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COMMUNICATION

An efficient conjugation method between hydrophilic and hydrophobic components using a solid-phase assisted disulfide ligation

Kyohei Muguruma, Takuya Shirasaka, Daichi Akiyama, Kentarou Fukumoto, Akihiro Taguchi, Kentaro Takayama, Atsuhiko Taniguchi, Yoshio Hayashi*

Abstract: Chemical conjugation between hydrophilic and hydrophobic components is a difficult challenge due to their extremely different solubilities. We here report a new versatile method with a "solid-phase assisted disulfide ligation" for overcoming the difficulty of conjugation attributed to the solubility. The method involves two steps in one-pot: 1) loading a hydrophobic molecule on the resin in an organic solvent, and 2) releasing the solid-supported hydrophobic molecule as the conjugate with a hydrophilic molecule into an aqueous solvent. This strategy allows the use of a suitable solvent system for the substrates in each step. Indeed, the conjugates of a water-insoluble drug, plinabulin, with hydrophilic carriers which were impossible to be prepared by general solution phase reaction were obtained in moderate yields (29-45%). This strategy is widely applicable to the conjugation of compounds with solubility problems.

Conjugation of functional molecules with carrier molecules is an attractive strategy for the efficient delivery of the former to target biomolecules or cells in chemical biology[1] or medicinal chemistry[2]. For the preparation of such conjugates, a selective crosslinking reaction of the two components is necessary. For example, a copper-catalyzed alkyne azide cycloaddition (CuAAC) is widely used because linkage with 1,2,3-triazole is easily created by mixing the azide with an alkyne and a copper (I) catalyst in a solvent.[3] However, general conjugation reactions, including click chemistry are hampered when the reaction solvent is not suitable to solubilize two components which may have extremely different solubilities.[4] This problem becomes serious when a hydrophobic functional molecule is linked with a hydrophilic carrier in an attempt to improve water solubility or targeting ability (Figure 1A).

As an alternative method, we report a one-pot solid-phase conjugation method consisting of two reactions (Figure 1B). The first step involves a hydrophobic component loaded on the solid support in an organic solvent. Then, after the exchange of the organic solvent for an aqueous solvent, the hydrophilic component is conjugated with the loaded hydrophobic component to generate the desired product. This procedure allows use of two suitable solvent systems, which can solubilize the respective reaction components in each step. In this way, the procedure overcomes the difficulty in the conjugation of hydrophobic and hydrophilic components.

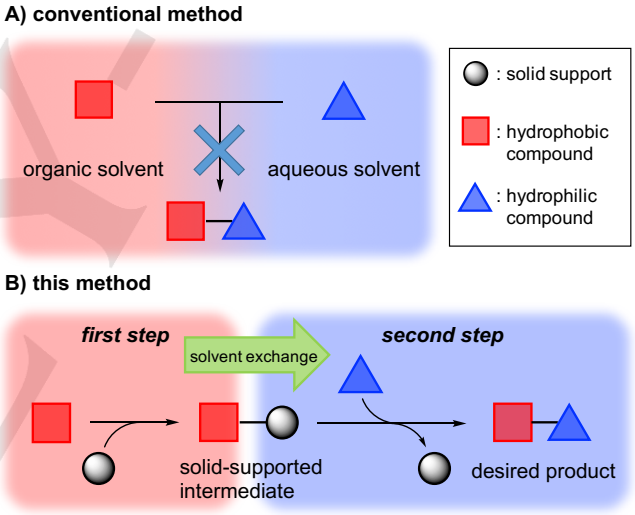

Figure 1. Conjugation reaction between hydrophobic and hydrophilic components: A) in solution-phase and B) *via* solid-phase.

Our group previously reported an anti-microtubule agent, plinabulin (**1**, Phase III) for treatment of non-small cell lung cancer.[5,6] Plinabulin has an extremely poor water-solubility (< 0.1 μg/mL) notwithstanding its use in injections, and it is an appropriate model drug for the conjugation with a water-soluble carrier. As a carrier, we adopted a highly water-soluble[2c] and cell penetrating[7] octaarginine peptide that is often used as a carrier peptide for drug delivery[2d]. We attempted the conjugation using a CuAAC reaction, which we had previously used successfully to prepare a water-soluble plinabulin prodrug (**2**) with a hydrophilic amino acid moiety (Figure 2).[8] However, the reaction failed to proceed because no solvent could sufficiently dissolve both plinabulin and peptide moieties simultaneously (Scheme S1). Consequently, we performed a two-step solid-phase conjugation using our recently developed "solid-phase assisted disulfide ligation (SPDSL)"[9,10] whose details are described below. The ligation has been successfully applied to the synthesis of a

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plinabulin-Z33 (IgG-binding peptide)[11] conjugate for a noncovalent-type antibody-drug conjugate.[9] It is a versatile method for the chemical conjugation between two molecules with very different solubilities. We synthesized conjugates of plinabulin with several water-soluble carriers by this method, through optimization of the reaction conditions.

