CHAPTER 7

Hybrid Systems Engineering: Polymer–Peptide Conjugates

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Contents

1. INTRODUCTION

Synthetic polymeric materials are normally designed with properties that are determined by the nature of the monomers making up their molecular structure. Improvements of function can be achieved by synthesizing block copolymers, which generally rely for their properties on the phase separation and differential solubility's of the blocks concerned (Park et al., 2003). For example, linear A-B block copolymers might incorporate hydrophilic A blocks with hydrophobic B blocks to achieve controllable degrees of crystallinity, water swelling, or rates of hydrolytic degradation. In designing such materials, gross material characteristics can be modified by altering the relative lengths of the blocks, creating branched structures or cross-linking. The introduction of a third block provides further control of properties through the increase in combinations available (Volker Abetz, 2000),

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including the orders (Hückstädt et al., 2000) and ratios of the three blocks involved. Even so, the ability to tune the properties of synthetic, amorphous copolymers at the nanoscale level is limited.

By comparison, nature makes use of an astonishing array of biopolymers with properties that appear to be finely tuned to their biological functions, not only in terms of their chemical makeup but also in terms of their structural organization. Although a number of monomers are utilized in nature, including nucleotides and saccharides, the amino acid polymers, or peptides, are the most interesting for use as designed materials because of the range of properties available and the relative stability of amide bonds. The 20 "natural" amino acids found in nature can be combined in N^{20} ways in a linear chain, where N is the number of monomers in the chain. By adding branched structures and using "unnatural" or noncanonical amino acids, the combinations become virtually limitless. Moreover, peptides can be produced with precise sequences and uniform molecular weights by harnessing the power of the protein synthesis machinery of living cells through genetic manipulation. For example, Tirrell and his colleagues have introduced noncanonical amino acid analogs into designed polypeptides and proteins by this means, including fluorinated amino acids (Wang et al., 2003). With this addition to the chemists' synthesis toolkit, the boundary between "synthetic" and "bio" polymers is becoming blurred. Nevertheless, it should be understood that the term "biopolymer" herein refers to polymers that are made up of the building blocks used by nature (or closely related ones) however they are synthesized and be they inspired by naturally occurring biopolymers or designed de novo.

In contrast to synthetic polymers, which are usually biologically inert, biopolymers are generally bioactive. Bioactivity can convey both advantages and disadvantages. Proper biological signals in biopolymers can promote cell and protein adhesion or elicit biological responses and can allow enzymatic degradation. On the other hand, peptides can be allergenic, immunogenic, or even highly toxic, as the combinatorial cocktail of peptides present in spider venoms attests (Sollod et al., 2005). Biorecognition factors can be built into synthetic polymers, for example, the arginine–glycine–aspartic acid (RGD) sequence that promotes cell adhesion in biomaterials conjugated to the surface of a polycarbonate polyurethane material for human monocyte adhesion (Ernsting et al., 2007). However, the biological inertness of synthetic polymers may have advantages over biopolymers in avoiding toxic, immunogenic, or allergic responses in biomaterials and also can offer resistance to biodegradation.

An interesting approach to the design of biomaterials and biologically inspired materials is to combine the best features of synthetic and biological polymers into copolymers (Klok, 2005; Vandermeulen and Klok, 2004). Thus, peptide sequences may be used to create self-assembled structural motifs at the nanoscale, most commonly β-sheets or α-helices, while the amorphous (or in some cases liquid crystalline) synthetic polymers can be used to provide inert, hydrophilic, or hydrophobic regions and to limit gross aggregation of the peptides. Cells contacting artificial fibers with diameters measuring in the 10's of micrometers behave as though they are on a flat surface, taking on a flat, nonphysiological morphology. Much smaller diameter fibers can now be made, with diameters of 10's of nanometers, but it is difficult to produce these in a network of fibers with pore dimensions appropriate for cellular migration. Therefore, such networks must be formed around cells without damaging them, which is a challenging task. Instead, the use of supramolecular assembly of nanofibrillar and coiled-coil (CC) (nanotubular) structures can be advantageous, particularly if coupled with amphipilic polymer blocks which can gel under normal physiological conditions (Lutolf and Hubbell, 2005).

