly of block copolypeptides has been under extensive investigation in recent years, typically in aqueous media to mimic biological conditions. In the following sections, the assembly of block copolypeptides into different types of supramolecular assemblies and their development toward medical applications is described.

Sidebar title: Drug delivery Vehicles

In addition to the potential for achieving controlled delivery and release, drug delivery vehicles have been investigated for several years due to many challenges associated with administering a naked drug.[20,21] For example, if the drug is a protein therapeutic, it can be susceptible to enzymatic and proteolytic degradation. Accordingly, protein drugs are administered intravenously to avoid the harsh environment of the digestive system. However, intravenously delivered drugs also need to overcome hurdles, such as catabolism in the liver, clearance by the kidneys, and recognition by the immune system. If the chemotherapeutic is a nucleic acid, it has the barrier of not being able to cross the cell membrane. In the case of hydrophobic drugs, their solubility in blood may be an issue. Regardless of the type of drug, they all have the problem of side reactions or toxicity resulting from affecting normal cells in the body. Drug encapsulation is an approach that can address these challenges, since it can protect the drug from enzymatic degradation and immune system recognition. Encapsulation can also decrease the amount of drug lost in the kidneys, allow the slow release of a drug, and protect the normal cells from cytotoxins. Moreover, ligands or other functionalization on polymeric carriers can provide targeting and internalization in cancerous cells. However, a major limitation in these strategies is the tedious and difficult process of preparing and assembling synthetic polymers to the level of intricate complexity as found in biological systems.[20]

BLOCK COPOLYPEPTIDE VESICLES

There has been an abundance of research in recent years on polymeric amphiphiles as drug carriers.[20] These materials, formulated as micelles, vesicles and emulsion droplets, can exhibit greater stability and improved control over release compared to conventional lipids or surfactant based carriers, and thus show great promise for encapsulation and delivery applications. While much has been accomplished in polymeric nanocarriers,[20,21] there remains a need for new biomimetic materials, where individual components are capable of performing multiple functions as seen in virus particles, where capsid proteins are involved in oligonucleotide protection, cell binding, endosomal release, and oligonucleotide release.[21] Addition of such functionality can be challenging since many synthetic polymer based systems have limited capability for modification, and when functionalized, finely balanced self assembly properties may be significantly altered or impaired. For these reasons, there is a need for amphiphilic polymers that can be prepared using versatile methods allowing for fine tuning of chemical composition and structure, and using building blocks that are biocompatible, biodegradable, and easily functionalized. Polypeptide amphiphiles are appealing since they are reproducibly prepared metal and pyrogen free in large quantities,

chain lengths and compositions are easily modified, and most importantly, their chain conformations can be used to guide assembly into well-defined nanoscale assemblies independent of many other parameters.[22]

Current challenges for polypeptide vesicle carriers include incorporation of multifunctionality into these amphiphiles for biological responsiveness, controlled release, and targeting to specific cell-surface biomolecules, as well as tuning of amphiphile components for controlled membrane stability, permeability and degradability. Incorporation of polypeptide domains has been widely used as a powerful way to introduce functionality, structural stability, and stimuli responsiveness in polymeric vesicles.[23] To date, many types of block copolypeptide amphiphiles that form stable vesicular assemblies have been developed. The first of these utilized diethylene glycol modified lysine residues that impart both non-ionic water solubility as well as ordered α-helical conformations to the hydrophilic polypeptide domains.[24] A majority of other materials utilize highly charged polyelectrolyte segments to impart both functionality and fluidity to the membranes. More recently, these copolypeptides have included increasingly complex functionality to assist in cargo loading, vesicle targeting, and vesicle disruption.

In 2004, Deming's lab studied the roles of chain length and block composition on the assembly of uncharged diblock copolypeptide amphiphiles of the general structure: poly(N_ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-L-lysine)-*block*-poly(L-leucine), or $K^P{}_xL_y$.[24] These diblock copolypeptide amphiphiles associate very strongly and essentially do not exist as single chains in aqueous solution. This property, in most cases, results primarily in the formation of irregular aggregates if the polymers are simply dispersed in deionized water. A protocol was developed, using organic solvent (THF) and a denaturant (TFA) that allowed annealing of these materials when water is added. Dialysis of the samples allows one to obtain regular assemblies in pure water.

