Reversible Immobilization of Protein into Hydrogel Using Designed Coiled-coil Peptides

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By using a heteromeric coiled-coil interaction between peptides, both immobilization of a protein into polymer hydrogel and an efficient release of the protein from the hydrogel were achieved.

Hydrogels have a variety of functional properties, such as swelling, mechanical, permeation, surface, and optical properties.[1,2] Stimuli responsive hydrogels containing biomolecules have been extensively studied in the context of biomedical applications.[3,4] Among them, the hydrogels that release the pre-loaded biomolecules in response to changes in the local environmental conditions such as pH, temperature, concentration of particular molecules, are useful materials in the field of drug delivery and release systems.[4] However, in pre-loaded protein systems, only a few kinds of stimuli have been utilized.[5,6] A proposition of a novel method for immobilization and release of proteins will expand the utility of such hydrogels. Along with these aspects, we attempted to develop a new protein immobilization method by using hetero-stranded coiled-coil peptides, which is one of the common folding motifs in native proteins and has been widely used in the self-assembly of biological and smart materials.[7,8]

In the present study, one of the hetero-stranded coiled-coil peptide chains is conjugated on a polymer hydrogel and the other chain is fused to a target protein (Figure 1a). By mixing these two, the protein should be immobilized into the hydrogel by the non-covalent and specific peptide–peptide interaction. Selective release of the protein from the hydrogel in response to the externally added counterpart peptides is also highly expected for this system. The design of coiled-coil forming peptide sequences was described in our previous paper.[7] Briefly, the peptide 1αK and 1αE contain hydrophobic (Leu, Val), basic (Lys), and acidic (Glu) residues along with a buried Asn residue to direct a heterodimeric parallel coiled-coil structure (Figure 1b).[9] An acryloyl group was introduced at N-terminus of 1αK via the flexible –Ahx–Gly–Gly– spacer (Ahx; 6-aminohexanoic acid) to give a monomer NAcr–1αK. We also designed NTrp–1αK, NDns–1αEY, CDns–1αEY, and NTrp–1αKA, in which Trp residue or dansyl group was placed at the N- or C-terminal as a fluorescence probe. In NTrp–1αKA, all the Leu and the Val residues in NTrp–1αK were substituted with Ala to perturb the coiled-coil interaction. Peptides were synthesized by solid-phase method using Fmoc-strategy. The peptides were purified with reversed-phase HPLC and identified by MALDI-TOFMS analysis. Circular dichroism (CD) and fluorescent resonance energy transfer analysis of NTrp–1αK, NDns–1αEY, and CDns–1αEY revealed that the 1αK and 1αE formed a parallel heterostranded coiled-coil with 1:1 stoichiometry (affinity constant; 8.8×10^6 M^{-1}).

As a model protein, green fluorescent protein (GFP) was chosen to demonstrate the utility of the specific peptide interaction for the immobilization of a large biomolecule into a hydrogel. Because of its own fluorescence, GFP is useful in estimating the amount of bound protein.[10] The 1αE sequence was fused into both the N- and the C-termini of GFP via a 6-residue linker to generate a construct, 1αE–GFP–1αE, (Figure 1c). To simplify the protein purification, we also appended a 6 × His tag to the C-terminal end of the construct. The plasmid containing the gene of the 1αE–GFP–1αE was prepared using standard methods. The protein was overexpressed in *E. coli* BL21(DE3)pLys and purified with Ni–NTA and Sephadex G-25 columns.