In this chapter, some methods for peptide–polymer conjugate synthesis are described and a number of examples are reviewed to illustrate the use of self-assembling peptide sequences to drive nanoscale organization. Conjugation of polymers with proteins or peptides for reasons other than self-assembly, for example, therapeutic protein or peptide PEGylation to prolong in vivo circulation half-lives, are not covered.

2. PEPTIDE–POLYMER CONJUGATION

Klok (2005) described a number of synthetic pathways for peptide–polymer conjugates, classed first by liquid- or solid-phase synthesis methods. Within these, synthesis was further classified as being either convergent or divergent. In convergent synthesis, a preformed polymer is attached to a preformed peptide, most frequently by site-specific conjugation, although nonspecific conjugation can be used. Nonspecific conjugation is usually achieved conveniently under mildly basic conditions by amine coupling between an *N*-hydroxy succinimidyl group on the polymer and the ε-amino group available on lysine (K) residues of the peptide. Lysine is one of the most abundant amino acids in proteins so in longer, biologically inspired peptide sequences, it is likely to occur several times in the sequence, thus leading to a number of positional isomers. For shorter sequences, a single lysine residue may be available for amine coupling. Alternatively, the N-terminal amine can be targeted somewhat selectively by reductive alkylation using an aldehyde-functionalized polymer. Because the pKa values of the N-terminus amine group ranges from 7.6 to 8.0 and those of lysine residues ranges from 10.0 to 10.2, the reaction can be made more selective by carrying it out under slightly acidic conditions (pH 5) (Klok, 2005).

The most common chemoselective conjugation is achieved through thiol coupling to free cysteine (C) groups via maelimide chemistry. In proteins, cysteine residues make up only 1.7% of amino acids, and because they tend to form disulphide bonds, it is rare to find a free cysteine group. In designed peptides, this limitation does not exist and a single cysteine can be engineered into the sequence to provide a specific conjugation site. Moreover, the cysteine can be located away from parts of the sequence that determine the structural organization so that the conjugated polymer does not sterically hinder self-assembly of biological function (Kopecky, 2006). Yang et al. (2008) used this technique to synthesize hydrogels by self-assembly.

Another biologically compatible, site-specific method for poly(ethylene glycol) (PEG) conjugation to proteins was described by Sato (2002), who reported the use of the transglutaminase enzyme to catalyze the acyl transfer between the γ-carboxyamide group of glutamine and alkylamines under mild conditions. This method could easily be adopted for peptide–PEG conjugation.

Solid-phase peptide synthesis, first introduced by Merrifield (1963), utilizes a system in which an amino acid is tethered to an insoluble particle by a cleavable link, followed by addition of amino acids one step at a time with protection and deprotection of reactive groups other than the conjugation site. It has the advantage of allowing high conversions at each step when adding each peptide to the sequence since large excesses of reagents can be used and then washed away at the end of each step. Felix and coworkers used this technique extensively during peptide synthesis to conjugate a PEG molecule at a precise location in the sequence (Felix, 1997). It can be used with either convergent or divergent conjugation strategies and has the advantage that it can be automated. However, the method is limited by economic considerations to sequences no longer than about 20–30 residues because the overall conversion is given by X^{N}, where X is the average fractional conversion at each step. Therefore, even with $X = 0.98$, the cumulative effect leads to overall conversion rates of about 55% for 30 residues, which drops drastically to just 21% with a (still respectable) fractional conversion per step of $X = 0.95$.

