博士論文

(要約)

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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Abbreviations

3D	three-dimensional
ABNO	9-azabicyclo[3.3.1]nonane-9-oxyl
Ac	acetyl
AuNC	gold nanocluster
BCN	bicyclo[6.1.0]nonyne
BSA	bovine serum albumin
BP	by-product
CBB	Coumassie brilliant blue
CHAMPO+	dicyclohexyl (4-acetamidopiperidin-1-yl)oxammonium
CuAAC	copper-catalysed azide alkyne cycloaddition
cryo-EM	cryogenic electron microscopy
Da	dalton
DCM	dicholoromethane
DEAC	diethylaminocoumarin
DIBO	dibenzocyclooctyne
DIPEA	diisopropylethylamine
DMSO	dimethylsulfoxide
DMT-MM	4-(4,6-dimethoxy-l,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	deoxyribonucleic acid
DOL	degree of labeling
EA	ethyl acetate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
eq	equivalent
Et	ethyl
EM	electron microscopy
Fab	antigen-binding fragment
Fc	fragment crystallizable region
FITC	fluorescein isothiocyanate
FM	fluorescein-maleimide
Fmoc	9-fluorenylmethyloxycarbonyl
GSH	glutathione
HILIC	hydrophilic interaction liquid chromatography

HER2	human epidermal growth factor receptor 2
hex	hexane
HPLC	high-performance liquid chromatography
keto-ABNO	9-azabicyclo[3.3.1]nonan-3-one-9-oxyl
keto-ABNOH	9-hydroxy-9-azabicyclo[3.3.1]nonan-3-one
LCMS	liquid chromatography-mass spectrometry
LED	light-emitting diode
LRMS	low resolution mass spectrometry
M	molar, mol per litter
MALDI	matrix-assisted laser desorption/ionisation
*p*MBA	4-mercaptobenzoic acid
Me	methyl
MQ	Milli-Q water
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
NAC	*N*-acetyl cysteine
NaTFMS	sodium trifuloromethanesulfinate
NBS	*N*-bromosuccinimide
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
*o*Nv	*ortho*-nitroveratryl
PAGE	polyacrylamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pH	potential of hydrogen
*p*HP	*para*-hydroxyphenacyl
PI	photoisomerization
pI	isoelectric point
PPG	photoremovable protecting group
QY	quantum yield
RNA	ribonucleic acid
RT	room temperature

sCC	strained alkyne
scFv	single chain antibody fragment
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SM	substrate material
SPAAC	strain-promoted azide-alkyne cycloaddition
STD	protein standard
Sulfo-NHS	sulfo-*N*-hydroxysuccinimide
TAD	triazolinedione
TBHP	*tert*-butyl hydroperoxide
TBTA	Tris((1-benzyl-4-triazolyl)methyl)amine
TCEP	tris(2-carboxyethyl)phosphine
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
TEMPO+	2,2,6,6-tetramethyl-1-oxo-piperidinium tetrafluoroborate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIPS	triisopropyl silane
TM	target material
TOF	time-of-flight
Tris	tris(hydroxymethyl)aminomethane
TLC	thin layer chromatography
UV	ultraviolet
Vis	visible

Amino acid	Three letter symbol	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Introduction

Gold nanoparticles and stable gold nanoclusters in chemical biology

Colloidal gold has been known since ancient times[1]. Currently, we can distinguish different types of nanoparticles based on their size and atomic composition. Colloidal gold nanoparticles can be defined as particles of size between 5 and 250 nm. Scientists have predicted that the nanoparticles of a smaller size – between 1 and 10 nm could potentially display very specific features whose origin is directly related to their electronic structure (Figure 1). Their physical properties would thus be different from those of bulk gold as well as atomic/ molecular gold and would depend on size, shape and protecting ligand shell.

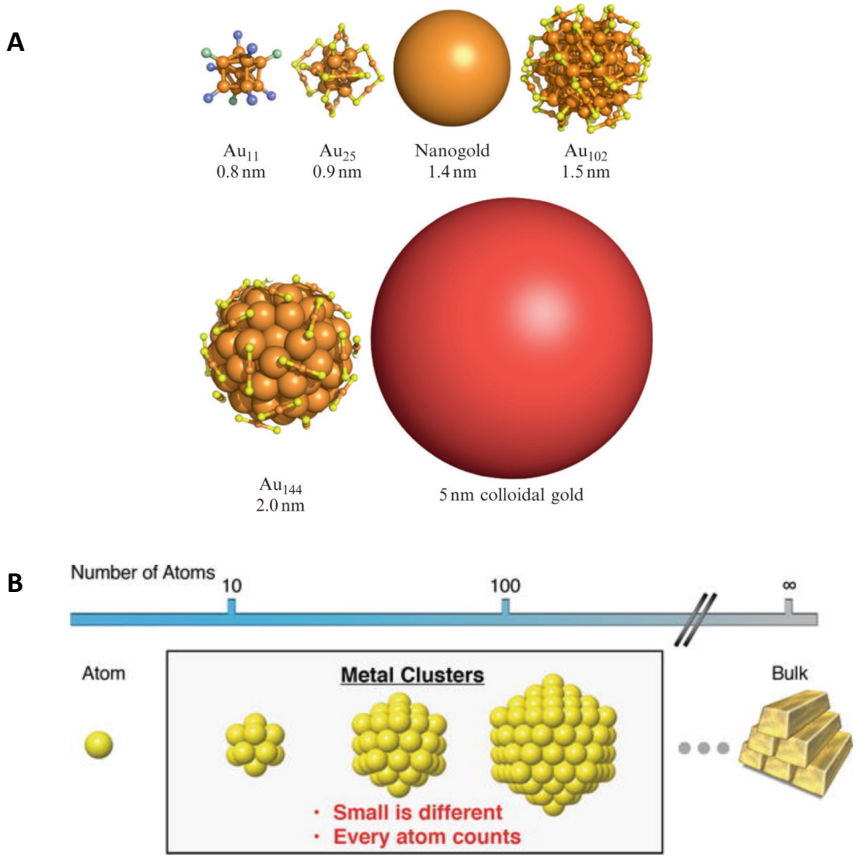

Figure 1. A: Gold nanoclusters and gold nanoparticles compared [2]. B: Relation of gold clusters, gold atoms and bulk gold [3].

[1] Daniel, M. C.; Astruc, D. Chem. Rev. 2004, 104 (1), 293–346.
[2] Ackerson, C. J.; Powell, R. D.; Hainfeld, J. F. Methods in Enzymology; Academic Press Inc., 2010; Vol. 481, pp 195–230
[3] Ruano, P.; Delgado, L. L.; Picco, S.; Villegas, L.; Tonelli, F.; Merlo, M.; Rigau, J.; Diaz, D.; Masuelli, M. Intech 2016, DOI: 10.5772/67833.