Using this procedure, a number of amphiphilic copolymers were studied where the hydrophilic domains were varied from 60 to 200 residues in average length; and the hydrophobic domains were varied from 10 to 75 residues in average length.[24] All block copolypeptides were expected to adopt rod-like conformations due to the strong α-helix forming tendencies of both the leucine and ethylene glycol-modified lysine residues.[25] These rod-like conformations provided a flat amphiphile interface upon association in water, thus directly tying polymer conformation to supramolecular structure. Circular dichroism spectroscopy of the copolymers in water confirmed that all samples were α-helical. Using differential interference contrast (DIC) optical microscopy, TEM, laser scanning confocal microscopy (LSCM), and DLS as initial methods to study the assemblies, some trends were identified.[24] When the hydrophobic poly(leucine) domains were less than 20 residues in length, a significant fraction of oblong or irregular micelles (*ca*. 100 nm diameter) were observed to form by DLS and TEM. When the size of the hydrophilic domain was 100 residues, unilamellar vesicles were observed to form with a size range of approximately 2 μm to 15 μm diameter. When the hydrophilic block was increased to 150 residues, the vesicles were much larger in size, approaching 50 μm in diameter. Finally, when the hydrophilic segments were increased to 200 residues long, membrane curvature was hindered such that the major structures formed were flat membrane sheets.

These block copolypeptides, where both hydrophilic and hydrophobic segments were α-helical, gave rise to very stiff membranes, as suggested by the large vesicle diameters and lack of fluidity in the sheets that were formed. Further investigation revealed that these membranes were completely insensitive to osmotic stress, a consequence of their impermeability to water, ions or other small molecules.[24] They also could not be reduced in size by liposome-type extrusion techniques, and could only be made smaller by more aggressive sonication methods. The inability of the uncharged vesicles to pass through small pore diameter filters was likely due to membrane rigidity and virtual absence of chain flexibility. One advantage of these materials for many applications is the media insensitivity of the ethylene glycol coating on the membrane surface. These vesicles were inert toward different ionic media, variations in pH, and the presence of large macromolecules, such as proteins in serum. However, the rigidity of these chains created drawbacks in sample processing, namely the need to use denaturants for vesicle formation, which may be problematic for encapsulation of sensitive materials, and difficulty in preparing nanoscale vesicles due to high membrane rigidity.

In 2005, Lecommandoux's group reported on the self assembly behavior of a short, zwitterionic diblock copolypeptide, poly(L-glutamatic acid)-*b*-poly(L-lysine), $E_{15}K_{15}$.[26] This polymer has the interesting characteristic that in aqueous solutions near neutral pH ($5 < pH < 9$), both segments are charged and the polypeptide is dispersed as soluble chains. However, if pH is lowered to values below pH = 4 or raised above pH = 10, one of the segments is neutralized and the chains self assemble into small vesicles. By adjustment of pH, vesicles with either anionic (high pH) or cationic (low pH) surfaces could be prepared, hence their description as "schizophrenic" vesicles. It is notable that these chains are soluble in water when both segments are highly charged, considering that the formation of water insoluble polyion complexes between poly(L-lysine) and poly(L-glutamatic acid) is well documented.[27] A key feature of this work is the utilization of short polyelectrolyte segments, which limits such polyion complex formation in dilute solutions.

Deming's group also reported in 2005 on the assembly of charged diblock copolypeptide amphiphiles, utilizing the structure directing properties of rod-like α-helical segments in the hydrophobic domains. Specifically, the aqueous self-assembly of a series of poly(L-lysine)-*b*-poly(L-leucine) block copolypeptides was studied: K_xL_y, where *x* ranged from 20 to 80, and *y* ranged from 10 to 30 residues, as well as the poly(L-glutamatic acid)-*b*-poly(L-leucine) block copolypeptide, $E_{60}L_{20}$.[28] In other work, it was found that samples with high K to L molar ratios (e.g. $K_{180}L_{20}$) could be dissolved directly into deionized water, yielding transparent hydrogels composed of twisted fibrils (*vide infra*) [85]. It was reasoned that use of shortened charged segments would relax repulsive polyelectrolyte interactions and allow formation of charged polypeptide membranes. Samples were processed by suspending the polymers in THF/water (1:1) followed by dialysis. Analysis of these assemblies using DIC optical microscopy revealed the presence of large, sheet-like membranes for $K_{20}L_{20}$, and thin fibrils for $K_{40}L_{20}$. The $K_{60}L_{20}$ sample was most promising, as only large vesicular assemblies were observed by DIC.[28]

