Protein–Polymer Hybrids

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Protein-Cross-Linked Polymeric Materials through Site-Selective Bioconjugation**

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Well-defined hybrid materials constructed from proteins and polymers offer significant opportunities for the construction of sensors, actuators, and drug-delivery systems. The enabling concept underlying these materials is the fusion of the specific biological function of proteins with the bulk properties and processability of synthetic polymers. Recent reports have capitalized on this concept to produce materials that undergo a dynamic change based on a number of different inputs. Examples include changes in response to ions,[1] peptides,[2] antigen-[3] and carbohydrate-binding interactions,[4] cell surface receptors,[5] and temperature.[6] In a particularly well-defined example, Murphy et al. demonstrated the covalent attachment of polymers to two specific protein sites, effectively generating a biomolecular cross-link. Ligand-induced conformational changes in the protein then afforded a significant change in volume.[2]

While these pioneering studies promise great opportunities, they have relied on coupling techniques that are difficult to generalize to all proteins and polymers. Site-selective bioconjugation is at the heart of such materials, and utilizing modern methods for protein activation[7–10] should provide access to a wider range of materials. In particular, it could be advantageous to attach at two sites, which requires the challenge of modifying proteins selectively at two locations. Ideally, the method for accessing these hybrids would not rely on the primary sequence and would be applicable to a wide array of proteins.

We therefore targeted the protein termini because they provide two site-specific, chemically distinct modifications that are independent of any one protein and yet common to all. This attachment strategy also represents an optimal way to relate the folded state of the protein to the properties of the polymer backbone. Herein, we report a method for the construction of protein–polymer hybrid materials utilizing orthogonal chemical reactions to link polymer chains to the two termini of a protein. We also report the initial characterization of a new material, a fluorescent hybrid hydrogel, constructed using this strategy.

To develop this methodology, we selected a protein with a unique set of properties to allow facile characterization of the resulting gels. Enhanced green fluorescent protein (eGFP) is a 26.6-kD protein with an internal chromophore that is sensitive to many factors that affect the protein's structure.[11] When incorporated into a material through termini cross-linking, the fluorescence and size properties of the material should depend on the protein's folded state. The application of different environmental stimuli was expected to change the protein, and thus the bulk material properties (Figure 1).

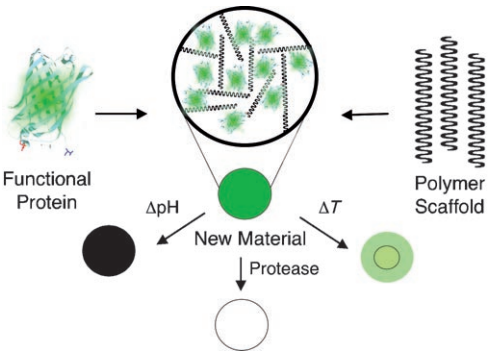

Figure 1. New materials can be constructed by cross-linking polymer chains with proteins that display a structure-dependent function. The unique properties of a material cross-linked by eGFP are illustrated: sensitivity to pH, heat, or degradation by proteases.

Recently, we reported a method for the transamination of the N terminus of proteins to afford aldehydes or ketones for subsequent oxime formation.[12] We found that this reaction works well for Gly, Asp, and others under mild reaction conditions. Herein, we have combined this reaction with the well-established technique of expressed protein ligation (EPL)[7,13,14] to provide a protein with similarly reactive groups for polymer attachment.[15]

The protein was first expressed in *Escherichia coli* as an intein–chitin fusion (IMPACT-CN). A cysteine piperidone amide (**3**) was attached by EPL (Figure 2). For analytical purposes, alkoxyamine **6**[12] was mixed with activated protein, which resulted in a shifted protein band upon analysis by SDS-PAGE. Following the EPL step, a second batch of protein was subjected to 10 mM pyridoxal 5′-phosphate (**4**; Figure 2) as previously described.[12] While both of these reactions have previously been shown to work on eGFP, they

