An enzymatic method for site-specific labeling of recombinant proteins with oligonucleotides†

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DNA was site-specifically conjugated to a substrate peptide of microbial transglutaminase fused to the N- or C-terminus of target proteins without the loss of the proteins' functions of interest.

Protein–oligonucleotide (DNA) conjugates are versatile molecular tools for a variety of applications in biotechnology and materials science.[1–3] In particular, oriented immobilization onto a surface displaying the complementary DNA (cDNA) has been a potential application of protein–DNA conjugates. This basic concept can be seen in *in situ* hybridization techniques in the field of molecular biology, and more recently the utility of protein–DNA conjugates has emerged in DNA-directed immobilization (DDI) techniques[4–9] for the fabrication of protein microarrays.

Despite the potential advantages of the DDI method, the synthesis of protein–DNA conjugates is often cumbersome. By chemical manipulation, depending on the functional groups available on the target proteins, the conjugates to be obtained represent a heterogeneous molecular species due to the uncontrollable and unpredictable attachment of DNA. This results in partial inactivation and/or heterogeneous orientation of the immobilized proteins. Hence, site-specific attachment of DNA to proteins is crucial when preparing protein-DNA conjugates. In this context, a technique was developed involving site-specific conjugation of PNA or DNA derivatized with an N-terminal cysteinyl group, to a C-terminal α-thioester of recombinant proteins prepared by expressed protein ligation (EPL).[10–13] Although this method has been shown to be capable of site-specific conjugation of a protein with DNA, the expression of a target protein as an intein-fusion protein could limit the generality of this methodology. Furthermore, the incorporation site of DNA is limited to the C-terminus of target proteins, indicating that this strategy is inadequate toward such proteins of which the C-terminus is functionally important.

In the present study, an alternative strategy for site-specific and covalent conjugation of DNA to a peptide tag of a recombinant protein was investigated, using microbial transglutaminase (MTG) from *Streptomyces mobaraensis*. Transglutaminase is an enzyme that catalyzes the acyl transfer reaction between a primary amine and the γ-carboxyamide group of Gln residues in peptides and proteins. When the ε-amino groups of certain Lys residues act as acyl-acceptors, proteins are site-specifically conjugated through the formation of covalent ε-(γ-Gln)Lys bonds.[14,15] A practically important characteristic of transglutaminases is that they also accept a range of primary amine-derivatized organic molecules and attach them to a specific Gln residue that fits the substrate specificity of this enzyme. Since MTG shows attractive catalytic features compared with transglutaminases from other origins,[16] it has been utilized for protein labeling with small organic molecules,[17–19] and for protein immobilization.[20–22] Therefore, one can imagine that MTG is applicable for the labeling of proteins with DNA. However, no report has yet shown the preparation of protein–DNA conjugates by MTG or any other transglutaminases.

Since the incorporation of primary amine-derivatized synthetic polymers such as polyethylene glycol[23] and polysaccharide[24] onto a specific Gln residue of proteins has been investigated by MTG and guinea pig liver transglutaminase, respectively, we first attempted MTG-mediated labeling of *N,N*-dimethylated casein, a good proteinaceous substrate of MTG possessing reactive Gln residues, with an 5′-end aminated 24-mer DNA. However, virtually no conjugation was observed by SDS-PAGE analysis (see ESI†). This implies low affinity of aminated DNA for MTG, possibly due to electrostatic factors such as an intrinsically low affinity of MTG for anionic acyl-acceptor substrates,[25,26] and additional steric factors such as intramolecular interactions between the cationic amino group and polyanionic DNA.

To increase the affinity of the polyanionic substrate for MTG, we converted the DNA from an acyl-acceptor to an acyl-donor substrate. *N*-Carbobenzyloxy glutaminyl glycine (Z-QG) is a neutral dipeptide substrate that is well recognized by MTG.[27] Successful conversion of an amine substrate of MTG, monodancyl cadaverine, to an acyl-donor substrate was previously reported by coupling the C-terminal carboxyl group of Z-QG with monodancyl cadaverine.[28] This finding prompted us to couple an aminated DNA with Z-QG in a similar manner, and the resultant Z-QG-modified DNA (Z-QG–DNA) was used as an acyl donor substrate for the subsequent MTG-mediated conjugation with proteins (Scheme 1).