Gold nanoclusters exist as a series of homologous structures with different number of Au atoms constituting their core. Every gold atom in the cluster is surrounded by 12 neighbouring Au atoms, hence the smallest stable cluster is composed of 13 gold atoms. The number of gold atoms in following larger clusters can be described by the equation $10n^2+2$, where n is the layer, and these numbers are often referred to as 'magic numbers'. Gold nanoclusters with filled super atomic orbitals display molecule like properties. Their stability is exceptional and can be compared to the stability of noble gases. The origin of stability is not only related to the number of gold atoms constituting the core, but also to the presence of organothiolate ligands, whose main functions are to protect the clusters form aggregation by providing steric hindrance and to adjust the number of valence electrons inside the gold core. The interaction of gold and thiols is very important for nanoscience[4]. Other types of ligands such as alkynes, phosphines or selenides have been also reported. Interestingly, selenoate clusters are more stable than thiolate clusters because gold-selenium bond is more covalent[5], however, due to toxicity of selenium compounds their use for biological experiments and medical applications should be limited. Doping with other metals has been successfully applied to create alloy or doped clusters (Figure 2). Ag, Cu, Hg, Cd, Ir or Pd are often applied, and their presence can lead to modified properties such as stability towards oxidation, stronger fluorescence or enhanced catalytic properties[6,7].

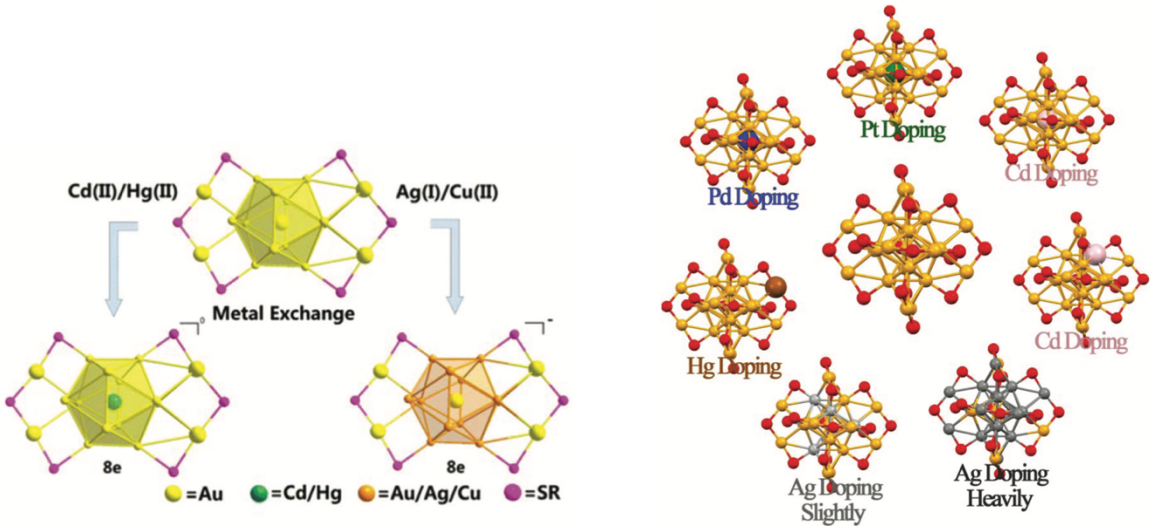

Figure 2. Doping of gold Au_{25} nanoclusters with metals. [6,14]

[4] Negishi, Y.; Nobusada, K.; Tsukuda, T. J. Am. Chem. Soc. 2005, 127 (14), 5261–5270.
[5] Zhong, J.; Tang, X.; Tang, J.; Su, J.; Pei, Y. J. Phys. Chem. C 2015, 119 (17), 9205–9214.
[6] Ghosh, A.; Mohammed, O. F.; Bakr, O. M. Acc. Chem. Res. 2018, 51 (12), 3094–3103.
[7] Takano, S.; Ito, S.; Tsukuda, T. J. Am. Chem. Soc. 2019, 141 (40), 15994–16002.

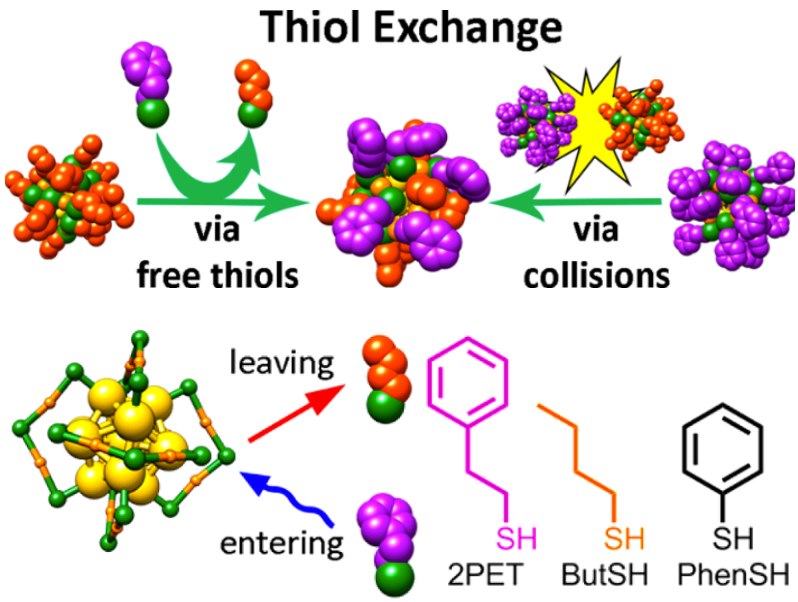

Figure 3. Thiol exchange reaction on Au_{25} nanocluster[8].

The organic ligand layer surrounding the gold nanocluster core is a dynamic structure. Thiolate ligands can be easily replaced in thiol exchange reaction – Murray place exchange reaction – to obtain modified compounds with functional ligands (Figure 3). Murray reaction is a type of S_N2 substitution[9] and a certain excess of exogenous, target thiol ligand[10] is needed for this modification to proceed. By applying a suitable ligand, the gold nanocluster can be made biocompatible. Up to date, more than 40 gold nanoclusters have been reported in the literature (Figure 5), with $Au_{25}SR_{18}$ being the most stable and one of the most popular for cluster studies. $Au_{25}SR_{18}$ has been first obtained in 1998[11] and its structure was determined in 2008[12]. This cluster consists of a gold core composed of 13 Au atoms, surrounded by a layer of 12 gold atoms and 18 thiolate ligands. Water soluble, biocompatible ligands such as glutathione, captopril or thiopronin and even bovine serum albumin have been reported (Figure 4). ADME (Adsorption-Distribution-Metabolism-Excretion) of Au_{25} nanoclusters by living organisms has been also extensively studied with distribution and excretion of clusters found

[8] Salassa, G.; Sels, A.; Mancin, F.; Bürgi, T. ACS Nano 2017, 11 (12), 12609–12614.
[9] Heinecke, C. L.; Ni, T. W.; Malola, S.; Mäkinen, V.; Wong, O. A.; Häkkinen, H.; Ackerson, C. J. J. Am. Chem. Soc. 2012, 134 (32), 13316–13322.
[10] Hostetler, M. J.; Templeton, A. C.; Murray, R. W. Langmuir 1999, 15 (11), 3782–3789.
[11] Gregory Schaaff, T.; Knight, G.; Shafigullin, M. N.; Borkman, R. F.; Whetten, R. L. J. Phys. Chem. B 1998, 102 (52), 10645–10646.
[12] Heaven, M. W.; Dass, A.; White, P. S.; Holt, K. M.; Murray, R. W. J. Am. Chem. Soc. 2008, 130 (12), 3754–3755.

to be dependent on surface charge[13]. Applications of $Au_{25}SR_{18}$ ligands include biosensing, drug delivery, optoelectronic devices, photodynamic therapy of cancer and cell labelling[14].