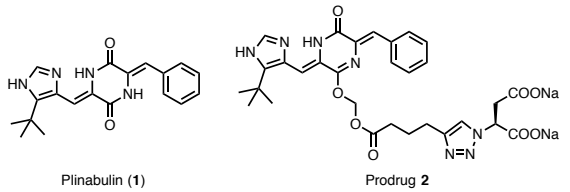
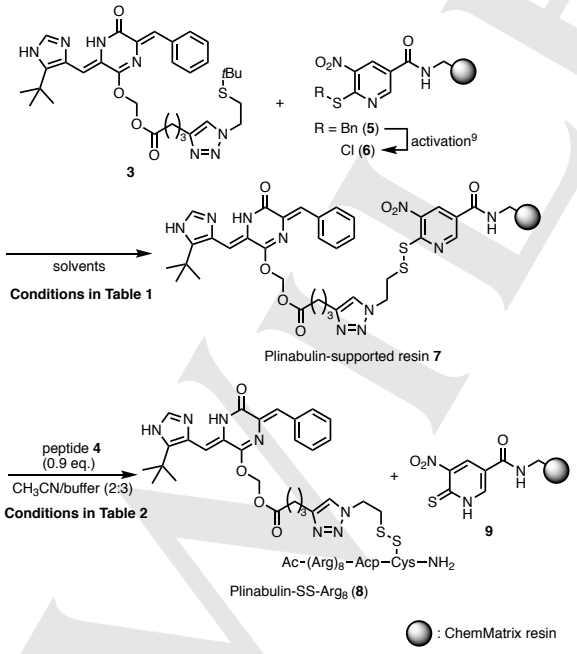


Figure 2. Structure of plinabulin and a water-soluble prodrug.

As starting materials, two types of building blocks, the sulfide derivative of plinabulin (**3**) and a cysteine-containing octaarginine peptide, Ac-Arg_8-aminocaproic acid (Acp)-Cys-NH_2 (**4**) were prepared according to a previously reported method[8,9] and by general Fmoc-based solid-phase peptide synthesis, respectively. As depicted in Scheme 1, the solid-supported 3-nitro-2-pyridine sulfenyl (Npys) group was activated from its benzyl form (**5**) to a chloride form (**6**) by chlorosulfenylation with 2% SO_2Cl_2 in 1,2-dichloroethane.[10] The Npys-Cl resin (**6**) prepared in this way was allowed to react with the hydrophobic sulfide (**3**) in an organic solvent. This resulted in the formation of drug-supported resin (**7**) through an active disulfide. The resin was washed twice with CH_3CN and four times with water to quench the residual Npys-Cl. In a second step, the organic solvent was replaced by an aqueous solvent of the hydrophilic thiol-containing peptide (**4**). As a result, the drug-component on the resin (**7**) was transferred to the peptide by a disulfide exchange reaction. Subsequently, the desired product (**8**), a disulfide-bridged peptide-drug conjugate (PDC), was released into the aqueous phase. We adopted an amphiphilic solid-support, ChemMatrix® resin[12] as the resin, which swells in both organic and aqueous solvents. The SPDSL is a useful one-pot method to prepare the conjugate between materials possessing different solubilities, for the following reasons: 1) the solid-phase assisted method allows a simple solvent exchange by simply washing the resin, 2) the disulfide formation enables a selective linkage between thiol groups of both components with no need for protecting groups, and 3) the conjugation reaction occurs simultaneously with the removal process of the plinabulin segment from the resin. The resultant conjugate can produce the active plinabulin via enzymatic degradation of the linker part.[8]



Scheme 1. Synthesis of plinabulin-SS-Arg_8 **8** by SPDSL.

To investigate the reaction conditions in the first step of our method, the sulfide **3** was mixed with resin **6** in various organic solvents and the loading of **3** was monitored by HPLC which tracks the decrease of **3** in the reaction solution. When CH_2Cl_2, $CHCl_3$ or CH_3CN were used as solvent, **3** disappeared from the reaction solution within 1 h (Table 1), indicating that it was completely loaded on the resin to afford the Npys-assisted active disulfide **7**. It was found that for each solvent, the disappearance level of **3** was inversely correlated (correlation coefficient (R) = –0.96, Figure 3) with Gutmann's donor number (DN)[13], a quantitative measure of Lewis basicity (see Supporting Information for the details).

