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A modular approach to enzymatic ligation of peptides and proteins with oligonucleotides

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Joining peptides and oligonucleotides offers potential benefits, but current methods remain laborious. Here we present a novel approach towards enzymatic ligation of the two modalities through the development of tag phosphoramidites that readily couple onto oligonucleotides. This simple and highly efficient approach paves the way towards streamlined development and production of peptide/protein-oligonucleotide conjugates.

Peptides and oligonucleotides (oligos) are widely utilized as chemical and molecular biology tools, and they have emerged as viable and effective drug classes in recent years.[1] Joining the two modalities would provide an opportunity to harness and combine the benefits of both,[2, 3] and to expand the scope of their activity. For instance, peptides can carry oligos, and conversely, aptamers[4] can carry peptides, over to their desired sites of action *in vivo* or in cells to serve as therapeutics or imaging tools. However, due to limited compatibility in chemistry between the solid-phase (SP) synthesis of peptides and oligos, generation of peptide-oligo conjugates (POCs) remains an ad hoc and laborious process.[2, 5] A streamlined method towards the conjugation of peptides and oligos would thus offer a means to expand the chemical biology toolbox and facilitate the production of POCs for therapeutic development.

Two general strategies are available for the synthesis of POCs.[2, 5] The first involves in-line synthesis through sequential addition of amino acid and nucleotide monomers onto the same solid support.[6] Albeit straightforward, this strategy is constrained by limited choices of compatible protecting groups, given that contrasting chemistries are employed in the stepwise coupling and global deprotection of the two entities. On the other hand, post-synthetic conjugation involves synthesis, deprotection, and purification of peptide and oligonucleotide separately before their conjugation.[7, 8] While this circumvents the compatibility issue, multiple synthesis and purification steps often render this approach tedious and yield-limiting. Moreover, the conjugation chemistry chosen must be exclusively orthogonal to prevent side reactions with reactive side chains of the peptide.

Peptide ligases catalyse the joining of two peptide ends that contain matching sequence or chemical motifs, and they have been extensively applied in protein engineering.[9, 10] The use of an enzyme with high regioselectivity ensures a clean reaction under mild aqueous condition. Adaptation of such enzymatic approaches towards post-synthetic generation of POCs would thus be highly desirable. However, few reports of POC creation utilizing this approach are available,[11, 12] and there remains a need for a streamlined process to the creation of diverse POCs.

Here we present a phosphoramidite tag-based approach to enzymatic ligation of peptides and oligos that greatly simplifies the preparation of POCs (Fig. 1). A short peptide recognition motif (≤ 3 amino acids) was converted to a tag phosphoramidite that can be readily incorporated onto an oligo during automated synthesis (Fig. 1a, top), followed by ligation with the peptide counterpart containing the matching ligation handle by the cognate ligase (Fig. 1a, bottom). We demonstrated the utility of our method with sortase[13, 14] and peptide asparaginyl ligase[9, 15, 16] (PAL), two distinct classes of ligase that have seen widespread use in protein engineering. The ligation strategy was successfully applied across a diverse range of oligo and peptide constructs, and was further extended to the ligation of a protein with oligo. The modular nature of tag incorporation further provides opportunity for the conjugation of multiple peptides/proteins onto an oligo in a controllable manner. Thus, our approach provides a straightforward path towards the streamlined development and production of POCs.

Sortase is a family of transpeptidases that anchor proteins onto bacterial cell wall.[13] Sortase A from *Staphylococcus aureus* ligates N-terminal poly-glycine onto a C-terminal end containing the consensus sorting motif LPXTG. Accordingly, we synthesized a phosphoramidite tag (**1**) containing the Gly-Gly-Gly ligation