As model proteins, bacterial alkaline phosphatase (AP) and enhanced green fluorescent protein (EGFP) were selected. A short substrate peptide containing a reactive Lys residue for MTG (Met-Lys-His-Lys-Gly-Ser = K6-tag[20]) was fused to the N-terminus of these proteins. The resultant recombinant proteins were

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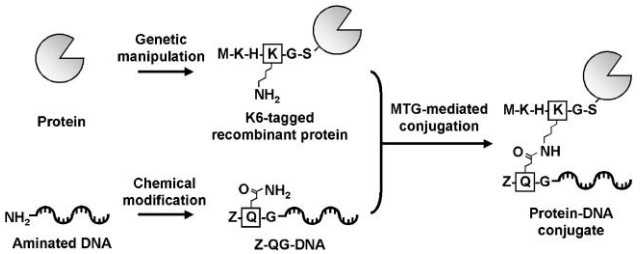
† Electronic supplementary information (ESI) available: Detailed protocols of chemical synthesis of Z-QG–DNA, protein expression and purification, MTG-mediated conjugation of proteins with DNA, and DNA-directed immobilization. See DOI: 10.1039/b613793h



Scheme 1 MTG-mediated peptide tag-specific labeling of a recombinant protein with Z-QG–DNA.

abbreviated as NK6-AP and NK6-EGFP, respectively. In the case of AP, the same substrate peptide was also tethered at the C-terminus (the resultant AP was abbreviated as CK6-AP).

Fig. 1 shows the SDS-PAGE analysis of the conjugation reactions. The bands of both NK6- and CK6-APs were shifted, indicating higher molecular weight, after the reaction with Z-QG–DNA in the presence of MTG, indicating that the Z-QG moiety coupled to the 5′-end of DNA *via* a C6 alkyl linker was recognized by MTG. Importantly, Z-QG–DNA was easily tethered to the K6-tag at both the N- and C-termini of AP (lanes 3 and 5 in Fig. 1). The conjugation between NK6-EGFP and Z-QG–DNA was also evident and protein function was virtually all retained after conjugation in all cases (see ESI†). These results indicate the generality of the present strategy in terms of the labeling sites as well as target proteins.

According to a hypothetical catalytic mechanism of MTG,[29] Cys64 in the active site nucleophilically attacks an acyl donor (*e.g.* Z-QG–DNA), and the acyl–enzyme intermediate is formed. The amino group of an acyl acceptor (*e.g.* NK6-AP) subsequently attacks the acyl–enzyme intermediate, and the reaction is completed with the release of the AP–DNA conjugate. On the basis of the protein bands in SDS-PAGE analysis, conversion rates were estimated to be $\sim$30% when the conjugation reaction was conducted with the equimolar amount of Z-QG–DNA and K6-tagged APs. About 4-fold increase in the Z-QG–DNA concentrarion improved conversion rates to be about 50–60% (Fig. 1). With respect to AP, which forms dimers in solution,[30] this means that there will be on average one DNA molecule per dimeric AP molecule. Since the subsequent increase in the concentration of Z-QG–DNA to 8-fold of that of the proteins failed to increase the yield of the conjugate, the deacylation step of the acyl–enzyme intermediate between MTG and Z-QG–DNA would be the rate-limiting step of this reaction.

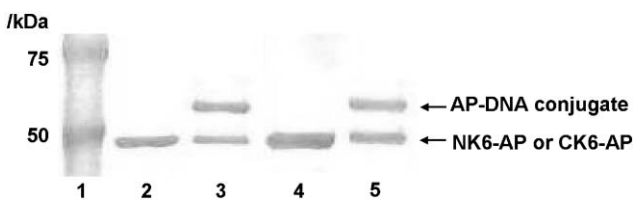


Fig. 1 SDS-PAGE analysis of MTG-mediated conjugation of NK6-AP or CK6-AP (6 μM) to Z-QG–DNA (50 μM) (lane 1, molecular weight markers; lane 2, NK6-AP; lane 3, NK6-AP and Z-QG–DNA treated with MTG; lane 4, CK6-AP; lane 5, CK6-AP and Z-QG–DNA treated with MTG).