Examples of divergent addition strategies are given by Lutz and coworkers (Lutz and Hoth, 2006; Lutz et al., 2006) and Loschonsky et al. (2008), specifically via atom transfer radical polymerization (ATRP). This method may lead to a distribution in the synthetic polymer molecular weight, though this might not necessarily be any broader than might be present in a preformed polymer attached via a convergent method. Divergent addition can be combined with either solid phase synthesis or genetic engineering techniques to introduce amino acids with unusual side groups for targeted conjugation.

3. PEPTIDE-DIRECTED FORMATION OF GELS

Natural extracellular matrices in vivo consist of various protein fibrils and fibers, interwoven within a hydrated network of glycosaminoglycan chains and may require less than 1% solid materials to form mechanically robust structures (Lutolf and Hubbell, 2005). Yang et al. (2008) synthesized a hydrogel from hydrophilic synthetic *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymerized separately with oppositely charged pentaheptad CC peptides. Such peptides possess the tendency to form CC conformations if their primary heptad sequences "abcdefg" have hydrophobic residues at positions a and d. In this case, one pentaheptad sequence (CCK) had mostly lysine (K) residues at positions e and g, while the other (CCE) had glutamic acid (E) residues in the same positions. By mixing equimolar quantities of these two peptide–polymer hybrids, a hydrogel was formed, driven by the antiparallel alignment of the CCK and CCE peptides. When modified versions of the two peptides were used, designed to form random coils by replacing glutamic acid with tryptophan (W) and lysine with tyrosine (Y), no hydrogel formed. Also, the hydrogel formed with the CCK and CCE peptide-conjugated HPMA hybrids collapsed after addition of a chaotropic agent (guanidine hydrochloride) or pure CCK or CCE peptides. This evidence supports the conclusion that the CCK and CCE portions of the hybrid molecules self-assembled as antiparallel coil–coil heterodimers, acting as physical cross-linkers to form the hydrogel.

Tonegawa et al. (2004) created a cationic polylysine with a tetrapeptide end sequence (glycine–tyrosine–glycine–lysine), which is a motif common to the consensus sequences of mussel adhesive proteins. They then cross-linked this with the anionic polysaccharide, gellan, enzymatically. The polyionic complexation between the cationic peptide and the anionic polysaccharide formed a hybrid fiber at the aqueous solution interface that, when cross-linked, mimicked the byssus gel that marine mussels use to adhere to surfaces, despite the presence of water and salt.

4. CYCLOPEPTIDE NANOTUBES

Unlike cyclopeptides that consist of peptides with uniform chirality, cyclic peptide sequences of 8–12 residues with an alternating arrangement D- and L-α-amino acids adopt a flat (planar) ring conformation (Ghadiri et al. 1993; Hartgerink et al., 1996). These (D-alt-L)-peptide rings have a high tendency to assemble into tubular ring stacks, occurring by β-sheet-like antiparallel arrangements. Such an arrangement has the H-bonds following the tube axis, with the amino acid side chains oriented outward, so that the exterior is functionalizable and the interior is hollow. ten Cate et al. (2006) noted that cyclopeptide nanotubes grafted with polymers after assembly can suffer from cross-ring linking or disruption with high side-chain grafting densities so investigated the assembly of (D-alt-L)-cyclopeptide-polymer conjugates. Poly(*n*-butyl acrylate) chains were conjugated to opposite sides of a preformed cyclic (D-alt-L)-α-octapeptide, thereby forming a coil–ring–coil conjugate through a convergent approach. Atomic force microscopy of the

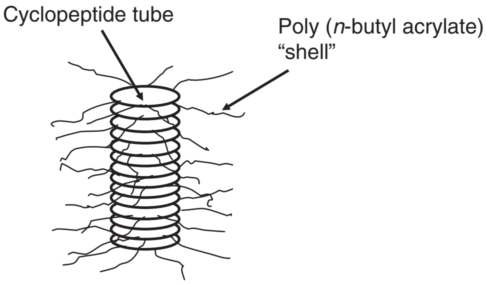

Figure 1 Nanotube-shell conformation of cyclopeptide–polymer coil copolymers (ten Cate et al., 2006).