The $K_{60}L_{20}$ copolypeptide vesicles obtained directly from dialysis are polydisperse and range in diameter from *ca*. 5 μm down to 0.8 μm as determined using DIC and DLS. For applications such as drug delivery via blood circulation, a vesicle diameter of *ca*. 50 to 100 nm is desired. It was observed that aqueous suspensions of $K_{60}L_{20}$ vesicles could be

extruded through nuclear track-etched polycarbonate membranes with little loss of polypeptide material. After two passes through a filter, reductions in vesicle diameter to values in close agreement to filter pore size were observed. These results showed that the charged copolypeptide vesicles are readily extruded, allowing good control over vesicle diameter in the tens to hundreds of nanometers range. DLS analysis revealed that the extruded vesicles were also less polydisperse than before extrusion and contained no micellar contaminants. The vesicular morphology was also confirmed through TEM imaging of the sub-micron $K_{60}L_{20}$ suspensions. Thus, it appears that the membranes of the $K_{60}L_{20}$ vesicles are more flexible and compliant than those of purely rod-like uncharged polypeptides. The extruded vesicles were monitored for 6 weeks using DLS and were found to be stable. The vesicles were also found to have high thermal stability. An aqueous suspension of 1 μm vesicles was held at 80 ℃ for 30 minutes, after which no vesicle disruption could be detected.[28] Only after heating to 100 ℃ for 30 minutes were the vesicles disrupted, yielding large flat membrane sheets.

Stability of these highly charged polypeptide vesicles in ionic media is important for use in most applications ranging from personal care products to drug delivery. Although the $K_{60}L_{20}$ vesicles are unstable at high salt concentrations (>1 M), they are stable in 100 mM PBS buffer as well as serum-free DMEM cell culture media.[28] Addition of serum, which contains anionic proteins, resulted in vesicle disruption, most likely due to polyion complexation between the serum proteins and the oppositely charged polylysine chains. Accordingly, it was observed that the negatively charged polypeptide vesicles prepared using $E_{60}L_{20}$ are stable in DMEM with 10% fetal bovine serum. Based on these results, these charged polypeptide vesicles may have potential as encapsulants for water soluble therapeutics as an alternative to liposomes. These copolypeptides retain much of the stability of the uncharged polypeptide vesicles described earlier, but allow straightforward encapsulation and size control due to much simpler processing.[28] Another feature of these charged polypeptide vesicles is the potential for facile functionalization of the hydrophilic polypeptide chains at the vesicle surface through either chemical conjugation to amine or carboxylate residues,[29] or by careful choice of charged residues.

Addressing this point, Deming's lab reported the preparation of arginine-leucine (i.e. $R_{60}L_{20}$) vesicles that are able to readily enter cells due to the many guanidinium groups of the arginine segments (Fig. 2).[30] In this case, the arginine residues played a dual role, where they were both structure directing in vesicle formation, as well as functional for cell binding and entry. Studies on endocytotsis and intracellular trafficking of these vesicles revealed that they enter HeLa cells primarily via macropinocytosis.[31] They were found to then primarily reside in early endosomes, but not in lysosomes, and although some manage to escape into cytoplasm many are trapped within these compartments. Regardless, another study showed that $R_{60}L_{20}$ vesicles were effective at condensing plasmid DNA and transfecting it into a variety of cell lines, showing the vesicles do have potential for intracellular delivery.[32] These DNA carriers are advantageous over many other transfection agents due to their low cytotoxicity.

In another study, targeted cell uptake capability was added to vesicles using transferrin (Tf). Deming's lab had previously developed negatively charged $E_{60}L_{20}$ vesicles, which were shown to be minimally toxic toward cells. However, their negative character also inhibits these vesicles from effectively being internalized by cells, which is problematic as many therapeutics have intracellular targets. To overcome this limitation of $E_{60}L_{20}$ materials, Tf was

conjugated onto the vesicle surfaces, and was chosen since the receptor for Tf is overexpressed on cancer cells.[29] The enhanced uptake of Tf-conjugated vesicles into cells expressing Tf receptors was verified through confocal microscopy. Furthermore, endocytosis and immunostaining experiments confirmed that Tf conjugated on vesicle surfaces plays a critical role in the internalization and subsequent intracellular trafficking behavior of the vesicles. This proof of concept study showed that addition of targeted uptake ability to polypeptide vesicles is feasible and promising.