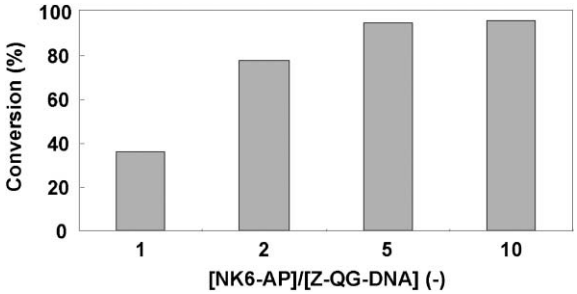


Fig. 2 Conversion rates of Z-QG–DNA in MTG-mediated conjugation with NK6-AP at different molar ratios of NK6-AP to Z-QG–DNA. The concentration of Z-QG–DNA was kept constant at 1.6 μM.

To verify this idea experimentally, MTG-mediated conjugation of NK6-AP and Z-QG–DNA was conducted by varying the concentration of NK6-AP while keeping the Z-QG–DNA concentration constant. Fig. 2 shows the marked increase in the conversion rates of Z-QG–DNA to the AP–DNA conjugate, and $\geqslant$95% of Z-QG–DNA was successfully converted to the conjugate in the presence of 5-fold excess amount of NK6-AP to Z-QG–DNA. When the concentration of NK6-AP was less than 5-fold, the conjugation reaction did not proceed sufficiently, possibly due to the competitive hydrolysis of Z-QG–DNA by water molecules.

To prove the functionality of the DNA moiety of the protein–DNA conjugates obtained in Fig. 1, each protein–DNA conjugate was first separated from free Z-QG–DNA, then immobilized on a surface displaying the cDNA, *via* DNA hybridization. Fig. 3 shows a fluorescence image of the avidin-coated 96-well microplate displaying the biotinylated cDNA after hybridization with the AP–DNA conjugates, followed by the subsequent AP activity detection with a fluorescent substrate. Control experiments with intact APs (lane 1 in Fig. 3) and AP–DNA conjugates (lane 2 in Fig. 3) provided the background signal, while the combination of AP–DNA conjugates and the cDNA-display microplate exhibited the substantial fluorescent signal (lane 3 in Fig. 3), indicating that the biological functionalities of both the DNA and the protein moieties were retained, showing the potential of MTG in the preparation of protein–DNA conjugates. It is worth noting that under the experimental conditions of Fig. 2, the reaction products were composed of the AP–DNA conjugate, an excess amount of free NK6-AP, and the residual Z-QG–DNA, which makes it possible to directly apply the reaction products to DDI because

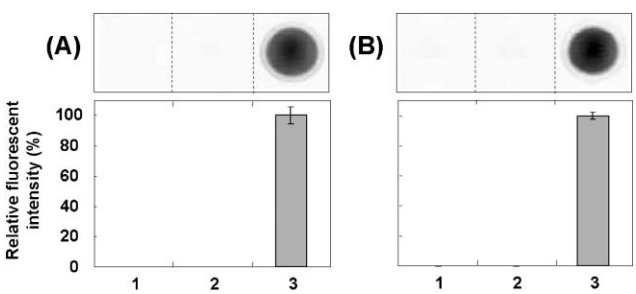


Fig. 3 DNA-directed immobilization of (A) NK6-AP–DNA conjugate, (B) CK6-AP–DNA conjugate on the cDNA-display microplates (lane 3). As controls, intact NK6- or CK6-AP was applied to the cDNA-display microplate (lane 1) and protein–DNA conjugates were applied to an intact avidin-coated microplate (lane 2).

competitive hybridization of free Z-QG–DNA will be significantly reduced. This was experimentally verified, and the signal-to-noise ratio of DDI was comparable with that observed in Fig. 3. Moreover, we ensured that no immobilization was observed in a negative control experiment with an avidin-coated microplate displaying a non-complementary DNA (see ESI†).

In terms of the stability of the resultant conjugate, it is likely that the stability of partner proteins for conjugation with DNA is crucial. As for the NK6-AP–Z-QG–DNA conjugate, the performance in DDI was retained at least 3 months when it was stored at −20 °C.

In summary we have developed a new strategy toward site-specific DNA conjugation to proteins using an enzyme, transglutaminase. Although MTG is reluctant to accept an aminated DNA for conjugation with proteins, coupling an aminated DNA with Z-QG makes it possible to direct MTG for labeling of DNA to a short peptide tag of recombinant proteins. Since MTG-mediated protein conjugation proceeds at any position, in principle, if the substrate sequence is genetically introduced to or is intrinsically present in the target proteins, the present strategy will be quite useful in obtaining protein–DNA conjugates as well as in the fabrication of protein microarrays by DDI.

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