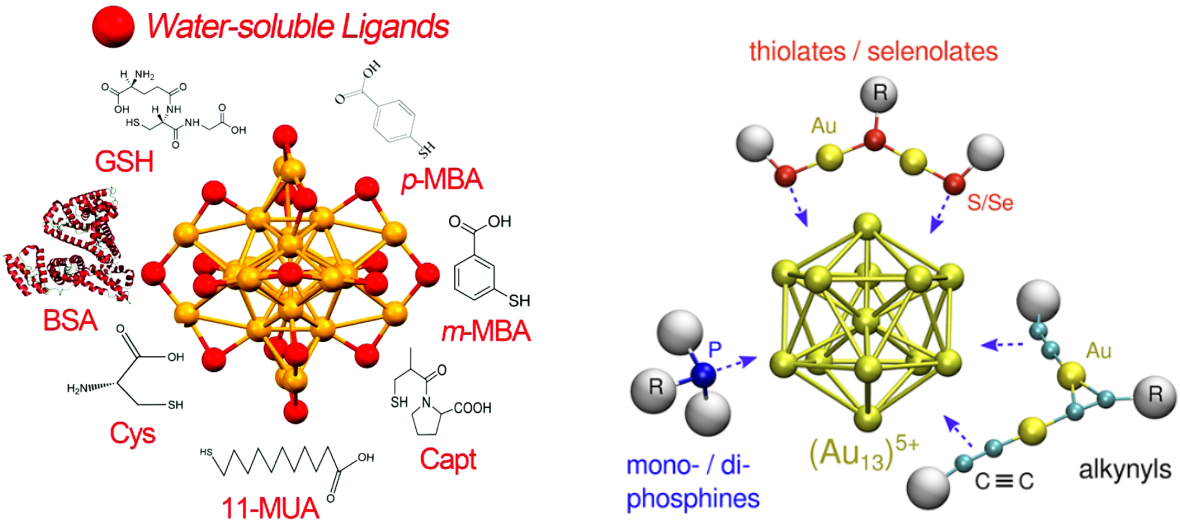

Figure 4. Ligands for Au_{25} gold nanocluster. Left: A selection of water-soluble ligands reported in the literature[14]. Right: Different classes of possible ligands for Au_{25}[15].

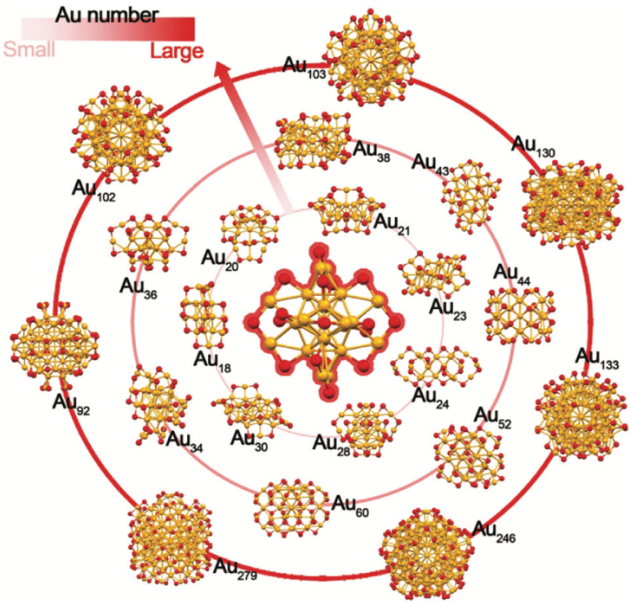

Figure 5. Stable gold nanoclusters with different core size indicated, reported until 2018. Central cluster, $Au_{25}SR_{18}$, consists of 25 gold atoms.[14].

[13] Wang, J. Y.; Chen, J.; Yang, J.; Wang, H.; Shen, X.; Sun, Y. M.; Guo, M.; Zhang, X. D. Int. J. Nanomedicine 2016, 11, 3475–3485.
[14] Kang, X.; Chong, H.; Zhu, M. Nanoscale 2018, 10 (23), 10758–10834.
[15] Narouz, M. R.; Takano, S.; Lummis, P. A.; Levchenko, T. I.; Nazemi, A.; Kaappa, S.; Malola, S.; Yousefalizadeh, G.; Calhoun, L. A.; Stamplecoskie, K. G.; Häkkinen, H.; Tsukuda, T.; Crudden, C. M. J. Am. Chem. Soc. 2019, 141 (38), 14997–15002.

Labelling of biomacromolecules with gold nanoparticles and nanoclusters

Bioconjugation of gold nanoparticles and gold nanoclusters is an attractive concept, as it can potentially lead to creation of a new type of nanomaterials with new properties. In particular, gold conjugates have found application in light and electron microscopy (EM). Gold nanoparticles, clusters and their bioconjugates can also be applied in cancer therapy[16,17]. Large gold nanoparticles are non-homogenous in nature but can be attached to biomacromolecules such as proteins via electrostatic bonds (physisorption). Such non-covalent labelling leads to random localisation of proteins on the gold nanoparticle and can be easily decomposed, which may cause loss of nanoparticle from binding site and poor analytical results. Nanoparticles also tend to aggregate very easily. Linker mediated or linker free approaches can be applied to covalently secure biomacromolecule on the surface of a gold nanoparticle. Free thiol or dithiane moiety is applicable for such modifications [18,19,20]. New examples of gold nanoparticle synthesis and bioconjugation continue to be reported.

Labelling of antibodies has found application in cancer diagnosis and treatment. Gold nanoparticles tend to accumulate in tumours due to enhanced permeability and retention effect. Nanoparticles functionalised with anti-epidermal growth factor receptor (anti-EGFR) antibody (cetuximab) or antibody fragments were successfully applied as contrast enhancing agents in computer tomography. Functionalisation with antibodies or antibody fragments allows for efficient delivery of smaller nanoparticles to tumours, with decreased non-selective off-target retention[18]. An increase in cytotoxicity towards non-small lung cancer cells expressing high levels of EGFR could be observed for cetuximab modified with gold nanoparticle in comparison with non-modified antibody[19]. Trastuzumab-gold nanoparticle conjugate was found to cytotoxic against metastatic gastric cancer cells with acquired resistance to trastuzumab. This type of cancer, unlike trastuzumab-resistant HER2 positive breast cancer, is also resistant to treatment with trastuzumab-DM1 conjugate[20]. 5 nm gold nanoparticles conjugated with trastuzumab via PEG linker were successfully labelled with radioactive astatine ^{211}At. Such gold nanoparticle radiobioconjugate exhibited higher cytotoxicity than

[16] Oh, M. H.; Yu, J. H.; Kim, I.; Nam, Y. S. Appl. Mater. Interfaces 2015, 7 (40), 22578–22586.
[17] Zhang, X. D.; Chen, J.; Luo, Z.; Wu, D.; Shen, X.; Song, S. S.; Sun, Y. M.; Liu, P. X.; Zhao, J.; Huo, S.; Fan, S.; Fan, F.; Liang, X. J.; Xie, J. Adv. Healthc. Mater. 2014, 3 (1), 133–141.
[18] Ashton, J. R.; Gottlin, E. B.; Patz, E. F.; West, J. L.; Badea, C. T. PLoS One 2018, 13 (11), 1–20.
[19] Qian, Y.; Qiu, M.; Wu, Q.; Tian, Y.; Zhang, Y.; Gu, N.; Li, S.; Xu, L.; Yin, R. Sci. Rep. 2014, 4, 1–8.
[20] Kubota, T.; Kuroda, S.; Kanaya, N.; Morihiro, T.; Aoyama, K.; Kakiuchi, Y.; Kikuchi, S.; Nishizaki, M.; Kagawa, S.; Tazawa, H.; Fujiwara, T. Nanomedicine Nanotechnology, Biol. Med. 2018, 14 (6), 1919–1929.

