論文の内容の要旨

Plural-Step Protein Modifications at Tryptophan
(トリプトファンを起点とする複数工程のタンパク質修飾法)

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I. Introduction

Protein modification is a dynamic field of protein science enabling creation of new protein-based medicines (biologics) and materials. Due to their structural complexity and essential function for all living organisms, proteins are a difficult target for modification. There is a constant demand for development of new protein modification methods to fulfil the needs of modern medicine and material science. Immunogold labelling was introduced in 1971 by Faulk and Taylor. This staining technique relying on non-specific conjugation of gold nanoparticles to antibodies is commonly applied for light and electron microscopy. However, gold conjugates obtained via non-covalent binding are unstable. Dissociation of gold nanoparticle from antibody can occur easily leading to competition at labelling site between antibody and gold conjugate and poor analytical results. On the other hand, site-selective conjugation of stable gold nanoclusters Au_nSR_m, which have been discovered in 1980s and continue to be intensely studied could solve one of the major problems of cryogenic electron microscopy (cryo-EM) - poor signal-to-noise ratio (SNR). Bioconjugation of Au_{102} or Au_{144} gold nanoclusters with the aim of studying protein structure via cryo-EM has already been reported, however, up to date there is no report on protein modification with smaller Au_{25} nanoclusters via thiol exchange, which are most studied one of the most stable among all known nanoclusters. In this work I am presenting development of multistep protein modification strategies for bioconjugation of gold nanoclusters Au_{25} and Au_{102} based on fine-tuned tryptophan (Trp)-selective bioconjugation.

II. 1-Step bioconjugation with ABNOH hydroxylamines and its limitations

Tryptophan is one of the least abundant amino acids in proteins, with surface exposure among the lowest, however, most proteins contain at least one Trp residue. In 2016 the group of Dr. Oisaki and Prof. Kanai reported transition metal free-bioconjugation of proteins. In this protocol, keto-ABNO radical could be oxidatively activated by NO_x species generated from sodium nitrite, $NaNO_2$ in the presence of a catalytic amount of acetic acid, AcOH. Important limitations of this method are: 1) the need for acidic conditions to initialize the reaction and 2) difficulty in determination of purity of ABNO radical derivatives. Nevertheless, I thought that this method could be applied for successful labelling of protein with Au_{25} gold nanocluster and bioconjugation could be realized by 1-step method using cluster-ABNO reagent. During the synthesis of thiol-ABNO ligand for cluster I discovered that hydroxylamine type reagent, thiol-ABNOH ligand, can be obtained instead of a radical. I found that bioconjugation with ABNOH reagents can be conducted under buffered conditions with good yield by using TEMPO+ oxidant. The method was further tuned by pre-activation of ABNOH reagent to ABNO radical with manganese(IV) dioxide, MnO_2. Bioconjugation of keto-ABNOH to model peptide could be realized with >90%

yield under aqueous conditions with addition of MeCN necessary to solubilise the peptide. Applicability of the reaction for protein modification was later confirmed experimentally.