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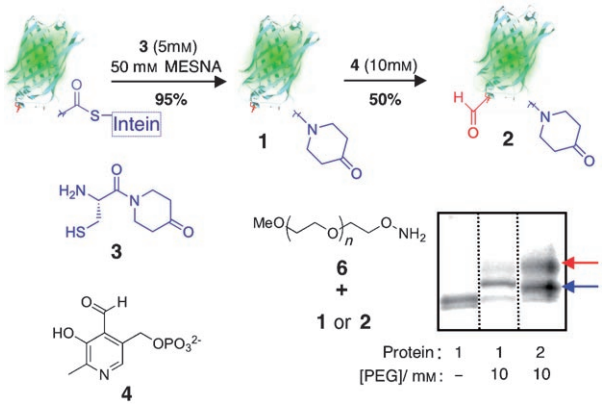
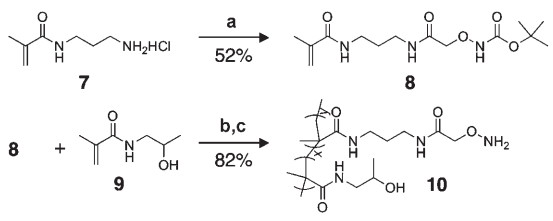


Figure 2. Activation of both eGFP termini for polymer attachment. eGFP was expressed as an intein–chitin fusion, purified by chitin affinity chromatography, and reacted with **3** (5 mM) and MESNA (50 mM) to yield **1** with 95% conversion. Then the product was reacted for 18 h with **4** (10 mM). Conversions were obtained by densitometry measurements of a gel-shift assay using 2-kD PEG-alkoxyamine **6**.[21] MESNA = mercaptoethanesulfonate sodium salt; PEG = polyethylene glycol.

have not to our knowledge been shown to work together to create a protein that has been modified at both termini. Although somewhat labor intensive, it is a technique that should be applicable to the incorporation of a large number of proteins.

With the activated protein prepared, it was necessary to create a polymer containing carbonyl reactive groups suitable for hydrogel formation. Alkoxyamine-bearing polymers have previously been synthesized for reaction with carbohydrates[16,17] and proteins.[18] Recently, the Maynard research group outlined several practical methods for obtaining alkoxyamine-bearing polymers.[19,20]

It has been shown that *N*-(3-aminopropyl)methacrylamide (**7**) can be functionalized and copolymerized to create protein hybrid hydrogels.[6] Starting from the same monomers, a similar polymer **10** was synthesized bearing an alkoxyamine (Scheme 1). The composition of the polymer was determined by ^{1}H NMR spectroscopy, which showed the predicted molar ratio of incorporated monomers.[22] Polymer size was determined as a number-average molecular weight of 56600 with a polydispersity index of 1.53 using polyethylene oxide as a reference in gel-permeation chromatography (GPC).[23]



Scheme 1. Conditions for the synthesis of alkoxyamine-*co*-HPMA (**10**): a) *N*-Boc-aminooxyacetic acid-NHS, Et_3N, CH_2Cl_2; b) 0.6% w/w AIBN, 86.9% w/w MeOH, 50 °C; c) HCl (4 M). HPMA = hydroxypropyl methacrylamide, Boc = *tert*-butoxycarbonyl, AIBN = azobisisobutyronitrile.

Protein–polymer conjugation was verified by two methods. First, a gel-shift assay was used to visualize conjugation (Figure 3a). A significant portion of the protein sample was