bare astatinated gold nanoparticle and could potentially be applied to local treatment of HER2 positive cancers.[21]

As mentioned earlier, gold nanoclusters can be considered as molecules of precise atomic composition and unique properties such as chemiluminescence or photostability. Introduction of a gold nanocluster to biomacromolecule via ligand exchange reaction leads to creation of more reliable probes, which should not be decomposed so easily, because the bond connecting cluster to biomolecule is covalent, not electrostatic in nature. Gold nanoclusters are also smaller in terms of size than nanoparticles which could lead to improvement of signal to noise ratio in instances where low power beam is used for cryo-EM studies. In addition to phase contrast characteristic to biomacromolecules, nanoclusters also possess amplitude contrast which makes them easy to distinguish from other objects in EM study[22]. Nanoclusters being molecule-like compounds are more stable than non-homogenous gold nanoparticles. Molecule-like behaviour and controlled ligand exchange can be utilised for synthesis of precise nanoprobes with distinct properties. Larger gold nanoparticles can be obtained easily by several well-established protocols and their use as contrast enhancers was reported many times. Their size can be a problem, if creation of small, precise probe is intended. If the particle is too big, it cannot possibly reach labelling site. Unfortunately, the smaller the gold nanoparticles the less stable such preparation becomes. For this reason, gold nanoclusters with diameters of gold core even below 1 nm are currently preferred. Gold nanoclusters can also be used for creation of conjugates other than protein, such as peptide or oligonucleotide conjugates, which are difficult to be reacted with gold nanoparticles. Due to small size and stability, purification by PAGE is possible for nanoclusters. The disadvantages of using nanoclusters include difficult synthesis of clusters and their conjugates. In addition to that, sometimes silver enhancement to increase the core size in the labelled sample may be needed to detect gold nanocluster labelling.[23]

Potential use of gold nanoclusters for electron microscopy studies of biological samples has been postulated by Bartlett in 1978[24] and Undecagold nanocluster is first reported. In 1992 Hainfeld and Furuya reported bioconjugation of Fab' antibody fragment with 1.4 nm gold nanocluster[25]. Direct or linker mediated approaches can be applied for installation of gold

[21] Dziawer, Ł.; Majkowska-Pilip, A.; Gaweł, D.; Godlewska, M.; Pruszyński, M.; Jastrzębski, J.; Wąs, B.; Bilewicz, A. Nanomaterials 2019, 9 (4) 632.
[22] Ackerson, C. J.; Powell, R. D.; Hainfeld, J. F. Site-Specific Biomolecule Labeling with Gold Clusters. In Methods in Enzymology; Academic Press Inc., 2010; Vol. 481, pp 195–230.
[23] Hainfeld, J. F.; Powell, R. D. J. Histochem. Cytochem. 2000, 48 (4), 471–480.
[24] Bartlett, P. A.; Bauer, B.; Sanger, S. J. Am. Chem. Soc. 1978, 100 (16), 5085–5089.
[25] Hainfeld, J. E.; Furuya, F. R. J. Histochem. Cytochem. 1992, 40 (2), 177–184.

cluster on biomacromolecule. Mono-sulfo-NHS and monomaleimido Nanogold and Undecagold clusters are examples of commercially available reagents, targeting lysine and cysteine residues of proteins. 'Click nanogold', 1.4 nm gold particles modified with azide or alkyne for attachment via click chemistry became commercially available recently.[26]

There are several ways of conjugating gold nanoparticle or nanocluster to protein, however, in this work special attention is given to conjugation by thiol exchange reaction. Gold nanocluster labelling of proteins (Figure 6) can be achieved if a cysteine thiol is available for thiol exchange reaction. The position of thiol within protein is important – residues buried inside protein or engaged in formation of disulphide bonds will not be reactive. Naturally occurring cysteine residues are rare, therefore often protein modification – genetic manipulation is used to introduce an additional cysteine residue to C- or N-terminus of protein of interest[27].

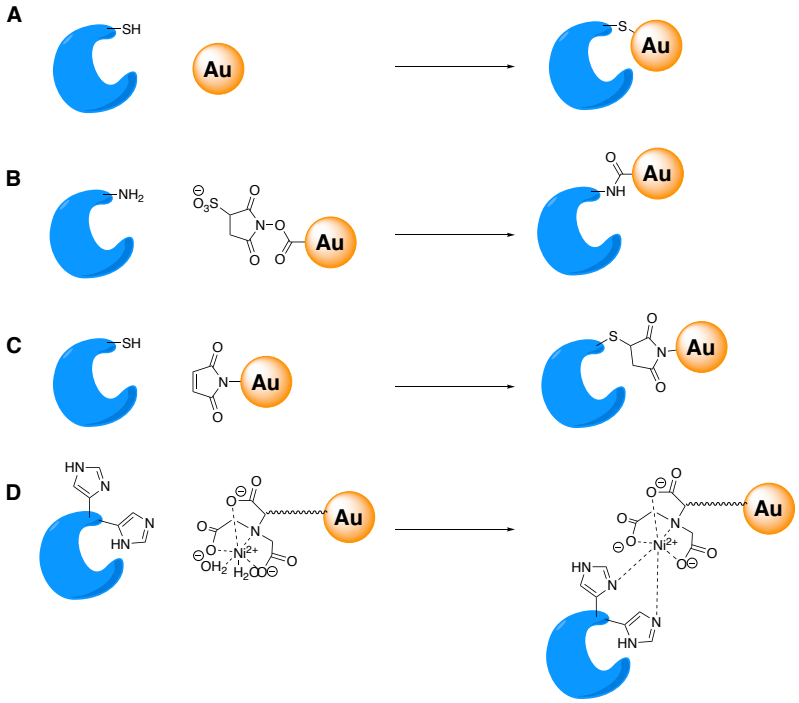

Figure 6. Most common gold nanoparticle / nanocluster protein bioconjugation strategies: A) Thiol exchange with cysteine residue. B) Sulfo NHS ester – amine coupling. C) Thiol – maleimide coupling. D) Ni–NTA Chelation approach.[28]

[26] http://www.nanoprobes.com/index.html; access 2021.05.24
[27] Ackerson *et al.,* 2010
[28] Heinecke C.L., Ackerson C.J. (2013) Preparation of Gold Nanocluster Bioconjugates for Electron Microscopy. In: Sousa A., Kruhlak M. (eds) Nanoimaging. Methods in Molecular Biology (Methods and Protocols), vol 950. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-137-0_17

Bioconjugation of Au_{71} gold nanocluster to single chain antibody fragment (scFv) was reported in 2006 by Kornberg[29]. The cluster was covalently attached via thiol exchange reaction with engineered cysteine residue. The reaction proceeded at pH ~9 and after oxidation of cluster, which made it more reactive towards thiolate. Ligands lost from the surface of cluster during bioconjugation were later supplemented by passivation of the conjugate with tiopronin. This work is an example of synthesis of a rigid cluster conjugate. The same group later reported labelling of an antibody fragment with larger cluster $Au_{144.}$[30]. In 2011, Kornberg's group published a paper about bioconjugation of Au_{102}*p*MBA_{44} gold nanocluster to scFv fragment of antibody against RNA polymerase II. In this work additional cysteine residue was introduced at *N*-terminus of protein and reacted with nanocluster in the presence of TCEP.[31] More recent interesting example of Au_{102} gold nanocluster bioconjugation and cryo-EM analysis of the conjugate was reported by Stark *et al.* in 2017[32]. In this work conjugation by direct thiol exchange or thiol-maleimide reaction with maleimide modified Au_{102} was realized to study the nanocapsid protein of hepatitis E virus. The protein contained artificially introduced, solvent exposed cysteine residue. The structure of conjugate and assembled higher order structure could be resolved by cryo-EM and provided insight to tuning of drug delivery system based on viral nanocapsids.