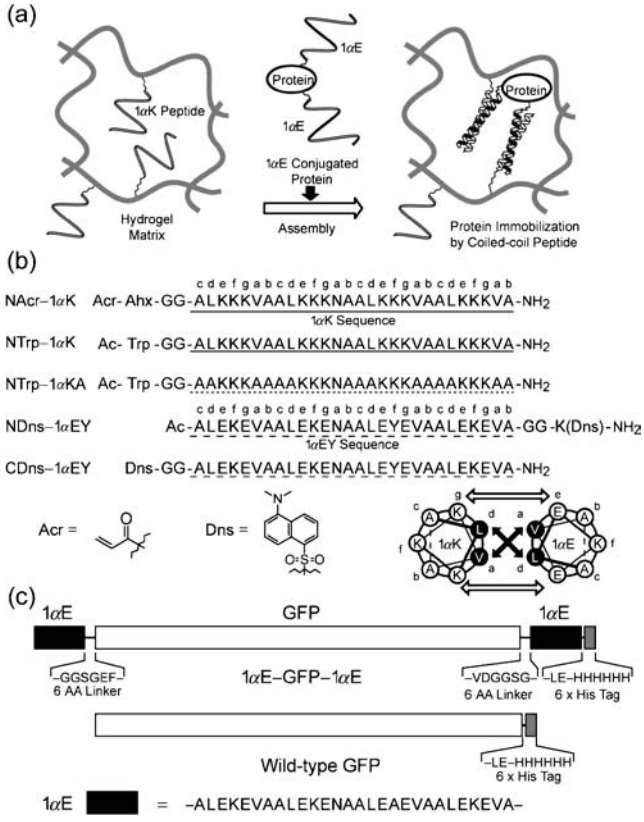

Figure 1. (a) Schematic illustration of the protein immobilization into a hydrogel using designed coiled-coil peptides; (b) Structure of designed peptides; (c) Structure of a designed protein, 1αE–GFP–1αE.

CD study revealed the interaction of 1αE–GFP–1αE and NTrp–1αK peptide via the coiled-coil formation (Figure 2). The 1αE–GFP–1αE showed a CD spectrum typical for the mixture of α-helix and β-sheet structure. The CD profile of wild-type GFP looked like that of purely β-sheet polypeptide; a negative and a positive maxima appear at 215 and 200 nm, respectively. These results indicate that the 1αE sequences in the designed protein should partially take α-helix structure. Interestingly, the α-helix content of 1αE–GFP–1αE was considerably increased by the addition of NTrp–1αK peptide (1.0

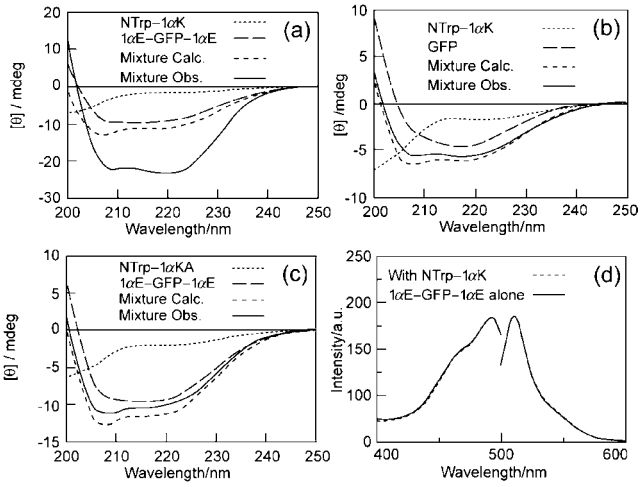

Figure 2. (a), (b), (c) CD spectra of 1αE–GFP–1αE in the presence and absence of NTrp–1αK (1.0 equiv.) (a) or NTrp–1αKA (c) and those of wild-type GFP/NTrp–1αK (b) in 20 mM Tris HCl/150 mM NaCl buffer (pH 7.4) at 25 °C. [Protein] = 5 μM. [Peptide] = 10 μM: (d) Fluorescence (right) and excitation spectra of 1αE–GFP–1αE in the buffer (pH 7.4) at 25 °C in the presence and absence of NTrp–1αK (1.0 equiv). [Protein] = 1 μM. λ_{ex} and λ_{em} were 480 and 510 nm, respectively.