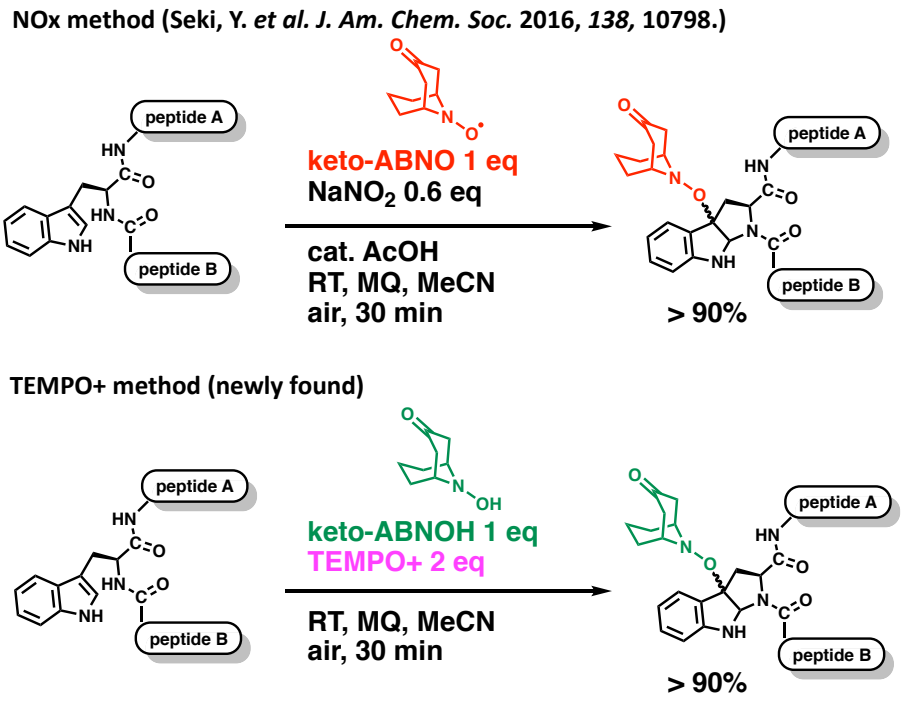


Figure 1. Tryptophan selective bioconjugation with keto-ABNO radical and keto-ABNOH hydroxylamine.

The thiol-ABNOH ligand for Au_{25} gold nanocluster was successfully applied for synthesis of several Au-ABNOH reagents which was performed by our collaborators Dr. Shinjiro Takano and Prof. Tatsuya Tsukuda from the Graduate School of Science, University of Tokyo. Bioconjugation of Au-ABNOH reagent to model peptide required excess TEMPO+ oxidant and Au-ABNOH reagent and was confirmed for 3 model peptides by challenging ESI-MS analysis performed by Dr. Takano. Due to various analytical limitations and apparent low yield of the reaction on both peptide and protein scale, I concluded that 1-step bioconjugation method was not optimal for this application.

III. Plural-step bioconjugation protocols

a. 3-Step bioconjugation with photoremovable protecting groups

Low yield of 1-step bioconjugation of Au_{25} gold nanocluster could be potentially related to oxidative damage of cluster or inhibition of oxidative bioconjugation step. Therefore, I imagined that separation of bioconjugation step and actual labelling with nanocluster could be more effective. Inspired by the reports on phototriggered thiol release on peptide and protein scale, I synthesised several thiol-ABNOH reagents with different photoreactive protecting groups (PPG) for 3-step bioconjugation strategy. Strategy was optimised using model Fmoc peptide and biotin maleimide reagent working as a thiol trap. I tested groups commonly applied for photodecaging of thiols: *o*-nitroveratryl (*o*Nv), *p*-hydroxyphenacyl (*p*HP) and 7-diethylaminocoumarin (7DEAC). *o*Nv-ABNOH and pHP-ABNOH required either long irradiation times or use of near UV light which resulted in messy HPLC charts. On the other hand, thiol release from C1-ABNOH (7-DEAC) proceeded smoothly.