Protein directed synthesis of fluorescent gold nanoclusters

Gold nanoclusters can be prepared by reduction of gold precursors such as tetrachloroauric acid, $HAuCl_4$ in the presence of thiols. $NaBH_4$ is a commonly applied reductant for this process. Similar technique can be used for preparation of gold nanoclusters, notably the stable Au_{25} cluster with proteins used instead of small molecule thiolates. Examples of such gold nanoclusters stabilized with proteins were reported in literature.

In 2009 Xie *et al.* reported synthesis of Au_{25} nanocluster stabilised with bovine serum albumin (BSA). The reaction proceeded after stock solutions of protein and gold precursor were mixed and pH of the solution was further adjusted to 12 by the addition of NaOH. $NaBH_4$

[29] Ackerson, C. J.; Jadzinsky, P. D.; Jensen, G. J.; Kornberg, R. D. J. Am. Chem. Soc. 2006, 128 (8), 2635–2640.
[30] Ackerson, C. J.; Jadzinsky, P. D.; Sexton, J. Z.; Bushnell, D. A.; Kornberg, R. D. Bioconjug. Chem. 2010, 21 (2), 214–218.
[31] Levi-Kalisman, Y.; Jadzinsky, P. D.; Kalisman, N.; Tsunoyama, H.; Tsukuda, T.; Bushnell, D. A.; Kornberg, R. D. J. Am. Chem. Soc. 2011, 133, 2976–2982.
[32] Stark, M. C.; Baikoghli, M. A.; Lahtinen, T.; Malola, S.; Xing, L.; Nguyen, M.; Nguyen, M.; Sikaroudi, A.; Marjomäki, V.; Häkkinen, H.; Cheng, R. H. Structural Characterization of Site-Modified Nanocapsid with Monodispersed Gold Clusters. Sci. Rep. 2017, 7. https://doi.org/10.1038/s41598-017-17171-x.

was found to be unnecessary, because BSA worked as an *in situ* reductant. The resulting BSA-Au_{25} nanocluster exhibited strong red fluorescence under irradiation with 365 nm light (UV lamp) and was found to be stable under buffered conditions as well as in solid state. The number of gold atoms was determined based on MALDI-TOF-MS analysis[33]. Insulin is another protein which was successfully applied for protein-directed synthesis of gold nanoclusters. Formation of clusters was achieved by mixing insulin with $HAuCl_4$ in Na_3PO_4 buffer at 4 °C. It is postulated that amino acid residues other than cysteine (tryptophan, tyrosine, arginine, lysine and aspartic acid) promote formation of clusters and the mechanism is different form previously cited BSA directed synthesis. The protein – cluster conjugate exhibited strong red fluorescence and retained its biological activity *in vivo*. Blood glucose lowering by insulin-AuNC in rats was comparable to that achieved by commercially available insulin[34]. Blue emitting Au_8 nanoclusters stabilised by lysozyme (type IV) with high quantum yield (QY ~ 56%) at an acidic pH. These clusters were capable of sensing GSH with nanomolar range limit of detection and would gradually form larger Au_{25} clusters upon raising of pH to 12. The mechanism of sensing was based on a drop of fluorescence intensity due to etching of Au_8 cluster by GSH[35]. Lysozyme capped gold nanoclusters functionalised with ampicillin revoked methicillin-resistant *Staphylococcus aureus* (MRSA) resistance to β–lactam antibiotic ampicillin. This bioconjugate was more effective than lysosome-cluster or ampicillin administered alone. Diabetic wounds in mice infected with MRSA could be effectively treated by this interesting gold cluster bioconjugate - drug formulation[36]. In 2011 Wang Y. *et al.* reported bioconjugation of BSA coated gold nanocluster to monoclonal antibody trastuzumab[37]. Cluster was synthesised with BSA working as a ligand. Subsequently BSA was carboxylated with glutaraldehyde and glycine and conjugation to trastuzumab was achieved by modification of lysine residues in the presence of EDC. Due to conjugation with antibody, small gold nanocluster (~2 nm) could be delivered to cancer cell nucleus more effectively than gold nanocluster alone. The reported trastuzumab gold cluster conjugate was more capable of localising in cell nucleus, where DNA damage leading to cancer cell death was achieved, than trastuzumab alone. This work is an example of non-linker mediated bioconjugation.

[33] Xie, J.; Zheng, Y.; Ying, J. Y. J. Am. Chem. Soc. 2009, 131 (3), 888–889.
[34] Liu, C. L.; Wu, H. T.; Hsiao, Y. H.; Lai, C. W.; Shih, C. W.; Peng, Y. K.; Tang, K. C.; Chang, H. W.; Chien, Y. C.; Hsiao, J. K.; Cheng, J. T.; Chou, P. T. Angew. Chemie - Int. Ed. 2011, 50 (31), 7056–7060.
[35] Chen, T. H.; Tseng, W. L. Small 2012, 8 (12), 1912–1919.
[36] Kalita, S.; Kandimalla, R.; Bhowal, A. C.; Kotoky, J.; Kundu, S. Sci. Rep. 2018, 8 (1), 1–13.
[37] Wang, Y.; Chen, J.; Irudayaraj, J. ACS Nano 2011, 5 (12), 9718–9725.

Small molecule florescent nanoprobes are often toxic to cells, therefore, protein-directed synthesis of fluorescent gold nanoclusters is especially attractive for imaging *in vivo*. Biocompatibility of strongly emitting gold nanocluster achieved by coating with proteins, high quantum yield of tuneable fluorescence, low toxicity and convenient synthesis are notable advantages.

Precedent works – bioconjugation at Tryptophan

Protein modification is a dynamic field of protein science, providing hybrid biomaterials of critical importance for medicine, pharmacy, and material science. Proteins are attractive yet difficult substrates for modification, due to their complex 3D structure and various chemical groups which directly contribute to their reactivity. Therefore, it is vital to develop modification methods which work site- and chemo-selectively, without interfering with protein reactivity and at the same time offer full control over the direction of desired modification. Tryptophan is an attractive amino acid target for protein modification as it is statistically the least abundant amino acid in proteins with surface exposure among the lowest of all amino acids[38,39] yet most proteins contain at least one Trp residue. There are many reports about protein selective modification with Trp used as bioconjugation handle.

Transition metal-based protocols

The method reported by Antos *et al.* in 2004 was based on generation of metallocarbenoid from $Rh(OAc)_2$, that could selectively react with Trp residues under aqueous conditions with small addition of polyethylene glycol. Addition of hydroxylamine hydrochloride significantly improved the yield of the reaction (Figure 7).