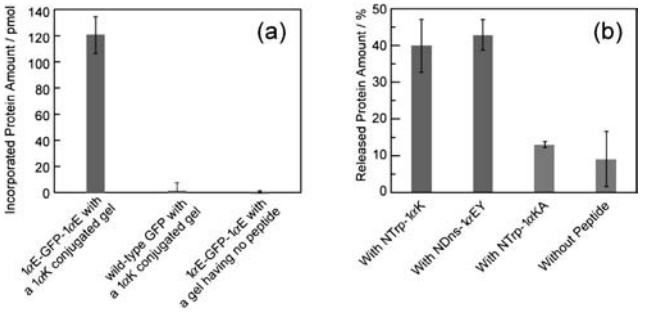

Figure 3. (a) Incorporation of protein into hydrogels. The gels were incubated with protein sample (350 pmol) for 5 h at 25 °C in a buffer (pH 7.4). Amount of bound protein was estimated by fluorescence assay of the supernatent. [Protein] = 10 μM: (b) Amount of protein released from the 1αK-conjugated hydrogel by the addition of designed peptides (1400 pmol). Amount of released protein was estimated by measuring the fluorescence intensity after incubation in a buffer for 5 h at 25 °C. [Peptide] = 14 μM.

equiv.) Taking into consideration that either the addition of NTrp–1αK to native GFP or NTrp–1αKA to 1αE–GFP–1αE did not affect their CD spectra (Figures 2b and 2c), it can be reasonably concluded that the increase in α-helicity is ascribed to the coiled-coil formation of 1αE and 1αK peptides on the protein surface. On the contrary, fluorescence and excitation spectra of 1αE–GFP–1αE in the presence of 1αK peptide were indistinguishable from those without 1αK (Figure 2d). The spectra were also identical with those of native GFP (data not shown). This means that the conjugation of 1αE sequences and the coiled-coil formation did not greatly alter the structure around the fluorophore in the GFP.

The binding study of 1αE–GFP–1αE was performed using the 1αK-attached polyacrylamide hydrogel which was prepared by polymerization of NAcr–1αK (700 pmol), *N*,*N*′-methylenebis(acrylamide) (17.5 nmol), and acrylamide (7 μmol) in a buffer at 25 °C in the presence of redox radical initiator (total volume: 10 μL).[11] Quantitative fluorescence analysis revealed that the 1αE–GFP–1αE was effectively incorporated into the 1αK-conjugated hydrogel (Figure 3a). After incubation of 1αE–GFP–1αE (350 pmol) with the gel for 5 h, ca. 30% of protein molecules were immobilized into the hydrogel material. On the other hand, wild-type GFP was not incorporated under the same conditions. Similarly, no detectable incorporation of 1αE–GFP–1αE was observed when the hydrogel lacks 1αK peptide moiety. These results clearly indicate that the coiled-coil peptides conjugated to protein and polymer chain play an essential role for the effective immobilization of the target molecule.

Release of 1αE–GFP–1αE in response to externally added peptide was also examined (Figure 3b). By the addition of NDns–1αE or NTrp–1αK, the 1αE–GFP–1αE was smoothly liberated into the solution. The amount of the released protein in the presence of these peptides was five times as large as that without the peptide. This implies that the externally added peptide formed coiled-coil structure with pre-existed peptide chains, leading to the cancellation of coiled-coil interaction between 1αE–GFP–1αE and 1αK-conjugated hydrogel. Such a postulation was further supported by the fact that the control peptide NTrp–1αKA, having the same number of basic Lys resides as NTrp–1αK does, showed much weaker protein-releasing ability. Thus, by employing coiled-coil-mediated immobilization of protein, highly selective release was realized.

In summary, we have successfully demonstrated a novel method for reversibly immobilizing a protein into the hydrogel via the specific peptide–peptide interaction. The release of protein was highly sensitive and selective against the externally added peptide sequence. Although detailed study on the interaction of the protein and hydrogel is needed, this strategy might be extendable to immobilization and release of a variety of proteins to/from hydrogels, and this will be potentially applicable to drug delivery systems.

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