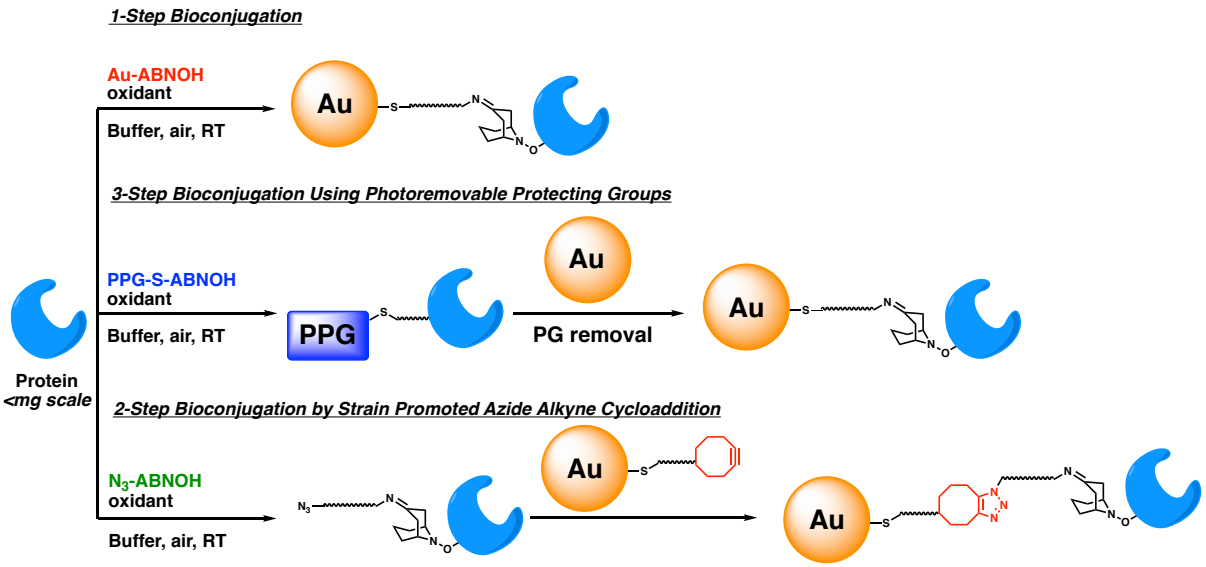


Figure 2. 1-Step and plural-step bioconjugation strategies for protein labelling with gold nanoclusters.

Thiol released from C1-ABNOH could be trapped with nearly 70% yield, however, approximately 34% of the peptide conjugate underwent photoisomerization instead of releasing a thiol. In order to suppress this side reaction, I synthesised 3-methyl-7DEAC protected compounds, however, screening revealed that photoisomerization could not be prevented by the introduction of steric hindrance. Thiol release yield for newly obtained compounds was comparable to or lower than in the case of C1-ABNOH.