From the pioneering studies on block copolypeptide vesicles described above, design criteria were established for successful vesicle formation, namely incorporation of an α-helical hydrophobic domain connected to a charged hydrophilic domain. Since this original work, many labs have prepared different variants of block copolypeptide vesicles based on this scheme. In 2007, Hadjichristidis reported lysine-PBLG-lysine (i.e. $K_xPBLG_yK_x$) triblock copolypeptides, where the helical PBLG core favors vesicle formation.[33] Jing and coworkers prepared vesicle forming lysine-phenylalanine (K_xF_y) copolypeptides, containing α-helical phenylalanine segments.[34] These vesicles were also found to be useful in encapsulating hemoglobin and acting as oxygen carriers. Deming's lab also reported the formation of vesicles from dual hydrophilic triblock copolypeptides composed of arginine-glutamate-leucine ($R_xE_yL_z$) or pegylated lysine-arginine-leucine ($K^P{}_xR_yL_z$) sequences.[35] The use of triblock architectures was intended to retain some homoarginine residues for cell uptake, but have the majority of the hydrophilic segments be anionic or uncharged to minimize cytotoxicity, all without disrupting vesicle formation. A number of different compositions were prepared and it was found that, although vesicles exhibiting low cytotoxicity could be formed with a $R_5E_{80}L_{20}$ copolypeptide, the R segments were unable to promote intracellular uptake. With the $K^P{}_xR_yL_z$ samples, the presence of the "pegylated" outer blocks was able to diminish cytotoxicity while still allowing the center R segments to promote cellular uptake.[35]

Using a different approach toward vesicle formation, Jan and coworkers prepared lysine-glycine (i.e. K_xG_y) copolypeptides, where the polyglycine segment does not adopt an α-helical conformation, and has inherently higher flexibility compared to helical segments.[36] Due to the lack of a rigid hydrophobic segment, and due to the hydrophilicity of glycine compared to leucine or phenylalanine, much longer "hydrophobic" segments were needed to drive self-assembly in water and vesicle formation. A $K_{200}G_{50}$ block copolypeptide was found to form vesicles in water using MeOH/H_2O processing, and was also mineralized with silica for entrapment of molecules.[37]

More recent developments of block copolypeptide vesicles have have focused on incorporation of functionality within one of the segments. In 2010, Deming's lab reported the preparation of lysine-dihydroxyphenylalanine (i.e. $K_{60}DOPA_{20}$) based vesicles, where the hydrophobic DOPA segments have the added feature of being sensitive toward oxidation.[38] DOPA residues are found naturally in mussel byssus and are important components in the ability of byssal threads to adhere underwater and to crosslink into rigid networks.[39] In a biomimetic process $K_{60}DOPA_{20}$ vesicles were oxidized in aqueous media resulting in crosslinking of the vesicle membranes. The resulting membranes were very robust, and stable to organic solvents, freeze drying and osmotic shock. Similar materials, in the form of glutamate-lysine/DOPA (i.e. $E_x(K_m/DOPA_n)_y$) copolymers were reported in 2012 by Qiao,[40] where the hydrophobic domains were statistical copolymers of different ratios (m:n) of lysine and DOPA that could be assembled and oxidized to crosslinked vesicles at high pH.

There is much current interest in synthesis of glycosylated polypeptides, and vesicle forming amphiphilic copolypeptides that contain sugars in the hydrophilic corona have now also been prepared. In 2012, Lecommandoux and Heise reported the preparation of benzyl glutamate-propargyl glycine (i.e. $PBLG_{20}PPG_{25}$) diblock copolymers.[41] The propargyl side-chains were then modified by copper catalyzed azide-alkyne cycloaddition with azide-functionalized galactose to give the amphiphilic glycopolypeptide. Since the PPG segment is racemic, it adopts a disordered conformation in glycosylated form. The resulting rod-coil amphiphile was found after DMSO-water processing to assemble into vesicles that were able to bind their complimentary lectin. Deming's lab, in 2013, reported a different system prepared from a galactosylated NCA, α,D-galactopyranosyl-L-cysteine (α-gal-C) NCA, and leucine of the composition $(\alpha\text{-gal-C})_{65}L_{20}$, which was able to form vesicles when the side-chain thioether functionalities were oxidized to sulfone groups and after THF-water processing.[42] The parent polymer, while water soluble, is α-helical, which prohibits formation of small spherical vesicles. The fully oxidized sulfone derivative, i.e. $(\alpha\text{-gal-C}^{O2})_{65}L_{20}$, is more polar, increasing its water solubility, and more importantly has a disordered conformation which assists in vesicle membrane formation.

