Fluorescent Labelling of Glycans by FRET-based Probes *in* a Gold(III)-mediated Three-component Coupling Reaction

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Abstract: A novel approach for fluorescent labelling of glycans by FRET-based probes *via* a gold(III)-mediated three-component coupling reaction with an alkyne-linked fluorescent dye and an amine-linked fluorescent dye has been developed. This glycan-labelling approach achieves multi-functionalization of glycans with high selectivity.

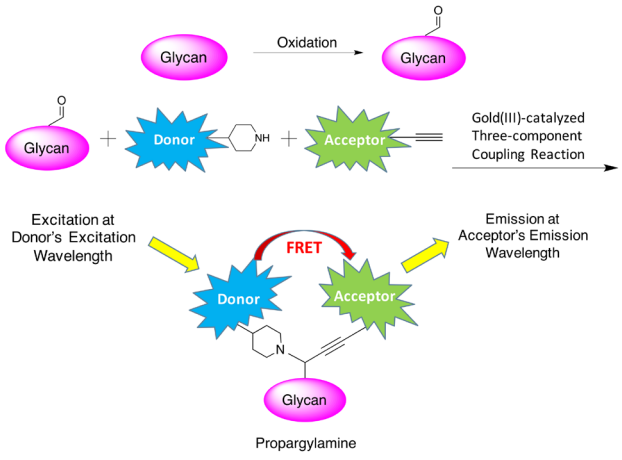
Glycosylation is one kind of post-translational protein modification in which oligosaccharide chains are covalently attached to the amino acid side chains of proteins.[1] Glycans play an important role in mediating various biological events, including immune response, cell regulation, inflammation, protein transportation, cellular division, pathogen-host interactions and signal transduction.[2-8] Specific glycosylation patterns are associated with numerous human diseases such as cancers, diabetes, Alzheimer's disease, hepatocellular carcinoma and kidney diseases.[9-12] To study the role of glycans in various biological events and disease developments, it is of importance to develop effective and selective methods for glycan modification.

Common approaches for glycan labelling usually involve chemical covalent recognition.[13] Through the introduction of aldehyde moiety to glycan by galactose oxidase or periodate-mediated oxidation, the carbonyl groups can then be ligated with aminooxy or hydrazine-based probes to give oximes and hydrazones, respectively.[14] However, the low reaction kinetics at neutral pH and low hydrolytic stability of oximes, hydrazones and imines have restricted their efficiencies for glycan labelling.

Fluorescence resonance energy transfer (FRET) has been a powerful technique in studies of biomolecules. FRET refers to non-radiative energy transfer from excited-state donor fluorophore to proximal ground-state acceptor fluorophore.[15] Contributed by their high sensitivity and selectivity, FRET-based probes have become an important tool for glycan labelling.[16] Compared with the glycan labelling approaches using one single dye, glycan labelling by FRET-based probes is much more advantageous as the latter approach can eliminate the interferences from various analyte-independent factors including local concentration of the probe, instrumental parameters and photo-bleaching by ratiometric analysis.[17] However, the general approaches of current methods do not allow single site multi-functionalization of glycans.

Single site multi-functionalization is important in biological studies as it provides a technological advance in developing novel bio-conjugates with high structural complexity, which have high potential in application of chemical proteomics and live cell surface engineering. Single site multi-functionalization could eliminate the problems arisen from the approaches using mono-functionalization, including non-specific labelling of glycans, which failed to address structure-activity relationship of the specific target glycans, and hence resulting in overestimation or underestimation of analysis. Through single site multi-functionalization, identification of specific glycosylation sites and glycan structures that modulate protein function in various biological processes can be achieved.[18] Besides, a diversity of functionalities can be introduced on the cell surface for bio-distribution studies together with cytotoxic drugs for therapeutic treatment, and biorthogonal linkers for cell-cell assembly and tissue engineering.[19] Therefore, development of new approaches for multifunctional labelling of glycans are important.