The sulfide **3** in CH_3CN was almost completely (98%) loaded even when the amount of resin **6** (corresponding to resin **5**) was decreased to 2 eq. (Table 1, entry 8 and Figure 4A), although the reaction was slower when using 1.1 eq. of **6** (80%, entry 9). We previously reported that the 5 eq. of resin **6** were required for the complete reaction when using 90% formic acid/H_2O as solvent.[10] This difference suggests that the water

Table 1. Reaction conditions for the first step.

Entry	Solvent	Resin (eq.)[a]	Disappearance level of **3** (%)[b]	DN[c] (kcal/mol)
1	CH_2Cl_2	5	100	1
2	$CHCl_3$	5	100	4
3	CH_3CN	5	100	14.1
4	MeOH	5	64	19
5	THF	5	74	20
6	DMF	5	42	26.6
7	DMSO	5	80	30.9
8	CH_3CN	2	98	14.1
9	CH_3CN	1.1	80	14.1

[a] Eq. of resin **5** against sulfide **3**, [b] The disappearance level was calculated by the following formula; (1-[peak area of **3** after 1h]/[peak area of **3** at 0 h] X 100), [c] Donor number.[13]

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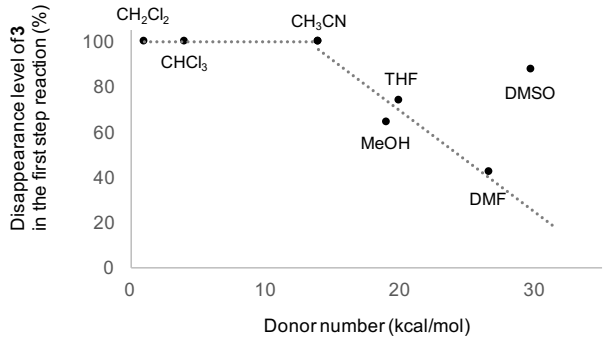

Figure 3. Correlationship between disappearance of **3** in the first step and DN[13] of solvent. The correlation coefficient (R) of approximation straight line between CH_3CN and DMF is –0.96.

molecule reacted with **6** to form an inactive form, while in CH_3CN, the reactivity of Npys-Cl resin (**6**) was maintained. Accordingly, we selected the conditions indicated in Table 1, entry 8 for the first step reaction.

In the second step, the reaction with a Cys-containing Arg_8 peptide (**4**) (0.9 eq. with respect to sulfide **3**) was performed using 40% aqueous acetonitrile as solvent, and the formation of conjugate **8** was tracked by HPLC analysis of the reaction solution.

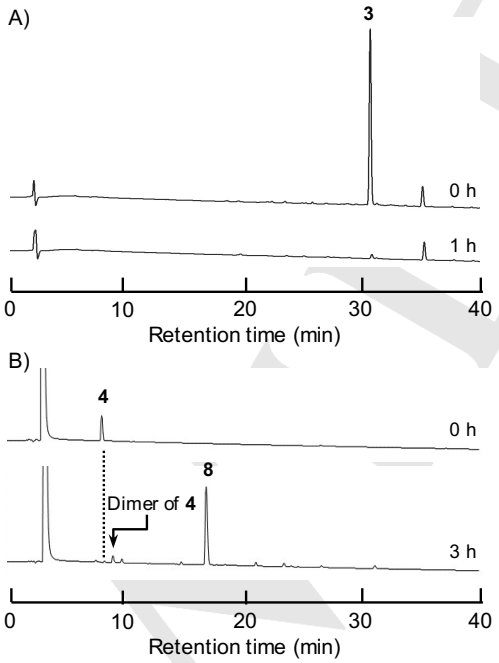

Figure 4. HPLC chromatograms of A) the first step reaction (Table 1, entry 8), and B) the second step reaction (Table 2, entry 4).

When using non-buffered water (Table 2, entry 1), the reaction was very slow and incomplete even after 5 days (data not shown). In addition, the parent drug (**1**) was produced by hydrolysis of the linker under these conditions. To enhance the reaction, we used aqueous buffer (pH 3.8–7.4) instead of water. At pH 5.0 (entry 4), the reaction proceeded efficiently and the peptide **4** completely disappeared within 3 h (Figure 4B). Subsequently, a disulfide-bridged PDC, plinabulin-SS-Arg_8 (**8**) was produced in a good HPLC yield (91%). The disulfide dimer of peptide **4** was produced at higher pH values (Table 2, entry 4, 5 and Figure S2). In addition, the loss of peptide **4** was observed when using pH 7.4 buffer (entry 6) due to a non-specific adsorption of Arg_8 by a weakly acidic thiopyridone moiety of resin **9** that was produced (Figure S3). Thus, this reaction is sensitive to pH. The tendency was remarkable in the requirement for 5 eq. of resin **5** (Table S1 and Figure S4). Moreover, an improved yield was observed in 1 M sodium acetate (43%) compared to a 50 mM solution (13%) (Table S1, entry 1 vs 5). This indicated that higher concentrations of buffer salt promote the reaction.