resulting self-assembled nanotubes on a mica surface revealed aggregates with a height of 1.4 nm and a width of 5 nm and lengths of 200–300 nm. These dimensions were explained by the formation of a tubular peptide core built via stacking of the cyclopeptides, with a poly(*n*-butyl acrylate) shell wrapped around the tube (Figure 1). Loschonsky et al. (2008) observed similar behavior with a cyclic (D-alt-L) octapeptide conjugated to butylacrylate chains at three positions in the rings. The hybrid molecules displayed a solvent-induced self assembly, yielding rod-like structures having a core shell morphology with an internal beta-sheet peptide assembly surrounded by a soft poly(*n*-butyl acrylate) exterior (Loschonsky et al., 2008). They used a divergent strategy with in situ polymerization of the butylacrylate and were able to control the final molecular weight between 5,000 and 30,000 g/mol by controlling the monomer conversion extent.

Although such nanotubes form an interesting structure with potential for exterior functionalization, they are currently limited to passive transport/release of molecules in the interior. Future work may focus on using non-canonical amino acids to impart functionalizable interior surfaces to allow orthogonal functionalization of the interior and exterior surfaces (ten Cate et al., 2006).

5. POLYELECTROLYTIC AND ORGANOMETRIC POLYMER–PEPTIDE CONJUGATES

Chiral conjugate polymers such as polythiophenes with an optically active substituent in the 3 position have been studied for use in optoelectronic devices, sensors, and catalysis (Nilsson et al., 2004). These materials show activity based on their chiral behaviors, which can be influenced by solvent or temperature. Helical biomolecules can be used to induce chirality in optically inactive polythiophenes (Ewbank et al., 2001). Nilsson et al. (2004) extended this to use a positively charged random-coil peptide conjugated with a negatively charged, optically inactive polythiophene to create a hybrid supermolecule with a three-dimensional ordered structure determined by the biomolecule and electronic properties determined by the polythiophene. The polymer backbone conformation was altered in the presence of the peptide to give a main-chain chirality with a predominantly α-helical structure due to acid–base complexation between the two components.

The incorporation of metal centers in polymeric structures creates molecules with interesting functions that have uses as precursors to carbon nanotubes, wire-like structures, or iron-rich ceramic materials (Vandermeulen et al., 2006). The oligotetrapeptide sequence found in silk, glycine–alanine–glycine–alanine (GAGA), was shown to retain the ability to form antiparallel β-sheets when conjugated with polyferrocenylsilane in both block and graft conformations. The block copolymer formed a fibrous network consisting of a core containing self-assembled antiparallel β-sheets with a corona of organometric polyferrocenylsilane. This result was similar to that of Rathore and Sogah (2001), who showed the conservation of GAGA-directed β-sheet formation when conjugated with PEG, as a structure inspired by spider silk.

6. β-SHEET SUPRASTRUCTURES

β-sheet structures are of significant interest, partly because of their ability to form fibrillar strands similar to those that are associated with the lesions seen in Alzheimer's Disease and partly because they form the basis of nanoscale architectures such as fibers and nanotubes. Smeenk et al. (2005) created a repetitive sequence of alanine–glycine interspersed with a glutamic acid–glycine block, conjugated with PEG at both the C and the N terminal cysteine residues via thiol coupling. The formation of β-sheets was observed, while the PEG decoration prevented the further aggregation that normally forms needle-like crystals in its absence.