Gold-catalyzed three-component coupling reaction between aldehydes, amines, and alkynes (A^3-coupling reaction) has become an important and convenient approach for the synthesis of propargylamines.[20] In the past few years, we have reported gold(III)-catalyzed three-component coupling reactions with different aldehydes,[21] including aldehyde-containing oligosaccharides.[22] We have demonstrated the application of A^3-coupling reaction for visual detection of formaldehyde in food.[23] In view of the versatility of the multicomponent A^3-coupling reactions,[24] it is worthy of exploring the application of the gold(III)-catalyzed three-component coupling reaction for the fluorescent labelling of glycans.



Scheme 1. Our strategy of fluorescent labelling of glycans by FRET-based fluorescent probes *via* a gold(III)-mediated three-component coupling reaction

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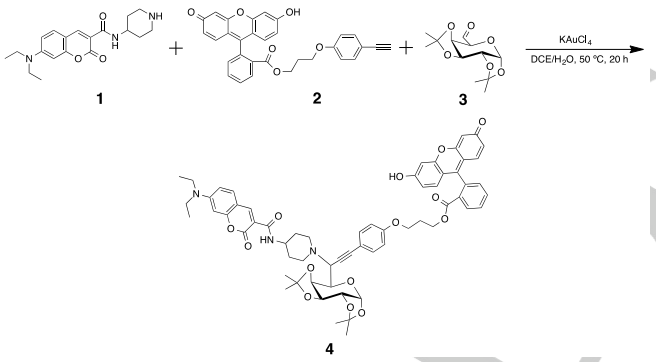
Herein we first introduce a novel method featuring the use of fluorescence resonance energy transfer-based (FRET-based) probes for fluorescent labelling of glycans *via* a gold(III)-mediated three-component coupling reaction. It involves oxidation of glycans into aldehydes by galactose oxidase, followed by a one-pot gold(III)-mediated three-component coupling reaction between the aldehydes, an amine-linked fluorescent dye and an alkyne-linked fluorescent dye (Scheme 1). It results in single-site formation of fluorescent propargylamine products exhibiting FRET from the donor fluorophore to the acceptor fluorophore. By ratiometric analysis of FRET, the conversion of labelled glycans can be revealed by FRET signals.

Two fluorescent dyes, coumarin and fluorescein, were chosen as the fluorophores for the synthesis of the FRET-based fluorescent probes. Regarding their photo-physical properties, the overlapping between the emission wavelength of coumarin and the excitation wavelength of fluorescein suggested that FRET would be exhibited upon linkage of these two fluorophores. Thus, coumarin-linked amine **1** and fluorescein-linked alkyne **2** were synthesized.

To examine the reactivity of the FRET-based fluorescent probes, we have performed the gold(III)-mediated three-component coupling reaction between coumarin-linked amine **1**, fluorescein-linked alkyne **2** and sugar aldehyde **3** (Scheme 2). The A^3-coupling reaction of sugar aldehyde **3** (0.2 mmol), coumarin-linked amine **1** (0.1 mmol) and fluorescein-linked alkyne **2** (0.1 mmol) in the presence of $KAuCl_4$ (2 mol%) in DCE/H_2O at 50 °C for 20 h was conducted. Propargylamine product **4** was obtained in 55% isolated yield.



Scheme 2. Gold(III)-mediated three-component coupling reaction of sugar aldehyde **3** with coumarin-linked amine **1** and fluorescein-linked alkyne **2**

Upon excitation at 430 nm (λ_{ex} for coumarin), propargylamine **4** showed FRET between the two attached fluorescent dyes. As shown in Figure 1, coumarin-linked amine **1** exhibited strong blue fluorescence at 470 nm, while fluorescein-linked alkyne **2** showed very weak green fluorescence at 554 nm. For propargylamine **4**, a great contrast between the fluorescence intensity of blue fluorescence and green fluorescence was observed. It showed weak fluorescence intensity at 470 nm but very strong fluorescence signal at 554 nm. No noticeable blue fluorescence at 470 nm indicated that FRET has occurred.