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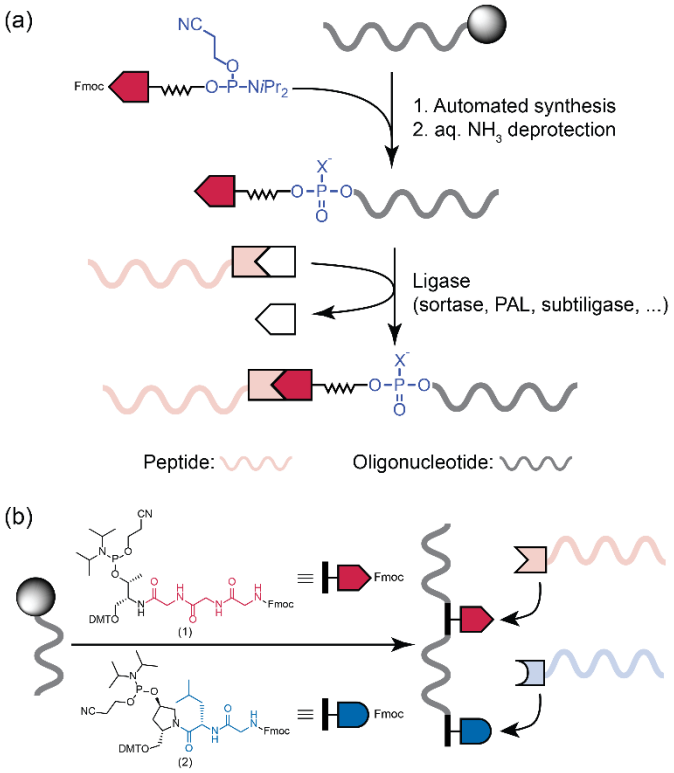

Fig. 1 Schematic illustration of a phosphoramidite tag-based approach to the enzymatic ligation of single or multiple peptide(s) with an oligonucleotide. (a) Coupling of a phosphoramidite tag (red) onto an oligonucleotide chain (grey ribbon) by solid-phase oligonucleotide synthesis, followed by enzymatic ligation of a peptide counterpart (pink ribbon) containing the cognate ligation handle with the tag-labelled oligonucleotide to produce the desired peptide-oligonucleotide conjugate. (b) Modular coupling of two distinct phosphoramidite tags (**1** and **2**) onto an oligonucleotide enables positional control on subsequent ligation of two peptides containing the matching ligation handles by the cognate ligases.

motif (Fig. 1b, red), starting from a threoninol (**S1**) scaffold (Scheme S1, ESI). Compound **1** is fully compatible with phosphoramidite-based solid-phase oligonucleotide synthesis (SPOS), hence allowing straightforward incorporation of the Gly-Gly-Gly tag onto oligonucleotides (Fig. 1a, top).

We proceeded with ligation of a 5'-(Gly-Gly-Gly)-tagged 16-nucleotide (nt) oligo sequence[17] (*ODN1*, Table 1) against a model peptide (*Pep1*, Table 2) containing the cognate ligation handle using recombinant sortase A.[18] The ligation reaction was performed in 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl at 4 °C for 16 h, at a peptide-to-oligonucleotide ratio of 1:5. The ligation product was isolated with reversed-phase (RP)-HPLC (Fig. S1, ESI), and its mass was verified by MALDI-TOF (Table 3). As a negative control, no ligation products were observed when sortase A was excluded from the reaction mixture, nor when the reference oligo (*ODNref*) with no tag was used for the reaction (Table 3). Ligation of a 5'-(Gly-Gly-Gly)-tagged fully phosphorothioate (PS)-modified oligo of the same sequence (*ODN2*, Table 1) with *Pep1* was similarly successful (Table 3 and Fig. S1, ESI), validating the applicability of our approach towards conjugation with therapeutic oligos, which have seen widespread use of PS backbone chemistry[19] for improved nuclease resistance. POC ligation was achieved regardless of the placement of the tag either at the 3'-

Table 1. Representative oligonucleotide sequences used in this study.