Deming's lab also redesigned vesicle forming copolypeptides to utilize methionine as the hydrophilic segment (Fig. 3).[43] In an effort to create enzyme responsive polypeptide assemblies that can rupture and release their cargos within cells, they envisioned creating amphiphilic copolypeptides containing segments of oxidized methionine residues, which occur naturally when methionine containing proteins are exposed to reactive oxygen species (ROS).[44] Methionine oxidation in proteins is well known and is believed to help maintain protein activity since these residues act as sacrificial substrates for ROS, preventing irreversible oxidation at critical active site residues such as cysteines.[45] The possibility to interchange methionine residues between hydrophobic (reduced) and hydrophilic (oxidized) states under biological conditions inspired the incorporation poly(L-methionine), M, segments into copolypeptide vesicle assemblies. Deming's lab designed amphiphilic copolypeptides containing segments of water soluble methionine sulfoxide, M^O, residues that were prepared by synthesis of a fully hydrophobic precursor diblock copolypeptide, poly(L-methionine)$_{65}$-b-poly(L-leucine$_{0.5}$-*stat*-L-phenylalanine$_{0.5}$)$_{20}$, $M_{65}(L_{0.5}/F_{0.5})_{20}$, followed by its direct oxidation in water to give the amphiphilic M^O derivative, $M^O{}_{65}(L_{0.5}/F_{0.5})_{20}$.[43] Assembly of $M^O{}_{65}(L_{0.5}/F_{0.5})_{20}$ in water gave vesicles with average diameters of a few microns that could then be extruded to nanoscale diameters. The M^O segments in the vesicles were found to be substrates for reductase enzymes, which regenerated hydrophobic M segments and resulted in a change in supramolecular morphology that caused vesicle disruption and release of cargos. In summary, the formation of vesicles has been one of the major applications of block copolypeptides. Early work developed guidelines for formation of these structures, while current work is aimed at increasing the potent functionality and biologically interactive properties of these materials.

BLOCK COPOLYPEPTIDE HYDROGELS

Hydrogels are a class of materials that have significant promise for use in soft tissue and bone engineering, as well as localized drug delivery.[46] The key feature of hydrogels that makes them attractive for these applications is their well hydrated, porous structure that can mimic natural extracellular matrices.[47] To replace natural materials, however, many structural and functional features must be built into synthetic hydrogels. Desirable features include:

biocompatibility; degradability to allow cell in growth; injectability and fast setting in the wound site; mechanical properties that can be tuned for different uses; control over cell adhesion to the hydrogel matrix; and tunable sustained release of growth factors and biologically active agents.[48] There are many examples where some, or even most of these features have been incorporated into hydrogels.[49] However, in many cases, hydrogel synthesis and formation becomes very complicated, which limits the practicality of such materials. More importantly, the complexity of these systems, combined with limited means for adjustment of molecular parameters, leads to the inability for independent adjustment of most of the features.

For example, it would be advantageous to be able to adjust scaffold rigidity while maintaining a constant hydrogel mesh size. Such a system would allow one to directly measure the effects of scaffold rigidity on cell proliferation. Also, since hydrogel degradation is commonly accomplished using degradable crosslinkers (e.g. in PEG based hydrogels),[48] it can be difficult to adjust degradation rate without also altering crosslink density, and hence initial gel mechanical properties.[48] It would be advantageous to have a hydrogel system where many of these desired adjustable features (e.g. gel strength, gel density, adhesive capability, degradation rate, growth factor release rate) could be controlled more or less independently so that meaningful evaluations of their roles in applications could be systematically evaluated. Currently, in many systems it is difficult to identify the most important gel characteristics, since many features are adjusted simultaneously.[49] Synthetic block copolypeptide hydrogels provide a platform that allows fine adjustment of many of these parameters as well as incorporation of the essential features required for tissue engineering and drug delivery applications.

The Deming lab has developed hydrogels based on amphiphilic block copolypeptides possessing many features that make them attractive as candidates for medical applications.[50] Foremost, through combination of chemical synthesis and structural characterization a detailed understanding of structure-property relationships in these materials has been established, allowing a high level of control over gel strength, gel porosity, gel functionality, and media stability; many which can be adjusted independent of each other.[17] Second, these physically associated gels are readily injectable through a 30G needle for facile application and filling of wound cavities.[50] Finally, the hydrogels can be prepared to limit their toxicity to cells in culture.[51] Hydrogel formation was first discovered in a series of diblock copolypeptides containing a long, charged, water solubilizing domain and an α-helical hydrophobic domain, e.g. $K_{180}L_{20}$ or $E_{180}L_{20}$ (Fig. 4).[50] Hydrogel formation is the result of self-assembly of these polymeric amphiphiles by direct dissolution in water, and the resultant gels possess a network structure composed of nanoscale to microscale porosity and significant material rigidity despite being composed of > 95% water. In order to determine the role played by each copolypeptide domain, a comprehensive study was performed using an array of samples where both overall chain segment lengths and hydrophilic to hydrophobic compositions were systematically varied. It was found that chain length modification of both the charged polyelectrolyte and hydrophobic segments had significant effects on properties.[50]