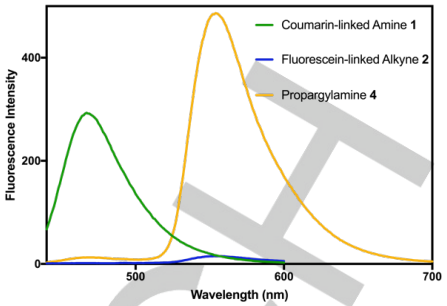


Figure 1. Fluorescence spectra of coumarin-linked amine **1**, fluorescein-linked alkyne **2** and propargylamine product **4** upon excitation at 430 nm

As propargylamine **4** has demonstrated FRET, we envisioned that coumarin-linked amine **1** and fluorescein-linked alkyne **2** can act as the donor fluorophore and the acceptor fluorophore in the fluorescence resonance energy transfer-based (FRET-based) fluorescent probes for glycan labelling.

After identifying the fluorophore components for the FRET-based fluorescent probes, we optimized the reaction conditions of the A^3-coupling reaction between coumarin-linked amine **1**, fluorescein-linked alkyne **2** and sugar aldehyde **3**. By measuring the fluorescent signal of the reaction mixtures, the conversion for the propargylamine formation was determined by FRET ratio ($R = 1 - F_{470\,nm}(T_{16} / T_0)$), which implied the extent of quenching of the donor fluorphore, coumarin-linked amine **1**, upon the formation of fluorescent propargylamine.[25]

We studied of the effect of reagent concentrations on the conversion of the coupling reaction. The A^3-coupling reactions of different concentrations of coumarin-linked amine **1**, fluorescein-linked alkyne **2** and sugar aldehyde **3** in the presence of $KAuCl_4$ in DCE/H_2O at 50 °C for 16 h were conducted. According to Figure 2, the conversion of the coupling reaction, interpretated by FRET ratio, increased with the reagent concentration. The FRET ratio reached its highest value and levelled off when the concentration of the FRET-based probes was beyond 5 mM (Table 1, Entry 4). As a result, the reagent concentration of coumarin-linked amine **1** (5 mM), fluorescein-linked alkyne **2** (5 mM), sugar aldehyde **3** (10 mM) and gold(III) catalyst (100 μM) would be the optimal concentrations for the following studies.

Table 1. Screening for the effect of reagent concentrations on the fluorescence ratio of propargylamine product **4**

Entry[a]	**1** (mM)	**2** (mM)	**3** (mM)	$KAuCl_4$ (mM)	$R = 1 - F_{470\,nm}(T_{16} / T_0)$ (%)
1	0.2	0.2	0.4	0.004	30
2	0.5	0.5	1	0.01	59
3	1	1	2	0.02	77
4	5	5	10	0.1	85
5	10	10	20	0.2	88

[a] Reaction conditions: coumarin-linked amine **1**, fluorescein-linked alkyne **2** (1 equiv.), sugar aldehyde **3** (2 equiv.) and $KAuCl_4$ (2 mol%) in DCE/ H_2O (5:1) (total volume = 3 mL) at 50 °C for 16 h

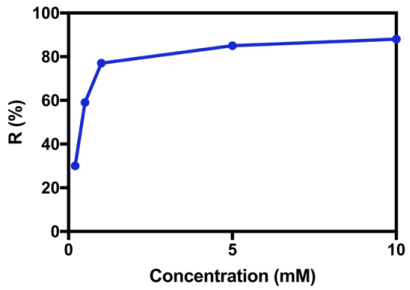


Figure 2. The relationship diagram between the concentration of FRET-based probes and the resulting FRET ratio ($R = 1 - F_{470\,nm}(T_{16} / T_0)$) at λ_{ex} = 430 nm