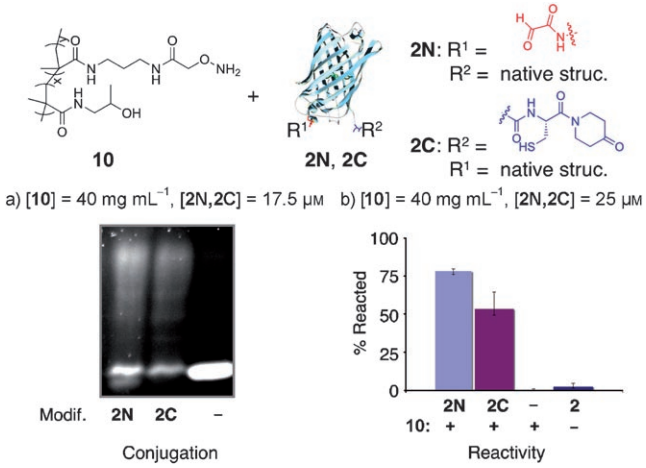


Figure 3. Assessing polymer–protein conjugation. a) Compound **10** was mixed with eGFP singly activated at the N terminus (**2N**), the C terminus (**2C**), or left unactivated (**2**). Conjugation was demonstrated by SDS-PAGE with SYPRO Orange staining. b) A portion of **10** (20 mg) was mixed with 25 μM eGFP (500 μL), and the samples were purified by ultrafiltration with a 100-kD MWCO filter. The graph indicates the percentage of total protein retained, which only occurs after polymer attachment; **2** denotes eGFP activated on both termini, but with no polymer present.

converted to a higher-molecular-weight species. To assess the overall reactivity in a more quantitative fashion, an ultrafiltration experiment was performed (Figure 3b). Samples of eGFP activated at either the N or the C terminus, as well as unactivated protein, were reacted and then spun in 100-kD molecular weight cutoff (MWCO) filters. The fluorescent protein species was retained in only the activated samples (Figure 3b). Good retention (ca. 75%) of eGFP indicated that oxime formation could be driven to high conversion with high concentrations of **10**.

Next, gels were formed by combining a solution of **10** (370 mg mL^{-1}) with a solution of **2** (92 mg mL^{-1}) in a 2:1 (w/w) ratio. These high concentrations were necessary to achieve relatively quick gel formation because the oxime reaction is concentration dependent. The gels were reacted for 24 h, separated from free protein by ultrafiltration, and drop-cast onto teflon sheets.

To confirm the cross-linking and gel properties, dried gels were swelled in phosphate-buffered saline (PBS; Gibco) to establish a swelling profile.[24] To verify that cross-linking was dependent on the activation of both protein termini, samples constructed of singly activated proteins were also swelled (Figure 4). In contrast to the doubly activated sample, these materials completely dissolved over a period of 6 h. Additionally, to ensure drying did not affect the stability of eGFP, nonactivated proteins were dried under gel-forming conditions and then redissolved. The eGFP stability, as monitored by fluorescence, was unaffected by the gel-forming conditions.[25]

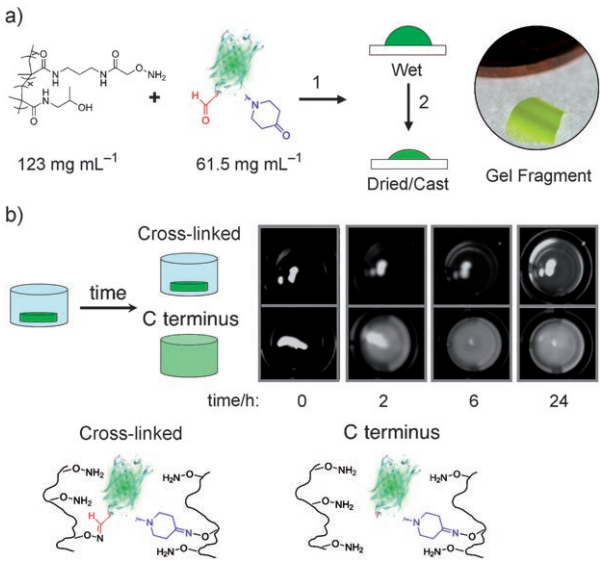


Figure 4. Material assembly and testing. a) 1) Compound **10** and activated eGFP were mixed in phosphate buffer for 24 h, then resuspended in buffer and purified by ultrafiltration to remove unreacted protein. 2) Wet gels were drop-cast onto teflon sheets and allowed to dry for 16 h. A picture of a gel fragment is shown next to the edge of a penny for scale. b) Gels were swollen in PBS. Gels cast with eGFP lacking N-terminal activation dissolved over 6 h. Gels cast with fully modified proteins were stable for > 24 h.