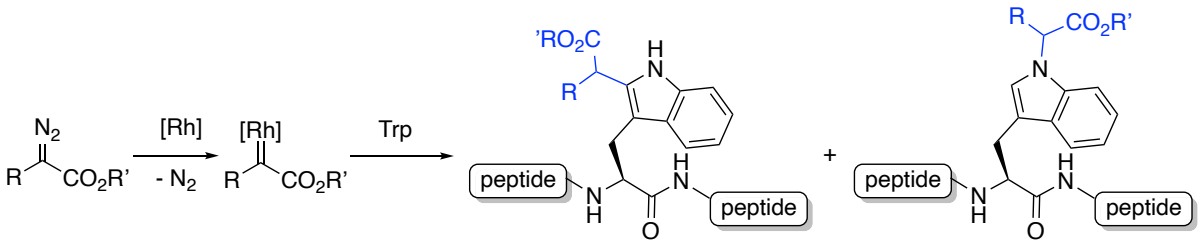

Figure 7. Modification of Trp residues with rhodium metallocarbenoids reported by Antos *et al.*

Successful modification of myoglobin could be achieved; however, this method led to generation of 2 regio-isomeric modifications on Trp[40] and acidic conditions were required for the reaction to proceed. In 2009 the same group reported improved protocol for Trp bioconjugation, in which protein modification could proceed under neutral conditions (pH 6-

[38] Moelbert, S.; Emberly, E.; Tang, C. Protein Sci. 2004, 13 (3), 752–762.
[39] Gilis, D.; Massar, S.; Cerf, N. J.; Rooman, M. Genome Biol. 2001, 2 (11), 1–12.
[40] Antos, J. M.; Francis, M. B. J. Am. Chem. Soc. 2004, 126 (33), 10256–10257.

7)[41]. The use of *tert*-butyl hydroxylamine instead of hydroxylamine was found to be essential for modification of peptide metillin at pH 6. Modification of lysozyme was achieved only after thermal denaturation of protein, to expose buried tryptophan residues. This limitation could be overcome by tryptophan mutagenesis to install solvent accessible Trp residue and thus general protocol for Trp selective protein labelling with rhodium carbenoids under neutral conditions could be established.

Rhodium catalyst in the form of dirhodium metallopeptide could also be applied for modification of tryptophan, phenylalanine, and tyrosine. Site selectivity was achieved due to molecular recognition between coiled coils of metallopeptide and substrate (Figure 8)[42].

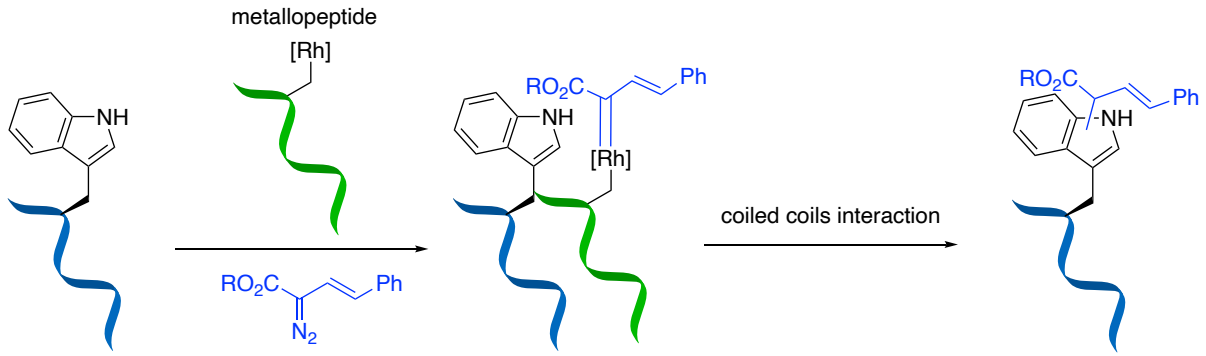

Figure 8. Trp modification in peptides driven by peptide-metallopeptide molecular recognition reported by Popp and Ball in 2010.

Ethynylation by hypervalent iodine reagent TIPS-EBX in the presence of gold catalyst was reported by Hansen *et al.*(Figure 9)[43] Catalytic amounts of [AuCl(SMe_2)] were sufficient on peptide scale while apomyoglobin modification required 5 eq of the catalyst. The method has serious disadvantages - apart from use of transition metal catalyst organic solvent acetonitrile and addition of TFA are required for the reaction to proceed, therefore, only robust proteins can be modified by this protocol. Nevertheless, successful introduction of fluorescent probe to polypeptide via copper catalysed azide-alkyne cycloaddition is reported.

[41] Antos, J. M.; McFarland, J. M.; Iavarone, A. T.; Francis, M. B. J. Am. Chem. Soc. 2009, 131 (17), 6301–6308.
[42] Popp, B. V.; Ball, Z. T. J. Am. Chem. Soc. 2010, 132 (19), 6660–6662.
[43] Hansen, M. B.; Hubálek, F.; Skrydstrup, T.; Hoeg-Jensen, T. Chem. - A Eur. J. 2016, 22 (5), 1572–1576.

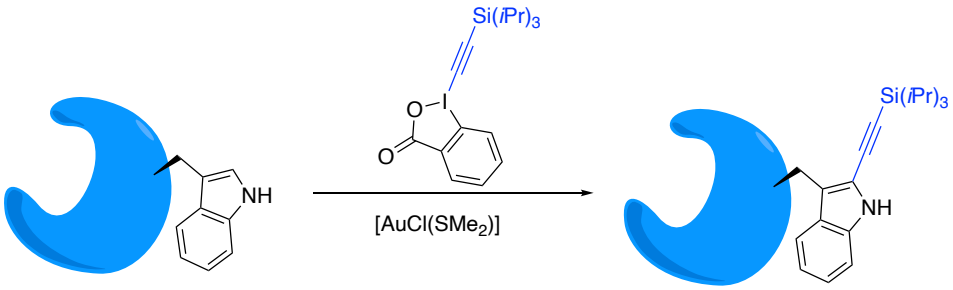

Figure 9. Trp ethynylation with hypervalent iodine reagent TIPS-EBX reported by Hansen *et al.* in 2016.

Yu *et al.* reported chemo selective photo assisted modification of Trp residues in peptides in the presence of iridium (Ir) photocatalyst. The method took advantage of the reactivity of Trp radicals which could be trapped by a broad scope of Michael acceptors (Figure 10)[44]. Interestingly, while most reported Trp selective conjugations were realized at C2 or C3 position of indole scaffold[45], in this protocol *β*-position of Trp is modified.

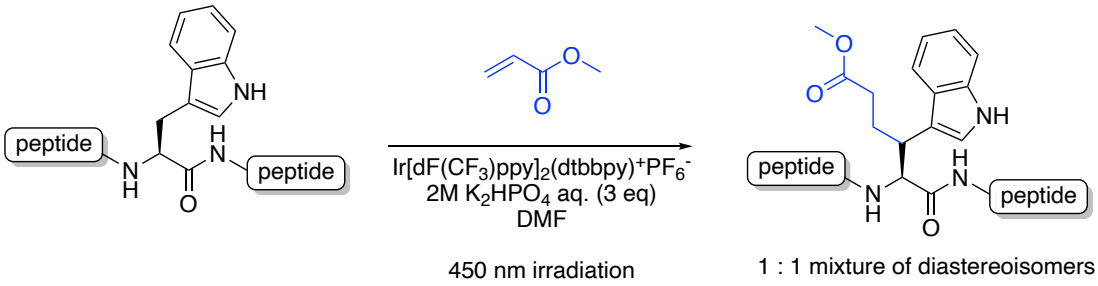

Figure 10. Trp modification in peptide catalysed by Ir photocatalyst reported by Yu, Y. *et al.*, in 2018.