Compositional studies with different copolypeptides revealed many trends relating molecular parameters to hydrogel properties. First, as oligoleucine composition was increased, gel strength was found to increase dramatically. Furthermore, only hydrophobic segments with α-helical conformations were found to form strong gels, as evidenced by the inability of a

K_{160}(*rac*-L)$_{40}$ sample, where the racemic residues yield a disordered conformation, to form strong hydrogels. It was found that longer polyelectrolyte segments increase interchain repulsions such that the packing of the hydrophobic helices, which prefer formation of flat 2D sheets,[24] must distort to minimize the overall energy of the system. The most efficient way to do this, while maintaining favorable helix packing, is to twist the sheets into fibrillar tapes, where tape width is determined by the degree of twist (Fig. 4).[52] In this model, the helices are still able to pack perpendicular to the fibril axis, but with a slight twist between planes of parallel packed helices. TEM imaging of the nanostructure in $K_{180}L_{30}$ does, in fact, reveal a more fibrillar, tape-like nanostructure constituting the hydrogel network. Overall, copolypeptide gel strength can be adjusted by many molecular parameters: overall chain length, hydrophilic to hydrophobic composition, and block architecture, in addition to the conventional method of varying copolymer concentration. By having many means to adjust gel strength, it is possible to optimize or adjust other hydrogel properties (i.e. mesh size, injectability, or surface functionality) while keeping gel strength constant.

To test their suitability for cell culture applications, hydrogel samples were also prepared in DMEM cell culturing medium, and DMEM with 5% fetal calf serum and penicillin.[53] Samples of $K_{170}L_{30}$ hydrogels were found to be stable and remained transparent in these media, which was somewhat surprising, since they contain numerous multivalent ions and anionically charged proteins. It is likely that the proteins coat the polylysine segments in the gel since it is known that polylysine homopolymer will complex with many serum proteins in solution.[54] Apparently, the resulting polyelectrolyte complexes retain enough charge or hydrophilicity to solubilize the hydrophobic gel scaffold and prevent precipitation and collapse of the network. The porous microscale morphology was found to persist in the $K_{170}L_{30}$ hydrogels in both the presence of 150 mM NaCl and in DMEM. Also, cryogenic TEM revealed that the porous nanostructure persists in the presence of salt as well. The presence of the porosity and the robustness of the nanostructure even in the presence of significant ionic concentration is a critical self-assembling material characteristic for medical applications. Overall, these copolypeptide hydrogels display remarkable stability in the presence of ionic species. Hydrogels formed from helical or β-sheet-forming proteins and peptides typically show some sensitivity to ions, either requiring them to form gels, or disrupting in their presence.[55,56] Likewise, hydrogels prepared from synthetic polyelectrolytes (e.g. crosslinked polyacrylic acid) are very sensitive to salts, shrinking dramatically as ionic strength is increased.[57] The gelation mechanism for these polypeptides, the association of hydrophobic helices, provides a robust structure that is unperturbed under a variety of conditions, including variation of pH, ionic strength, and temperature.

In effort to further understanding on hydrogel formation and tuning of mechanical properties, Deming's lab investigated $K_xL_yK_z$ triblock architectures, which were found to allow for additional tuning of hydrogel properties.[20] In particular, triblocks gave higher gel moduli and improved stability to ionic media compared to diblock copolymers of identical composition. These changes were found to be due to the increased density of K chains at the amphiphile interface, since each hydrophobic segment has a polylysine chain at both ends compared to only one end for the diblock samples, where this additional steric bulk acts to enhance copolymer assembly into the fibrillar morphology that gives strong networks. Deming's lab later studied pentablock copolypeptides of the structure $K_xL_yK_zL_yK_x$ that were expected to possess attributes similar to $K_xL_yK_z$ triblock copolymers, since both have associating L segments capped on each side by K segments.[21] Due to the presence of two α-helical L

segments per chain, the pentablocks also have the intriguing potential for organized intrachain folding, akin to natural proteins, in addition to intermolecular assembly.