Oligo[a]	Sequence (5' → 3')[b],[c],[d]
ODNref	CTATTTGGATGTCAGC
ODN1	/Tag$_{SORT}$/CTATTTGGATGTCAGC
ODN2	/Tag$_{SORT}$/*C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C
ODN3	CTATTTGGATGTCAGC/Tag$_{SORT}$/
ODN4	CTATTTGG/Tag$_{SORT}$/ATGTCAGC
ODN5	/Tag$_{PAL}$/CTATTTGGATGTCAGC
ODN6	/Tag$_{PAL}$/C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C
ODN7	/Tag$_{PAL}$/C*T*A*T*T*T*T*G*G*A*T*G*T*C*A*G*C
ODN8	CTATTTGGATGTCAGC/Tag$_{PAL}$/
ODN9	CTATTTGG/Tag$_{PAL}$/ATGTCAGC

[a] *ODN1–4* are labelled with (Gly-Gly-Gly) tag, while *ODN5–9* are labelled with (Gly-Leu) tag. [b] Ligation tags for sortase A (Gly-Gly-Gly) and *Oa*AEP1b (Gly-Leu) are represented as /Tag$_{SORT}$/ and /Tag$_{PAL}$/, respectively. [c] Phosphorothioate-modified inter-nucleotide linkages are marked with asterisks (*). [d] LNA nucleotides are underlined.

Table 2. Peptide sequences used in this study.

Peptide	Sequence (N → C)[a],[b]
Pep1	KALVILPETGLE
Pep2	H(A$_{ib}$)EGTFTSDVSSYLEGQAAKEFIAWLVKGRGLPETGLE
Pep3	KALVINGL
Pep4	H(A$_{ib}$)EGTFTSDVSSYLEGQAAKEFIAWLVKGRGNGL

[a] Cognate ligase sequence motifs of the ligation handles are underlined. [b] 2-aminoisobutyric acid substitution is represented as (A$_{ib}$).

Table 3. Ligation reaction conditions and POC characterization by MALDI-TOF mass spectrometry. Unless otherwise specified, all reactions were performed in 20 µL reaction mixture with peptide concentration of 200 µM and 5 eq. (for sortase) or 2.5 eq. (for *Oa*AEP1b) of oligonucleotide. Yield was estimated based on area of HPLC peak monitored at 260 nm.

Oligo	Peptide	Cond.[a]	Ligase	Mass (exp./det.)	Est. Yield
ODNref	*Pep1*	I	1 eq.	N.A.	N.A.
ODN1	*Pep1*	I	–	N.A.	N.A.
ODN1	*Pep1*	I	1 eq.	6190.6/6190.1	79%
ODN2	*Pep1*	I	1 eq.	6447.5/6447.8	100%
ODN3	*Pep1*	I	1 eq.	6190.6/6192.1	14%
ODN4	*Pep1*	I	1 eq.	6190.6/6190.7	49%
ODN2	*Pep2*	II	3 eq.	9274.5/9276.9	23%
ODNref	*Pep3*	III	0.01 eq.	N.A.	N.A.
ODN5	*Pep3*	III	–	N.A.	N.A.
ODN5	*Pep3*	III	0.01 eq.	5875.3/5874.2	93%
ODN6	*Pep3*	III	0.01 eq.	6116.2/6113.0	52%
ODN7	*Pep3*	IV	0.1 eq.	6310.2/6311.3	100%
ODN8	*Pep3*	III	0.01 eq.	5875.3/5873.8	94%
ODN9	*Pep3*	III	0.01 eq.	5875.3/5873.4	52%
ODN5	*Pep4*	V	0.1 eq.	8702.3/8701.9	43%
ODN6	*Pep4*	VI	0.3 eq.	8944.2/8947.3	17%