[44] Yu, Y.; Zhang, L. K.; Buevich, A. V.; Li, G.; Tang, H.; Vachal, P.; Colletti, S. L.; Shi, Z. C. J. Am. Chem. Soc. 2018, 140 (22), 6797–6800.
[45] DeGruyter, J. N.; Malins, L. R.; Baran, P. S. Residue-Specific Peptide Modification: A Chemist's Guide. Biochemistry 2017, 56 (30), 3863–3873.

Transition metal free protocols

Modification of tryptophan residue in peptides has been achieved with malonyl dialdehyde under strongly acidic conditions by the group of Lindner (Figure 11).[46].The tag could be further modified through Schiff base formation or removed, by pyrrolidine or hydrazine, depending on pH.

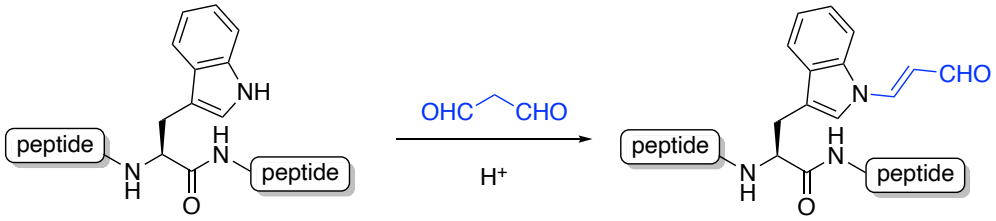

Figure 11. Modification of Trp residues by malonyl dialdehyde reported by Foettinger *et al.*

Photoinduced cyclo-addition of tetrazole appended with PEG to Trp residues in proteins was reported by Siti *et al.*(Figure 12). This reaction has been discovered accidentally during studies on modification of bovine *β*–lactoglobulin with artificially introduced allyl group by the same reagent. Trp selectivity of this method was confirmed by screening of the reaction on several proteins and peptides and possible structure of modified Trp is proposed, however, it was not confirmed experimentally.[47]

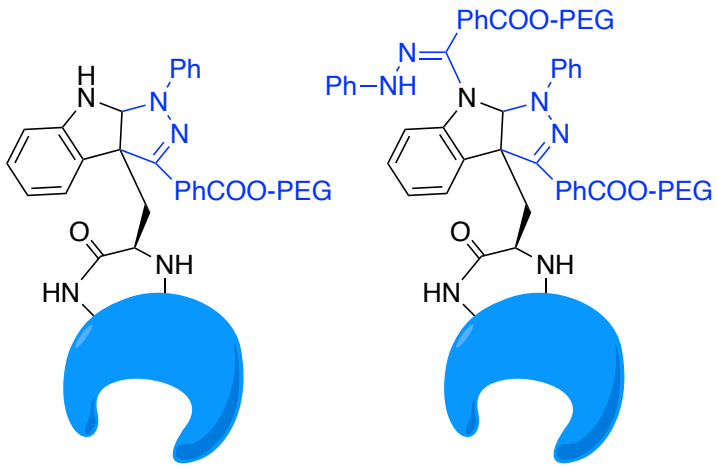

Figure 12. Proposed structures of PEG-tetrazole modified Trp residues reported by Siti *et al.* in 2015.

[46] Foettinger, A.; Melmer, M.; Leitner, A.; Lindner, W. Bioconjug. Chem. 2007, 18 (5), 1678–1683.
[47] Siti, W.; Khan, A. K.; De Hoog, H. P. M.; Liedberg, B.; Nallani, M. Org. Biomol. Chem. 2015, 13 (11), 3202–3206.

In 2016 the group of Oisaki and Kanai published a paper on transition metal free tryptophan selective protein bioconjugation (Figure 13)[48]. This method is based on the use of keto-ABNO, a stable nitroxyl radical, which is oxidized to reactive oxammonium species by NO_x generated from sodium nitrate under mild acidic conditions (pH 3). The ketone group of keto-ABNO can be easily modified to introduce various payloads via PEG linker. This method is compatible with mild reaction conditions, as opposed to previously mentioned reports which described bioconjugation with the use of transition metal catalysts and generally harsh reaction conditions. However, the method still requires an addition of catalytic amount of acid to proceed.

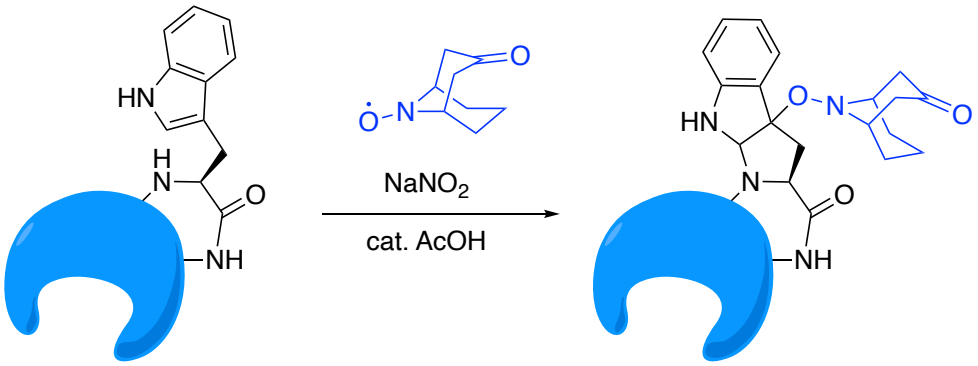

Figure 13. Transition metal free Trp-selective bioconjugation reported by Seki *et al.* in 2016.

An interesting upgrade of this mild protocol has been reported by the same group in 2019, with the use of ElectraSyn 2.0 equipment suitable for electrochemistry. Toyama *et al.* reported a method[49] in which oxidation of keto-ABNO radical to reactive oxammonium species achieved by direct anodic oxidation or indirectly by 4-oxo-TEMPO radical, which was found to be the best small molecule mediator of this reaction (Figure 14). A selection of peptides could be successfully modified. The reaction under neutral aqueous conditions, in the presence of supporting electrolyte $LiClO_4$, could also be applied to lysozyme and bovine serum albumin. In 2020 the group reported synthesis of folate peptide and protein conjugates by NO_x method with folate-ABNO/ABNOH reagent.[50] Synthesis of folate protein conjugates is problematic due to sensitivity of folate derivatives to harsh chemical conditions; therefore, this report demonstrates the applicability of the NO_x/ABNO method for challenging payloads.

[48] Seki, Y.; Ishiyama, T.; Sasaki, D.; Abe, J.; Sohma, Y.; Oisaki, K.; Kanai, M. J. Am. Chem. Soc. 2016, 138 (34), 10798–10801.
[49] Toyama, E.; Maruyama, K.; Sugai, T.; Kondo, M.; Masaoka, S.; Saitoh, T.; Oisaki, K.; Kanai, M. ChemRxiv, 2019, DOI: 10.26434/chemrxiv.7795484.v1.
[50] Maruyama, K.; Malawska, K. J.; Konoue, N.; Oisaki, K.; Kanai, M. Synlett 2020, 31 (8), 784–787.