Pentablock copolypeptides of the composition $K_{60}L_{20}K_zL_{20}K_{60}$, where *z* was varied from 10 to 200, were synthesized by stepwise linear block copolymerization using $(PMe_3)_4Co$ initiator in THF, followed by removal of protecting groups and purification. Deming's lab found that $K_{60}L_{20}K_{10}L_{20}K_{60}$ formed clusters of micelle-like aggregates with diameters ranging from 50 to 200 nm, which differed greatly from the fibrillar structures seen with diblock and triblock samples. On the other hand, the $K_{60}L_{20}K_xL_{20}K_{60}$ copolypeptides, when $z > 60$, self-assembled in water to form fibrillar hydrogel assemblies. Furthermore, adjustment of the central K segment length allowed tuning of assembly morphology and hydrogel properties where it was observed that G′ increased and minimum gelation concentration decreased as the pentablock central K segments were lengthened. The ability to control intramolecular versus intermolecular assembly of the two hydrophobic L segments in these pentablock sequences gave substantial enhancement of hydrogel properties compared to the corresponding diblock and triblock architectures.[21] The ability to tune intrachain interactions in these materials via molecular design also is a key advance in biomimetic assembly.

Inorganic-organic biocompatible composites have tremendous potential for therapeutic and diagnostic materials applications. Block copolypeptide hydrogels are promising templates for formation of porous composites, where the porous gel scaffold can serve as a template for mineral growth. In 2009, Mallapragada and coworkers reported use of $K_{170}L_{30}$ hydrogels as templates for assembly of calcium phosphate nanocomposites.[58] The porous nature of the hydrogels, and their ability to form gels at low concentrations, allowed composites to be formed that contained up to 50% inorganic material, approaching the inorganic content of bone. Furthermore, detailed characterization of the composites revealed the mineral phase to be carbonated hydroxyapatite, with elongated platelike morphology of nanoscale dimensions, similar to natural bone. In a similar study, Li's group studied the ability of a series of K_xL_y hydrogels ($170 < x < 440$; $10 < y < 30$) to direct silica morphology by sol-gel condensation of tetramethylorthosilicate in the presence of the hydrogels.[59] They found that both the polypeptide lengths, as well as nature of anionic counterions used, had significant effects on resulting silica morphology, where either plates or rods of silica could be formed.

Initial quantitative measurements of polypeptide cytotoxicity involved cell culture within three dimensional hydrogel substrates in cell culturing medium.[51] Although polylysine is known to be cytotoxic at when free in solution,[60] use of higher concentrations of polypeptide above gelation concentrations revealed that both cationic and anionic functionalized gels were promising substrates for short-term cell culture. It is likely that the hydrogel network prevents bulk diffusion of gel-bound lysine chains limiting the amount of polylysine that can interact with the cells. Although the cells remained viable, in neither gel was cell attachment or proliferation observed. The cells, in the presence of either hydrogel matrix, retain their spherical shape after 4 and up to 24 hours. Although it appears cell binding epitopes need to be incorporated into these hydrogels, their peptidic backbone provides many advantages for use of these materials as scaffolds. These include the straightforward incorporation of chemical functionality by use of functional amino acids, as well as enzymatic degradability.

Following up on this work, Sofroniew and Deming studied the biocompatibility of diblock copolypeptide hydrogels in vivo in mouse central nervous system (CNS) tissue.[61] This work was undertaken since biomaterials represent a major opportunity for developing novel CNS

treatment strategies based on site-specific delivery of scaffolds that promote the growth and migration of axons or cells derived from host or grafts, or as depots that release diffusible bioactive molecules to act in a locally restricted manner inside the blood brain barrier. A range of diblock copolypeptide hydrogel formulations with rheological properties similar to brain tissue were injected into mouse forebrain and examined after 1–8 weeks using light microscopy, immunohistochemistry and electron microscopy. Hydrogel deposits were found to elicit no more gliosis, inflammation, or toxicity to neurons, myelin or axons than did injections of physiological saline. The size, rigidity, and density of the hydrogel deposits could be varied subtly by altering sample composition and concentration. The $K_{180}L_{20}$ hydrogel was selected for detailed analyses because it formed deposits with desirable physical properties and since lysine is routinely used as a substrate for neural cell cultures. Deposits of unmodified $K_{180}L_{20}$ exhibited time-dependent in-growth of blood vessels and of certain glial cells, and limited in-growth of nerve fibers (Fig. 4). These findings showed that block copolypeptide hydrogels are injectable, re-assemble in vivo to form three dimensional deposits, exhibit little or no detectable toxicity in the CNS, integrate well with brain tissue and represent a new class of synthetic biomaterials with potential for applications as depots or scaffolds in the CNS.[61]