[a] Condition I: 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 4 °C, 16 h, 1 eq. *Sort-7M*. Condition II: 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 4 °C, 16 h, 3 eq. *Sort-7M*. Condition III: 20 mM KPi (pH 7), 37 °C, 30 min; Condition IV: 20 mM KPi (pH 7), 37 °C, 1.5 h, 0.1 eq. *Oa*AEP1b; Condition V: 20 mM KPi (pH 7), 37 °C, 16 h, 0.1 eq. *Oa*AEP1b; Condition VI: 20 mM KPi (pH 9), 37 °C, 16 h, 0.3 eq *Oa*AEP1b, peptide-to-oligonucleotide ratio of 1:5.

end (*ODN3*) or at an internal position (*ODN4*) of the oligo (Table 3 and Fig. S1, ESI). Furthermore, *ODN2* was also successfully ligated onto a 38-amino-acid (aa) long glucagon-like peptide 1 (GLP1) variant[20] containing the sorting motif on its C-terminus (*Pep2*, Table 2; Table 3 and Fig. S1, ESI).

We proceeded next to POC ligation using PAL, a family of asparaginyl-specific ligases found in cyclotide-producing plants, among which include butelase 1[9] and *Oa*AEP1b.[15] Native substrates of *Oa*AEP1b comprise the peptide sequences GL and NGL at the N- and C-terminus, respectively. In this case, we synthesized a phosphoramidite tag (**2**) containing the (Gly-Leu) ligation motif (Fig. 1b, blue), which is fully compatible with SPOS, starting from a hydroxyprolinol (**S4**) scaffold (Scheme S2, ESI). Native and PS-/locked nucleic acid (LNA)-modified version of *ODNref* 5'-tagged with (Gly-Leu) (*ODN5*, *ODN6*, and ODN7, respectively, Table 1) were all successfully conjugated onto a model peptide (*Pep3*, Table 2) containing the cognate ligation handle using recombinant *Oa*AEP1b in 20 mM potassium phosphate buffer (pH 7), at a peptide-to-oligonucleotide ratio of 1:2.5 (Table 3 and Fig. S2, ESI). The ligation products were obtained after only 30 min of incubation with *Oa*AEP1b, thanks to high efficiency of the PAL family. Ligation of *Pep3* with various oligo constructs, either consisting of different placement of the ligation tag (*ODN8* and *ODN9*) or comprising additional modification (*ODN S8* with a disulphide modifier; Table S1, ESI), was similarly successful. Lastly, ligation of a 34-aa GLP1 variant containing the ligation handle on its C-terminus (*Pep4*, Table 2) with *ODN5* and *ODN6* were also successful (Table 3 and Fig. S2, ESI) after incubation with the ligase for 16 h.

We next investigated application of the tag-based approach towards ligation of a protein with oligo. To that end, a cyan fluorescent protein (CFP) variant was engineered to harbour an LPETG (for sortase A; CFP_{SORT}) or NGL (for *Oa*AEP1b; CFP_{PAL}) ligation handle at the C-terminus (Fig. 2a). Both proteins were ligated against a panel of different oligos with sortase A and *Oa*AEP1b accordingly. For sortase A, we observed successful CFP ligation for all constructs in general (Fig. 2b and Fig. S3, ESI), with *ODN1*, *ODN2*, and a poly-dT oligo (*ODN S1*, Table S1, ESI) giving particularly high yields. For *Oa*AEP1b, we also observed successful CFP ligation for the tested constructs in general (Fig. S4, ESI), even with just 1 h of incubation time. Successful validation here showed that the approach should be readily adaptable to the ligation of oligos with other protein counterparts.

Chemical conjugation has been the prevailing approach towards development of POCs, from design of compatible in-line peptide and oligo synthesis, to functionalization on the peptide and oligo fragments[21] for post-synthetic conjugation. However, due to diverse functionalities present in peptides/proteins and oligos, these methods pose considerable difficulties and limitations, including the need for stabilizing ligands,[8] extensive modifications,[22] and harsh reaction conditions. In many cases, particularly for protein, functional groups can only be introduced after synthesis/expression, thus necessitating additional steps prior to conjugation. The current work brings forth an enzymatic dimension to POC generation by leveraging the high efficiency and specificity of enzymatic ligation, which is traceless and can