We then investigated the catalytic efficiency of $KAuCl_4$ and gold(III) catalysts **5a**-**5c** in the A^3-coupling reaction. The A^3-coupling reactions of coumarin-linked amine **1** (5 mM), fluorescein-linked alkyne **2** (1 equiv.), sugar aldehyde **3** (2 equiv.) and different gold(III) catalysts (2 mol%) in DCE/H_2O (5:1) at 50 °C for 16 h were conducted. The results of the studies are given in Figure S11 and Table 2. FRET was found from the decrease of blue fluorescence at 470 nm as well as the increase of green fluorescence at 554 nm (Figure S11). In Entry 1, $KAuCl_4$ exhibited the highest catalytic activity with the greatest FRET ratio of 85%. Meanwhile, comparable catalytic effects were exhibited by **5a**-**5c**, with fluorescence ratio of 68%, 76% and 79%, respectively (Table 2, Entries 2-4). The higher catalytic activity of $KAuCl_4$ than that of **5a**-**5c** could be attributed to their difference in steric bulkiness. $KAuCl_4$ has least bulky ligands among the gold(III) catalysts, and hence resulting in the greatest catalytic efficiency with least steric hindrance. Given the higher catalytic efficiency of $KAuCl_4$ than gold(III) catalysts **5a**-**5c**, it is concluded that $KAuCl_4$ would be the most suitable catalyst for our later studies.

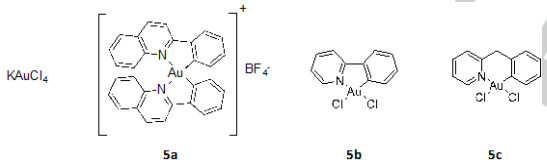


Table 2. Screening for the effect of different gold(III) catalysts on the fluorescence ratio of propargylamine product **4**

Entry[a]	Gold(III) Catalyst	$R = 1 - F_{470\,nm}(T_{16} / T_0)$ (%)
1	$KAuCl_4$	85
2	**5a**	68
3	**5b**	76
4	**5c**	79

[a] Reaction conditions: coumarin-linked amine **1** (5 mM), fluorescein-linked alkyne **2** (1 equiv.), sugar aldehyde **3** (2 equiv.) and $KAuCl_4$ or gold(III) catalyst **5a**-**5c** (2 mol%) in DCE/H_2O (5:1) (total volume = 3 mL) at 50 °C for 16 h

After the investigation of the efficiency of gold(III) catalysts, we moved on to study the effect of different solvents on the conversion of the $KAuCl_4$-mediated three-component coupling reaction. The A^3-coupling reactions between coumarin-linked amine **1** (5 mM), fluorescein-linked alkyne **2** (1 equiv.), sugar aldehyde **3** (2 equiv.) in the presence of $KAuCl_4$ (2 mol%) in different solvents at 50 °C for 16 h were performed. DCE, dioxane, THF and EA were chosen as the solvents, while H_2O was used for dissolving the gold(III) salt. As shown in Table 3 and Figure S13, it was found that the reaction was compatible in all solvents with FRET ratio ranging from 57 to 85%. The reaction worked best in DCE/H_2O (5:1), giving the highest FRET ratio of 85% (Table 3, Entry 1). The conversion was slightly lower with a FRET ratio of 77% when using EA/H_2O (5:1) as solvent (Table 3, Entry 4). However, dioxane/H_2O (5:1) and THF/H_2O (5:1) were found to be poor solvents in the reaction as the resulting FRET ratios were only 61% and 57%, respectively (Table 3, Entries 2-3). The highest resulting fluorescence ratio using DCE/H_2O (5:1) could be attributed to the best solubility of the reagents in DCE. It was observed that the coumarin-linked amine **1**, fluorescein-linked alkyne **2**, sugar aldehyde **3** have the best solubility in DCE. As a result, it was concluded that DCE/H_2O (5:1) would be the optimal solvent for the A^3-coupling reaction giving the highest conversion.