To extend the self-assembling properties of peptide–polymer conjugates into the organic domain, an organosoluble peptide–polymer conjugate comprising a sequence-defined peptide and a poly(*n*-butyl acrylate) was created with incorporation of structure-breaking switch defects in the peptide segment that allowed control of self-assembly (Hentschel and Borner, 2006). The defects consisted of substituting two amine bonds with esters, which were later restored by an O—N acyl transfer reaction with a pH change. Aggregation tendency was thus suppressed and then triggered by a pH change which restored the native peptide backbone, leading to formation of densely twisted tape-like microstructures. The helical superstructures underwent defined entanglement to form superhelices, leading to the formation of soft, continuous organogels. Again, the structural model proposed to explain the observed behavior was a core-shell tape in which the peptide segments form an antiparallel β-sheet surrounded by a polymer shell. Hentschel et al. (2007) used a similar switch defect approach to suppress aggregation for a range of peptide–polymer conjugates with constant peptide sequences but varying polymer molecular weights and found that once the switch defects had been repaired, the rates of self-assembly correlated with the molecular weights of the poly(*n*-butylacrylate) blocks. Oligopeptide contents of as little as 3.5 wt% could direct nanostructural arrangement.

Self-assembly in an organic phase, followed by in situ cross-linking to preserve the nanostructure, was achieved by a combining diacetylene macromonomers with a parallel β-sheet forming tetrapeptide. After self-assembly, in which the parallel arrangement of the β-sheets was critical to align the functional endgroups, the diacetylene macromonomers were cross-linked by UV irradiation (Jahnke et al., 2007). Hsu et al. (2008) followed a similar path to create a nanostructured, cross-linked polydiacetylene structure. Linear and branched conjugates formed gels at concentrations of 2.0 wt% in water in the presence of ammonium hydroxide vapor. Samples irradiated at 256 nm after gel formation resulted in a characteristic color change from colorless to an intense blue color. Irradiation of the diacetylene macromonomers in the absence of the conjugated peptide resulted in a purple color, indicating both some ordered (blue) and disordered (red) states in the polydiacetylene acids. After irradiation, the gels appeared more mechanically robust, a result of the formation of polydiacetylene backbones within the nanofibers. The β-sheet structure was able to induce a chiral structure in the backbone, reflecting a high degree of order.

7. ROD–COIL CONFORMATIONS

Rod–coil diblocks can be synthesized by conjugating an α-helical peptide sequence to a random coil synthetic polymer (Klok et al., 2000). The self-assembly is driven both by the microphase separation of the blocks and by a tendency for the rods to form anisotropic liquid–crystal domains, giving rise to a range of morphologies that are distinct from those found in coil–coil copolymers (Stupp et al., 1997). According to Klok et al. (2000), asymmetry in the stiffness of the rod and coil parts of the copolymer results in an increase in the heat of mixing (as indicated by the Flory–Huggins parameter) over that of coil–coil copolymers, yielding phase separation-driven self-assembly at lower degrees of polymerization than coil–coil copolymers, that is, at smaller length scales. These rod–coil copolymers may therefore show self-assembled domains with sizes of the order of a few nanometers, although coil–coil copolymers can take on similar dimensions at the lower end of their range (5–100 nm). Unlike the earlier work of Gallot et al. (1996), who worked with polypeptides, Klok et al. (2000) utilized this expected phase separation in studying the behaviors of oligopeptides with just 10–20 units of γ-benzyl-L-glutamate as the rod and 10 repeat units of styrene as the random coil. The resulting morphologies were distinct from the lamellar structures observed with high-molecular-weight components. At 120°C, (styrene)$_{10}$-b-(γ-benzyl-L-glutamate)$_{10}$ conjugates possess partly α-helical and partly β-sheet organization, whereas increasing the peptide length to 20 units increased the proportion of α-helices, resulting in a "double-hexagonal" organization (Figure 2). Increasing the temperature tended to disrupt the hexagonal structure, yielding only β-sheet assemblies.

The advantage of utilizing peptides as rod segments is that their conformation as either α-helices or random coils can be controlled by pH, solvent, ionic strength, or temperature, which may therefore allow reversible switching of the nanoscale organization since this is driven largely by hydrogen-bonding interactions between neighboring rods in the α-helical conformation.