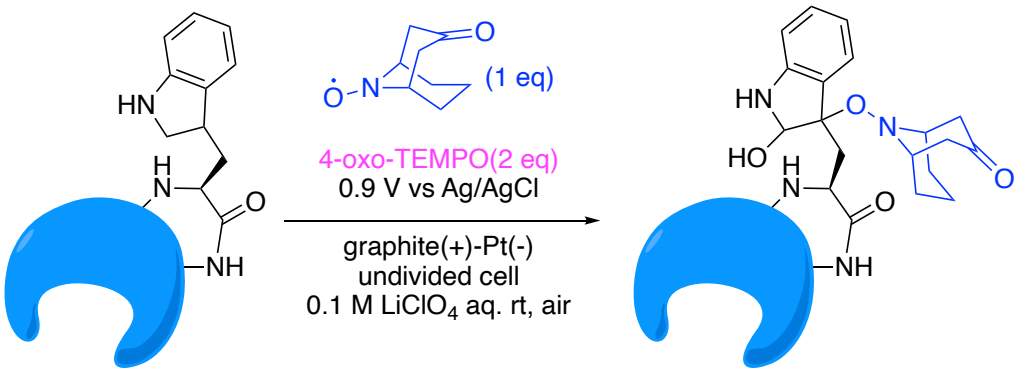

Figure 14. Electrochemical Trp selective bioconjugation of proteins reported by Toyama *et al.* in 2019.

In 2018 the group of Davis reported Trp selective trifluoromethylation. C2 position of Trp could be modified with Langlois' reagent (NaTFMS) which is a trifluoromethyl radical donor (Figure 15). Modification of peptide mellitin and horse heart myoglobin are described. Incorporation of fluoro tags into protein may be useful for studies of protein structure and catalysis. The group could confirm utility of this modification for ^{19}F NMR studies of several proteins.[51]

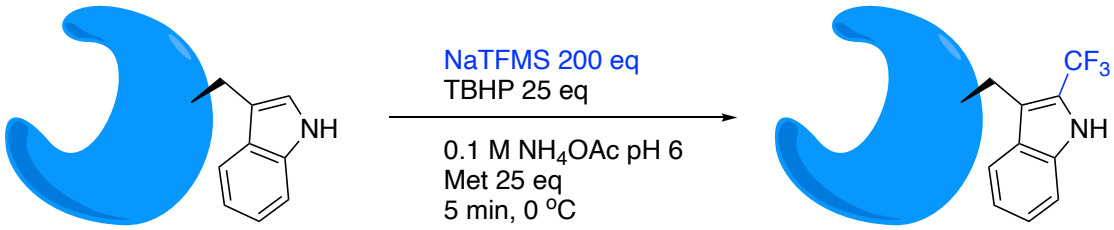

Figure 15. Trifluoromethylation of proteins reported by the group of Davis in 2018.

Recently Tower *et al.* reported *N*-substituted pyridinium salt mediated Trp-selective photo-bioconjugation (Figure 16)[52]. The reaction proceeded under irradiation with 302 nm light (UVB) in water or under buffered conditions. Addition of GSH was found to be beneficial, as GSH could scavenge reactive oxygen species generated during irradiation. Challenging peptide substrates such as octreotide or leuprorelin could be modified with good yields, bioconjugation

[51] Imiołek, M.; Karunanithy, G.; Ng, W. L.; Baldwin, A. J.; Gouverneur, V.; Davis, B. G. J. Am. Chem. Soc. 2018, 140 (5), 1568–1571.
[52] Tower, S. J.; Hetcher, W. J.; Myers, T. E.; Kuehl, N. J.; Taylor, M. T. J. Am. Chem. Soc. 2020, 142 (20), 9112–9118.

of lysozyme is also reported. Alkyne handle and several other tags could be introduced to peptides via this method with good yield.

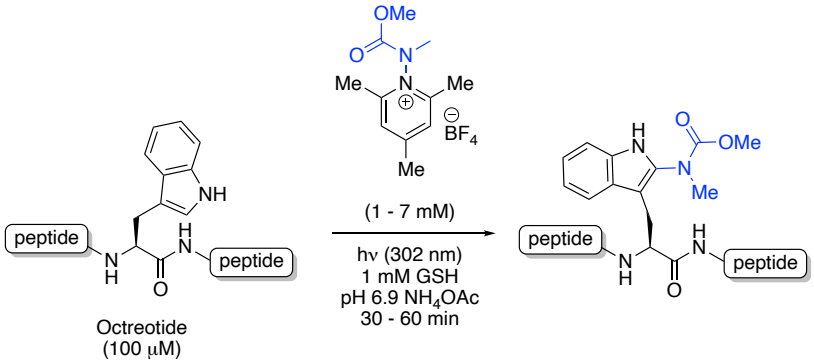

Figure 16. Modification of Trp with *N*-substituted pyridinium salts reported by Tower *et al.* in 2020.

Most recent example of transition metal free protocol for selective modification of Trp residues was reported by the group of Madder[53]. In this protocol modification can be realized with triazolidinedione (TAD) reagents (Figure 17). The use of TADs was previously reported for Tyr selective click reaction and Trp conjugation was found to be a side reaction. Modification conditions could be optimised to target Trp residues, with pH of the used buffer being important for this selectivity and the protocol could be successfully applied to several proteins.

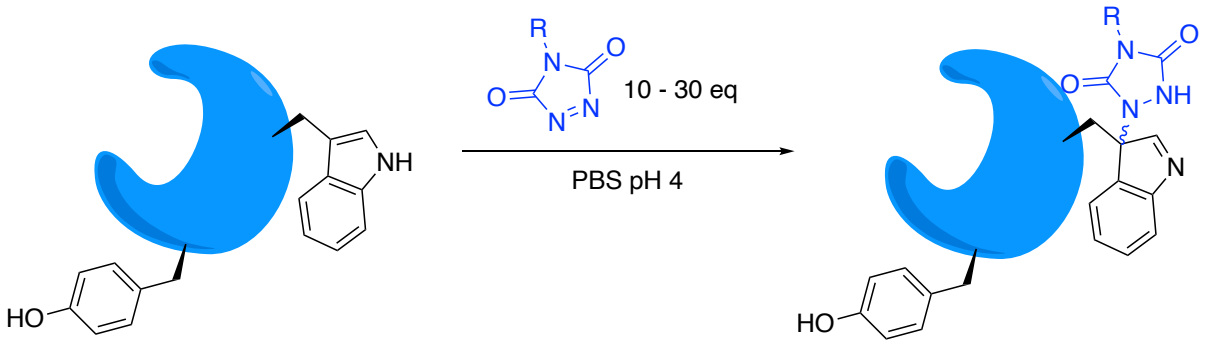

Figure 17. Modification of Trp residues in protein with TAD reagent reported by the group of Madder in 2021.

In summary, the currently available Trp bioconjugation protocols can be divided into 2 categories - metal assisted and metal - free protocols, with the latter being more explored and attractive, due to lack of heavy metal related toxicity and side reactions, as well as the

[53] Decoene, K. W.; Unal, K.; Staes, A.; Gevaert, K.; Winne, J. M.; Madder, A. ChemRxiv 2021, https://doi.org/10.26434/chemrxiv.13739320.v1.

possibility to conduct bioconjugation under mild, aqueous conditions. Based on the works cited in this part, the obvious trends in current Trp selective protein modifications seem to be the use of mild activating agents such as biocompatible oxidants or initiation of conjugation by photoirradiation or electrochemical reaction, which offer great spatio-temporal control over the process.

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