Table 3. Screening for the effect of solvents on the fluorescence ratio of propargylamine product **4**

Entry[a]	Solvents	$R = 1 - F_{470\,nm}(T_{16} / T_0)$ (%)
1	DCE/H_2O (5:1)	85
2	Dioxane/H_2O (5:1)	61
3	THF/H_2O (5:1)	57
4	EA/H_2O (5:1)	77

[a] Reaction conditions: coumarin-linked amine **1** (5 mM), fluorescein-linked alkyne **2** (1 equiv.), sugar aldehyde **3** (2 equiv.) and $KAuCl_4$ (2 mol%) in different solvents (total volume = 3 mL) at 50 °C for 16 h

Finally, the effect of the difference in the reagent ratio on the conversion of the gold(III)-mediated three-component coupling reaction were investigated. The A^3-coupling reactions between different concentrations of coumarin-linked amine **1**, fluorescein-linked alkyne **2** and sugar aldehyde **3** in the presence of $KAuCl_4$ in DCE/H_2O (5:1) at 50 °C for 16 h were conducted. According to Table 4 and Figure S15, different reagent ratios gave comparable responses with fluorescence ratio from 83% to 87% (Table 4, Entries 1-4). The reagent ratio was found to exhibit no significant effect on the conversion of the A^3-coupling reaction.

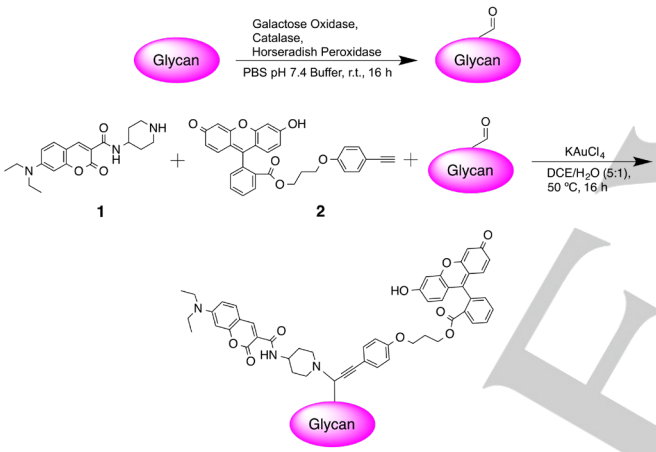
Table 4. Screening for the effect of reagent ratios on the fluorescence ratio of propargylamine product **4**

Entry[a]	**1** (mM)	**2** (mM)	**3** (mM)	$KAuCl_4$ (mM)	$R = 1 - F_{470\,nm}(T_{16} / T_0)$ (%)
1	5	5	10	0.1	85
2	5	5	2.5	0.1	83
3	5	5	5	0.1	87
4	5	5	25	0.1	84

[a] Reaction conditions: different concentrations of coumarin-linked amine **1**, fluorescein-linked alkyne **2**, sugar aldehyde **3**, and $KAuCl_4$ in DCE/H_2O (5:1) (total volume = 100 µL) at 50 °C for 16 h

Based on the above experimental results, we have optimized our reaction conditions that the conversion of the gold(III)-mediated three-component coupling reaction would be the highest when using $KAuCl_4$ as the catalyst and DCE/H_2O (5:1) as the reaction medium. In line with our previous studies on the reaction temperature optimization of the gold(III)-mediated three-component coupling reaction,[23] the reaction temperature of 50 ºC was chosen.