Mori et al. (2005) used a convergent method to fabricate rod–coil diblock copolymers of oligo(*p*-phenylenevinylene) (OPV) as a hydrophobic rod and poly(ethylene oxide) (PEO) as a hydrophilic random coil. Their aggregation behavior in a tetrahydrofuran/H_2O solvent was explored and parallel alignment of the OPV blocks was observed. Copolymers with PEO weight fractions of 41 and 62% formed cylindrical aggregates (diameter 6–8 nm and lengths of several hundred nanometers), but with higher PEO content (79 wt%), the conjugates formed distorted spherical aggregates of average diameter 13 nm. The aggregates were able to solubilize homo-oligomer OPV within the OPV cores of the aggregates, and their morphologies changed from "ambiguous" nanosized structures to cylindrical structures with

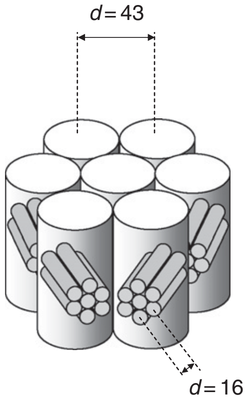

Figure 2 Klok et al.'s model of the self-assembly of rod–coil copolymer (styrene)$_{10}$-b-(γ-benzyl-L-glutamate)$_{20}$ at 120°C. Small cylinders represent the peptide rods. Styrene coils, not shown for clarity, are proposed to protrude randomly from both sides of the oligopeptide clusters (Klok et al., 2000).

diameters that depended on the mixing ratio of the OPV homo-oligomer. As suggested by the authors, solubilization inside the cores of aggregates may comprise a method of fine tuning the nanostructural organization.

8. PHYSICAL MANIPULATION OF NANOSTRUCTURAL ORIENTATION

In an attempt to mimic the process of biosilicification, Kessel and Borner recently developed an intriguing method for creating aligned polymer-silica fiber networks by 2D-plotting of PEO–peptide nanotape "ink" (Kessel and Borner, 2008). According to the authors, the PEO–peptide nanotapes mimic the functional structures of proteins like silaffin and silicatein that catalyze and guide the formation of biosilica from dilute silicic acid solution at neutral pH. The plotting process relied on local injection of the nanotape solution into a thin layer of a dilute solution of prehydrolyzed tetramethylorthosilicate (TMOS), a silicic acid equivalent. The "plotting" was achieved by moving a 250 μm inner diameter capillary connected to a syringe pump (flow rate 0.2 mL/min) with a computer-controlled (CNC) lathe, able to move at rates of between 0.5 and 2 m/min. The width of the nanotape fibers was directly related to the speed of plotting at a constant ink flow rate, giving fibers between 700 μm at the slowest plotting speed and 170 μm at the fastest plotting speed. Silicification was dependent on the presence of the β-sheet-aligned peptide blocks and increased with ageing of the fibers in the TMOS solution. The fibers were seen to align with the direction of plotting. Calcination at 450°C resulted in decomposition of the organic constituents, leaving the silica network intact and preserving the nanostructured morphology to give mesoporous silica fabrics with high surface areas and aligned cylindrical pores. The authors suggested that 3D plotting may provide more complex structures of importance for regenerative bone repair as active scaffolds for cell growth and directional cell organization in artificial tissues.

9. CONCLUSIONS

Peptides can be used to direct the nanoscale assembly of amphiphilic synthetic polymers. A common feature is that the self-assembly of the peptides proceeds as it would do in the absence of the polymer conjugates, with the peptide suprastructure forming a core, surrounded by the polymer random coil. The polymer shell acts to limit aggregation of the peptides beyond a certain size limit. A particularly striking example of this is the self-assembly of cyclopeptide–polymer composites, which form hollow nanotubes surrounded by a polymer shell, resulting in a uniform size distribution. The polymer shell could also be used to add functionality to the exterior of the nanotubes.

Potential applications of peptide–polymer conjugates include drug delivery materials, optoelectronics, biosensors, tissue scaffolds, tissue replacement materials, hydrogels, adhesives, biomimetic polymers, lithographic masks, and templates for metallic or silica nanostructures.

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