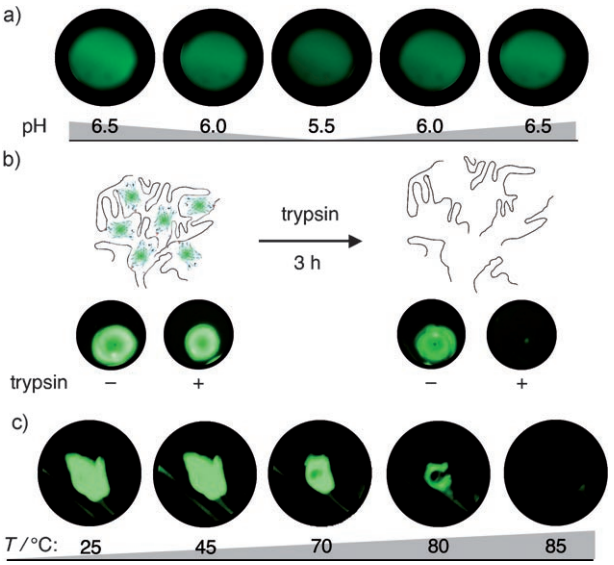


Figure 5. a) Swelled hydrogels were placed in successive solutions of PBS in which the pH was first lowered, then raised. Fluorescence was lost and then partially regained with negligible volume changes. b) Swelled hydrogels were placed in $CaCl_2$ (50 mM, pH 8.0) and trypsin (33 mg mL^{-1}). Gels with trypsin dissolved completely within 3 h. c) Gels were placed in a sealed vial filled with PBS and heated in an oil bath. A decrease in gel volume accompanied a loss of fluorescence with increasing temperature. Gels were illuminated with UV light in a darkened chamber and imaged by a CCD camera.

A unique aspect of these gels is the fact that the only cross-links are formed by the protein itself. As eGFP is sensitive to protease degradation,[26] this property should also be passed along to the hybrid gel, thus creating a rapidly biodegradable material. To verify this concept, swollen gels were exposed to high concentrations of trypsin. Gels with trypsin completely disintegrated in less than 3 h, while gels in only buffer did not (Figure 5b).[27]

To demonstrate the properties of the hydrogels that are imparted by the protein component, equilibrium-swelled gels were exposed to conditions known to affect eGFP fluorescence and/or structure and monitored by imaging with a digital charge-coupled device (CCD) camera (Figure 5). First, as eGFP fluorescence is pH sensitive with loss occurring from pH 7 to 4,[28] the gels containing it should be similarly environmentally sensitive. In this instance, a material was achieved that senses pH through fluorescence changes without a change in size as a result of the protein's minimal conformational change. Additional cycling of the pH values resulted in repeated fluorescence modulation with an overall loss of about 5% per round.

Finally, as eGFP undergoes a structural denaturation from 60 to 80 °C,[29–31] it should impart this function to the gel and create a material with a simultaneous change in volume and fluorescence around 80 °C. To test this possibility, gels were heated and their fluorescence and swelling were measured. Gels began to shrink, accompanied by a loss of fluorescence, at around 70 °C. This continued until a loss of fluorescence and a large size reduction occurred at 75 °C.

By combining proteins and polymers in this manner we were able to create a bulk material imbued with some of the properties of a protein. As the chemistry is independent of amino acid sequence, this method should serve as a general route to a wider number of protein-based materials, each providing a unique function in the resulting gel. An example would be metallothioneins,[32] whose metal-binding properties would be highly desirable in a bulk material, but whose reactive cysteines prohibit attachment by other methods. Additionally, the pH and protease sensitivity of this specific material could be adapted to applications that include drug delivery and environmental sensing. Finally, as it has been established that protein conformational changes can influence the shape and volume of materials, we propose that our strategy may be well-suited to demonstrate the converse: that strain could affect protein conformation, thus creating a material capable of visually reporting damage.

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