After the reaction condition optimization, we investigated the applicability of the FRET-based fluorescent probes on the glycan labelling *via* a gold(III)-mediated three-component coupling reaction (Scheme 3). Raffinose and galactose were used for the coupling reaction. The generation of aldehyde moiety was performed by the oxidation of glycans (100 mM) in the presence of galactose oxidase (7.4 U), catalase (15929 U) and horseradish peroxidase (64.3 U) in 50 mM phosphate-buffered saline pH 7.4 at room temperature for 16 h.[26] After that, the A^3-coupling reactions between the oxidized glycans (10 mM), coumarin-linked amine **1** (5 mM) and fluorescein-linked alkyne **2** (5 mM) in the presence of $KAuCl_4$ (2 mol%) in DCE/H_2O (5:1) at 50 ºC for 16 h were performed.



Scheme 3. Fluorescent labelling of glycans by oxidation in presence of galactose oxidase, catalase and horseradish peroxidase, followed by gold(III)-mediated three-component coupling reaction with coumarin-linked amine **1** and fluorescein-linked alkyne **2**

The success of glycan labelling by FRET-based probes *via* $KAuCl_4$-mediated three-component coupling reaction with coumarin-linked amine **1** and fluorescein-linked alkyne **2** was shown in Figure 3. According to Figure 3, the decrease of blue fluorescence at 470 nm as well as the increase of green fluorescence at 554 nm indicated the presence of FRET upon the labelling of raffinose and galactose, respectively. By ratiometric analysis, FRET ratios of 66% and 69% were resulted upon the labelling of raffinose and galactose, respectively, by FRET-based probes *via* $KAuCl_4$-mediated three-component coupling reaction with coumarin-linked amine **1** and fluorescein-linked alkyne **2**. In this study, we revealed that the FRET-based fluorescent probes can be a novel approach for fluorescent labelling of glycans.

The lower conversion of labelling glycans than that of propargylamine product **4** in the model reaction could be attributed to their differences in polarity and solubility. As the formation of propargylamine product in the A^3-coupling reaction was resulted from the linkage between the two fluorescent dyes and the sugar aldehyde substrate, it was hence suggested that the reaction would proceed better if the sugar aldehyde substrate was in the same phase with the fluorescent dyes, that was, the organic phase. Regarding the structural differences of the sugar aldehyde substrates, the oxidized raffinose and galactose involved multiple polar hydroxyl groups, while the hydroxyl groups of sugar aldehyde **3** was protected as acetals. The presence of free hydroxyl groups in the oxidized glycans contributed to higher polarity, and leading to much better solubility in the aqueous phase. On the other hand, the protection of hydroxyl groups in sugar aldehyde **3** would lead to much lower polarity, thus having greater solubility in the organic phase. As a result, it was suggested the fluorescent labelling of sugar aldehyde **3** was more efficient than that of glycans.

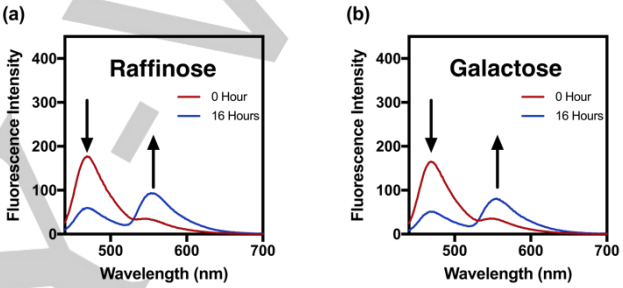


Figure 3. Fluorescence spectra of A^3-coupling reaction between **1**, **2** and (a) oxidized raffinose; (b) oxidized galactose, in the presence of $KAuCl_4$ in DCE/H_2O (5:1) at λ_{ex} = 430 nm

In summary, we first develop a novel approach of fluorescent labelling of glycans based on FRET-based probes *via* a gold(III)-mediated three-component coupling reaction between oxidized glycans, coumarin-linked amine **1** and fluorescein-linked alkyne **2** in the presence of $KAuCl_4$. This glycan labelling approach achieves multi-functionalization of glycans with high selectivity.

Experimental Section

The Experimental methods are summarized in the Supporting Information.

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Keywords: Gold • Fluorescence • FRET • Biomolecules • Glycan

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