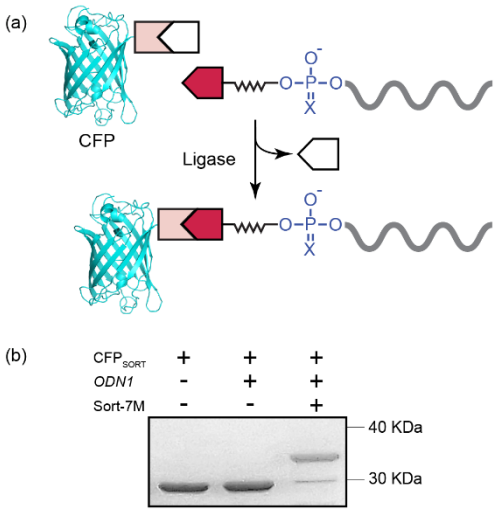

Fig. 2 (a) Schematic illustration of ligase-assisted ligation between a tag-labelled oligonucleotide and a protein containing the cognate ligation handle. b) SDS-polyacrylamide gel electrophoresis of the crude ligation mixture for sortase-assisted ligation of *ODN1* with CFP_{SORT}. CFP_{SORT} was ligated with *ODN1* to produce the POC in the presence of the cognate enzyme sortase.

be performed in mild aqueous condition compatible with most peptides and proteins. By implementing an in-line incorporation of ligation motif onto oligo through the design of custom phosphoramidite tags, simple incubation of the oligo and peptide/protein that contain matching ligation handles with the cognate ligase led to the desired POCs with minimal side products. No additional activation or preparative steps were needed aside from standard SP synthesis and purification of the peptide and oligo counterparts. Orthogonal nature of our approach further allows it to be combined with existing chemical methods.

Despite broad adoption of ligases in protein engineering, reports on their adaptation to POC generation were few and far between. Koussa *et al.* reported on construction of DNA-protein hybrids using sortase,[11] while Harmand *et al.* combined sortase A and butelase 1 to generate a C-to-C fusion protein through DNA linkers.[12] We note that in both cases, protein conjugation was localized to the 5'-end of the oligo, and that intermediate steps were required in order to prepare the oligo for ligation. In the current work, the phosphoramidite tag was directly coupled onto the oligo chain during automated SP synthesis and ready for ligation after standard cleavage, deprotection, and purification protocols. Furthermore, we showed that successful POCs were generated regardless of the point of tag incorporation on the oligo, be it at the 5'-/3'-end or at an internal position. Versatility of the approach was demonstrated through diverse nature of the oligo constructs employed (Table S1, ESI), ranging from fully backbone-modified to the presence of additional modifier (e.g. disulphide modifier: *ODN S8*) and adoption of non-canonical structural forms (e.g. G-quadruplex-forming motif: *ODN S3*/*ODN S4*/*ODN S9*). In principle, multiple copies of the same phosphoramidite tag can be coupled either in tandem or at discrete loci on an oligo for conjugation with multiple copies of the same peptide/protein (Fig. S5, ESI). Different phosphoramidite tags can also be combined to bring about conjugation

of two or more distinct peptide(s)/protein(s) onto a single oligo in an addressable manner (Fig. 1b and Fig. S5, ESI).

In recent years, there have been mounting interests in the conjugation of RNA therapeutics with peptides and proteins[23] to achieve targeted delivery or enhancement in efficacy. Here we showed that a GLP1 variant can be ligated with PS oligos at high yields with minimal steps, and also successfully applied the strategy in the context of a bigger protein component.

In conclusion, we have shown the use of two separate ligases for POC generation through the development of phosphoramidite tags tailored for each ligase. Diverse oligo and peptide/protein constructs were successfully ligated with high efficiency. The ligation approach provides a straightforward path towards the streamlined development and production of POCs.

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A patent on the technology has been filed by Nanyang Technological University with the authors as inventors.

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