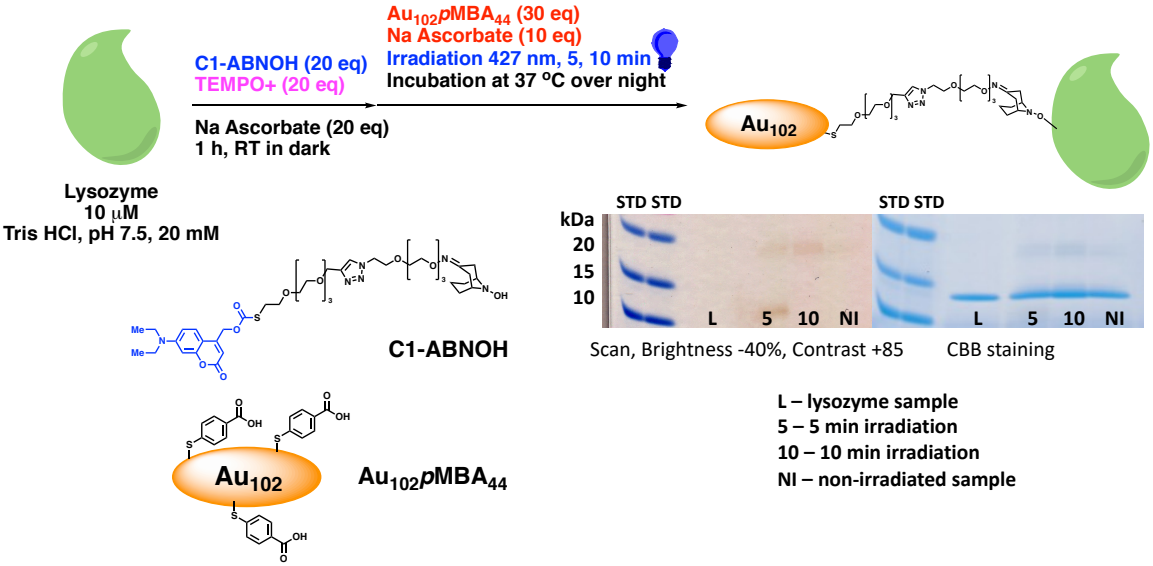


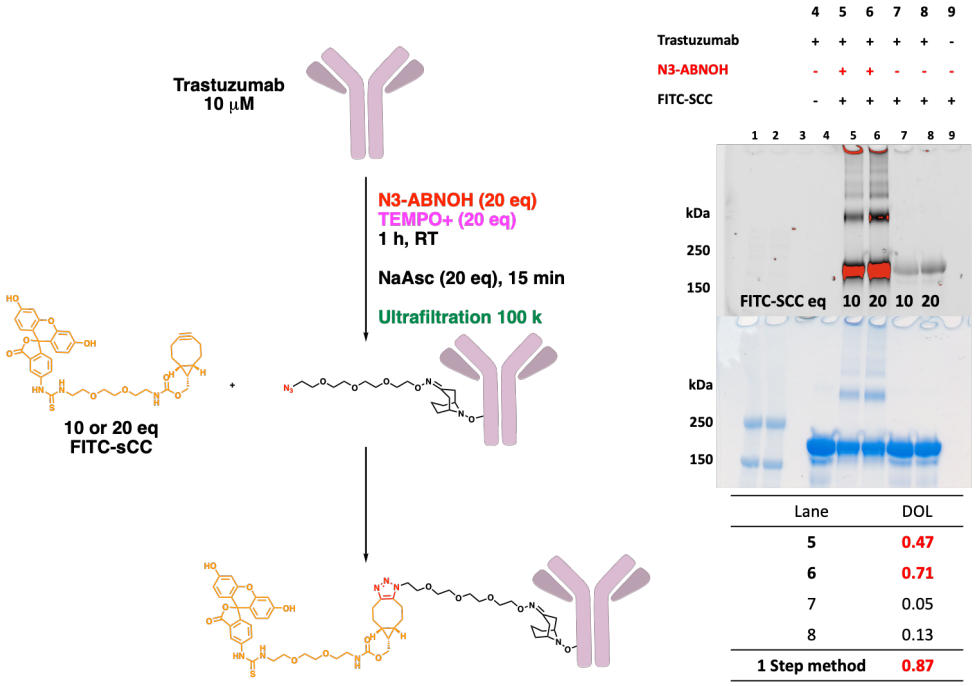
Figure 3. 3-Step bioconjugation experiment on lysozyme with C1-ABNOH and $Au_{102}(pMBA)_{44}$ gold nanocluster.

For protein scale thiol release experiments, I used C1-ABNOH and a larger gold nanocluster – $Au_{102}(pMBA)_{44}$. C1-ABNOH conjugate samples were irradiated for 5 or 10 min with blue LED and a non-irradiated sample control was also prepared. Initially, weak gold labelling band could be detected in SDS-PAGE result – a faint gold/brown stain visible without additional staining. Applying additional amount of sodium ascorbate after gold cluster stock was added to the reaction mixture in later experiments on trastuzumab and lysozyme resulted in a difference of labelling intensity, with the highest intensity visible for samples irradiated for 10 min and low intensity of

background labelling (Figure 3). I tried to optimise the thiol release reaction on protein scale with small molecule fluorescent thiol trap – fluorescein maleimide, however, the results were always hampered by strong background labelling.

b. 2-Step bioconjugation by strain-promoted azide alkyne cycloaddition (SPAAC)

In this strategy, protein modified site selectively with N3-ABNOH – a platform compound bearing azide moiety – reacts with payload decorated with strained alkyne, such as BCN, without any irradiation or special additives. The use of SPAAC for modification of the surface of a large gold nanoparticle and Au_{25} nanocluster has already been reported. Protein samples modified with N3-ABNOH showed intense fluorescence indicating successful SPAAC with FITC-sCC. A very low intensity of background labelling could be detected for samples of non-modified protein. Degree of labelling found for the most intense labelling was comparable to the degree of labelling achieved previously by 1-step method with FITC-ABNOH (Figure 4).



Lane	DOL
5	0.47
6	0.71
7	0.05
8	0.13
1 Step method	0.87

Figure 4. 2-Step bioconjugation experiment with N3-ABNOH and FITC-sCC on Trastuzumab.

To realize gold nanocluster bioconjugation using this strategy, a thiol ligand bearing strained alkyne is necessary, however, such useful molecule is impossible to obtain due to reactivity of thiol with strained alkynes. Therefore, surface modification of the *para*-mecraptobenzoic acid (*p*MBA) ligands of Au_{102} nanocluster was proposed by our collaborator, Dr. Takano. Carboxylic moiety of *p*MBA ligand could be modified with BCN-amine in the presence of DMT-MM reagent and a small amount of Au_{102} cluster bearing between 1 and 5 strained alkyne ligands was supplied by Dr. Takano (Au102-sCC). Bioconjugation trial on trastuzumab with 10 eq of the material gave poor results due to low solubility of the Au102-sCC in aqueous buffer. This result can hopefully be optimised by tuning of the linker structure or number of strained alkynes introduced to Au_{102}.

In summary, 3 different methods were applied to conjugate the gold nanocluster to protein. Each method gave a trace of bioconjugation on protein scale. Multistep bioconjugation protocols that separate oxidative ABNOH bioconjugation step from payload delivery to protein seem to be more reliable and currently the most promising method – 2-step bioconjugation by SPAAC is under investigation.