In a follow up study, Sofroniew and Deming examined the loading and release of bioactive hydrophilic molecules from $K_{180}L_{20}$ and $E_{180}L_{20}$ hydrogels in vitro and in vivo.[62] In vitro tests demonstrated sustained release from dialysis cassettes of the representative protein, lysozyme, dissolved in $K_{180}L_{20}$ or $E_{180}L_{20}$ hydrogels. Release times of molecules in vitro varied in relation to DCH charge and mechanical properties, and ionic strength of the media. To evaluate bioactive protein delivery in vivo, they used nerve growth factor (NGF) and measured the size of mouse forebrain cholinergic neurons, which respond to NGF with cellular hypertrophy (Fig. 5). In comparison with NGF injected in buffer, depots of NGF dissolved in either $K_{180}L_{20}$ or $E_{180}L_{20}$ provided significantly longer delivery of NGF bioactivity, maintaining hypertrophy of local forebrain cholinergic neurons for at least 4 weeks and inducing hypertrophy a further distance away (up to 5 mm) from injection sites.[62] These findings show that depots of block copolypeptide hydrogels injected into CNS can provide sustained delivery within the blood brain barrier of a bioactive protein growth factor that exerts a predicted, quantifiable effect on local cells over a prolonged subacute time.

Conclusion

The synthesis of block copolypeptides by ring opening polymerization is an area that has been under study for more than five decades. Over the last 15 years, vast improvements in NCA polymerizations now allow the synthesis of a variety of block copolypeptides of controlled dimensions (molecular weight, sequence, composition, and molecular weight distribution) and chemical functionality. Block copolypeptides have now been developed that can self-assemble into vesicles or hydrogels with unique properties. While many studies of their utility for medical applications are still in early stages, many of the results to date are promising. The recently developed capability to easily prepare copolypeptides with ordered chain conformations and functionality has opened up a new class of synthetic biomaterials with great potetnial for medical applications.

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Figure captions

Figure 1 (A) Polymerization of NCA monomers into polypeptides. (B) Formation of active species in transition metal initiated NCA polymerization. (C) Stepwise batch formatino of triblock copolypeptides by successive addition of different NCA monomers to a cobalt initiator.

Figure 2 (A) Structure and schematic of $R_{60}L_{20}$ block copolypeptides. (B) Schematic of proposed vesicle self-assembled structure. (B) LSCM image of 1.0 μm extruded vesicles (Bar = 5 μm). Adapted with permission from reference 30.

Figure 3 Schematic showing (A) structure and redox properties, (B) proposed self-assembly of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ copolypeptides into vesicles, and (C) proposed enzymatic rupture of vesicles. Adapted with permission from reference 43.

Figure 4 (A) Schematic representations of block copolypeptide hydrogel composition and structure. Block copolypeptides are composed of variable-length chains of hydrophilic (blue) and hydrophobic (red) amino acids. In aqueous solution, hydrophobic segments associate into elongated fibrillar assemblies that entangle to form 3D networks with hydrophilic segments exposed. (B) In aqueous solutions, hydrophobic segments associate to form elongated fibrillar tape-like assemblies that branch and entangle to form 3D networks with hydrophilic segments exposed. (C-F) Light-microscopic images of 3% $K_{180}L_{20}$ at 1 (C), 2 (D), 4 (E) and 8 (F) weeks after injection of 2 μl into the striatum in cresyl violet stained tissue sections showing time-dependent migration of cells into block copolypeptide hydrogel deposits in vivo. Essentially no cells are present in the deposits after 1 week in vivo (C). After 2 weeks in vivo (D), a number of cells have migrated into, and are scattered throughout the deposits and after 4 (E) and 8 weeks (F) the deposits are densely packed with cells. Arrowheads indicate the borders of deposit and host tissue. Scale bar: C-F = 25 mm. Adapted with permission from reference 61.

Figure 5 (A) Schematic of experimental design to evaluate release of NGF from $K_{180}L_{20}$ hydrogel (DCH) depots in vivo. NGF is known to induce hypertrophy of basal forebrain cholinergic (ChAT) neurons in the caudate putamen (CP) and medial septum (MS). Depots of DCH with NGF were injected into the CP on one side of the brain. (B) Effects of NGF released from DCH depots on local forebrain cholinergic neurons in ipsilateral caudate putamen. Graph shows mean cell area in mm^2 of cholinergic neurons in various treatment groups and at various treatment times as indicated. n = 4 per group, *$p < 0.01$ relative to carrier only, **$p < 0.01$ for group comparisons as indicated, ns non-significant, ANOVA with Newman-Keuls post-hoc pair-wise comparisons. Adapted with permission from reference 62.

Further Reading/Resources

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