Table 2. Reaction optimization in the second step.

Entry	Aqueous solvent	Yield (%)[a]	Dimer[b]
1	non-buffered water	7	n.d.[c]
2	50 mM sodium acetate (pH3.8)	82	n.d.[c]
3	50 mM sodium acetate (pH4.5)	81	0.8
4	50 mM sodium acetate (pH5.0)	91	1
5	50 mM sodium acetate (pH5.6)	75	1.3
6	50 mM sodium phosphate (pH7.4)	7	n.d.[c]

[a] HPLC yields are calculated from the area of the peak of **8** in the analytical HPLC measured after 3 h on the basis of the amount of peptide **4**, [b] The values are reported as a relative to the condition in entry 4, [c] not detected.

Finally, conjugate **8** was obtained in 44% isolated yield (Table 3, entry 1). The isolated yield was lower than the HPLC yield (Table 2, entry 4), as a result of the generally observed non-specific adsorption of octaarginine. Next, we applied the solid-phase assisted reaction to coupling with other functional hydrophilic peptides as shown in Table 3. The PDC, including D-octaarginine peptide, that has an ability to accumulate in tumors,[2d] was obtained in 37% isolated yield (entry 2). On the other hand, the non-ionized peptide, $(Gly\text{-}Ser)_4$, a repeated sequence, which is used as a control for cell-penetrating octaarginine peptide[7b], was conjugated with plinabulin in moderate yield (45%, entry 3). Although some by-products were observed in the conjugation of Z33 peptide in which the disulfide homo-dimer was the main by-product, the reaction with Z33 peptide was drastically accelerated (5 h, 32%: entry 4) by change of solvent, compared with the previously reported conditions which used 50% DMF/H_2O (20 h, 28%)[9]. In addition, we successfully synthesized a plinabulin-galactose conjugate with a yield of 29 % (entry 5). This suggests

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that the reaction can be applied to conjugations not only with peptides but also with other hydrophilic functional materials such as sugars. In Table 3, entries 4 and 5, the buffer at pH 4.5 was preferred to the one at pH 5.0, because of the slow homo-dimerization at lower pH values, resulting in higher isolated yields.

Table 3. Application of SPDSL to the production of other conjugates.

Entry	Thiol containing hydrophilic components[a]	Isolated yield (%)
1	Ac-(L-Arg)$_8$-Acp-Cys-NH$_2$ (Table 2, Entry 4)	44
2	Ac-(D-Arg)$_8$-Acp-Cys-NH$_2$	37
3	Ac-(GS)$_4$-Acp-Cys-NH$_2$	45
4	H-CG-Z33-NH$_2$ (pH 4.5)	32[b]
5	Galactose-SH (pH4.5)	29

[a] The sequence of the peptides and products are shown in SI, [b] 0.75 eq. of peptide was used to directly compare with the previous condition[9].

In conclusion, we successfully synthesized the conjugates of the hydrophobic plinabulin with several hydrophilic carriers using SPDSL. This method involves two steps: 1) loading a hydrophobic moiety on the resin in an organic solvent, and 2) conjugating the loaded hydrophobic moiety and a hydrophilic moiety in an aqueous solvent. This strategy allows the use of different solvent systems in each step, and the difficulty in conjugation between materials possessing opposite solubilities was overcome. The solubility problem in organic synthesis is not limited to peptides but also natural products,[4c] and our strategy would be productively applied to such instances. This study established the benefit of the solid-phase assisted disulfide coupling reaction. Optimisation of the reaction conditions revealed that the first step prefers low DN solvents and that a pH below 5.0 and high salt concentrations are important in the second step. High yields of the conjugations were obtained, which is difficult in solution-phase reactions. This strategy will provide a versatile system for the conjugation of materials with extremely different solubilities.

Acknowledgements

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Keywords: Peptide-drug conjugate • Solubility • Synthetic methods • Solid-phase synthesis

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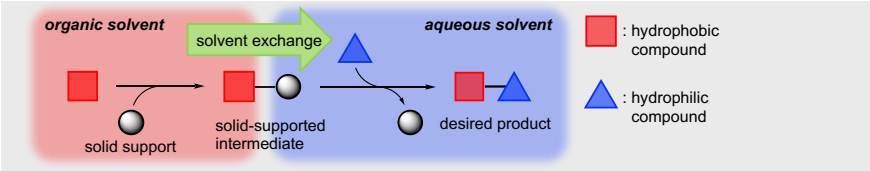

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