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Sortase-promoted synthesis of homooligomers from a monomeric protein

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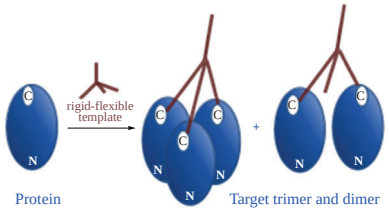
We demonstrated covalent cross-linking of two or three protein molecules specifically at the C-terminus by sortase-catalyzed conjugation of recombinant proteins with conformationally rigid-flexible tetraantennary templates, where structure of the antenna is [Gly-Gly-(*N*-CH_2COOH)Gly]$_n$$CH_2$–.



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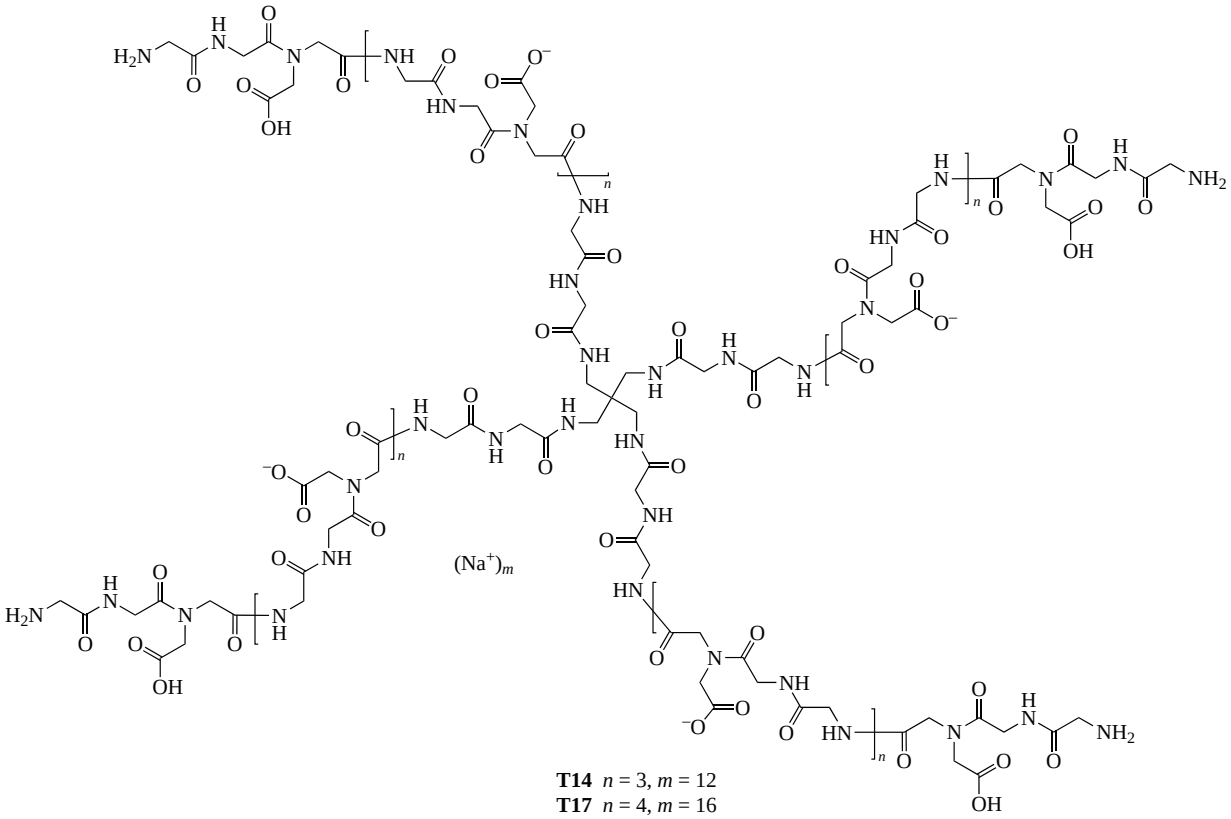
Many proteins, *e.g.*, immunoglobulins, lectins, viral receptor-recognizing proteins, function as di-, and tri- (or even more) homooligomers. The assembly of the quaternary structure does not always occur spontaneously in solution; for example, galectin-3 becomes an oligomer only in the course of binding to glycans.[1] A number of plasma membrane proteins dynamically oligomerize due to the assistance of the membrane. Notably, modern therapeutic strategies use recombinant monomers which are not inherent in the property to bind in pairs and triplets; they have to be oligomerized 'forcibly' by covalent linking directly or with the help of a template that can non-covalently (but firmly) bind two or three copies of a monomeric protein.[2] These phenomena would suggest the need to have tools for the routine assembly of homooligomeric proteins in such a way that their functional integrity should be obligatory preserved. This paper describes a positive attempt to solve this problem using sortase (calcium-independent sortase A), a specific trans-peptidase[3] capable of transferring a protein to an oligoglycine substrate, a substrate organized as a tetraantennary symmetrical molecule, generally speaking, capable of sortase-promoted attachment of



T14 $n = 3$, $m = 12$
T17 $n = 4$, $m = 16$

one to four protein copies. The recombinant protein to be attached by sortase is designed in such a way that it has a peptide region –LPETG–, which is specifically recognized by this enzyme.[4] It is obvious that the goal requires the solution of two specific tasks. First, to provide a distance between the ends of the antennas that allows the attachment of two or more rather large protein objects to a relatively small molecule, as well as such an oligomer architecture in which proteins-monomers can function as efficiently as in a natural di- or trimer. In this regard, we used two tetra-antennary templates of different sizes, **T14** and **T17**. Second, to find conditions for sortase conjugation that would allow selective synthesis of either di- or trimer. In this work, we have been solving only the first of the tasks.

The synthesis of tetraantennary templates **T14** and **T17** was described earlier.[5] They are attractive because, due to several negatively charged *N*-carboxymethyl substituents of the oligoglycine chain, each antenna tends to adopt an unfolded conformation, and, in addition, to distance itself from the other three. These templates have the necessary balance of flexibility and rigidity, which is confirmed by molecular dynamics simulation (the data will be published elsewhere). The presence of two glycine residues at the end of the antenna makes it a substrate for sortase, a significantly better substrate than the average proteinic amino group. Templates **T14** and **T17** were conjugated in molar ratios of 1 : 1 and 10 : 1 to recombinant protein A (PrtA),[6] whose polypeptide end contained a sortase-recognized pentapeptide (Figure 1). This small (15.8 kDa) protein is attractive since it is able to bind immunoglobulin G, which opens up the possibility of further constructing of clustered (*i.e.*, with elevated avidity) IgG.

Sortase conjugation products were not isolated, but analyzed by polyacrylamide gel electrophoresis (see Figure 1). The reaction products masses, as well as the mobility of the initial PrtA and SrtA (Sortases A have low activity and are not taken in the usual ‘catalytic amounts’, therefore SrtA is clearly visible on the foregram of the reaction mixture) were estimated due to reference proteins. Proteomic analysis of bands A, B and C from tracks 2 and 4 showed the presence of protein A in all of them.

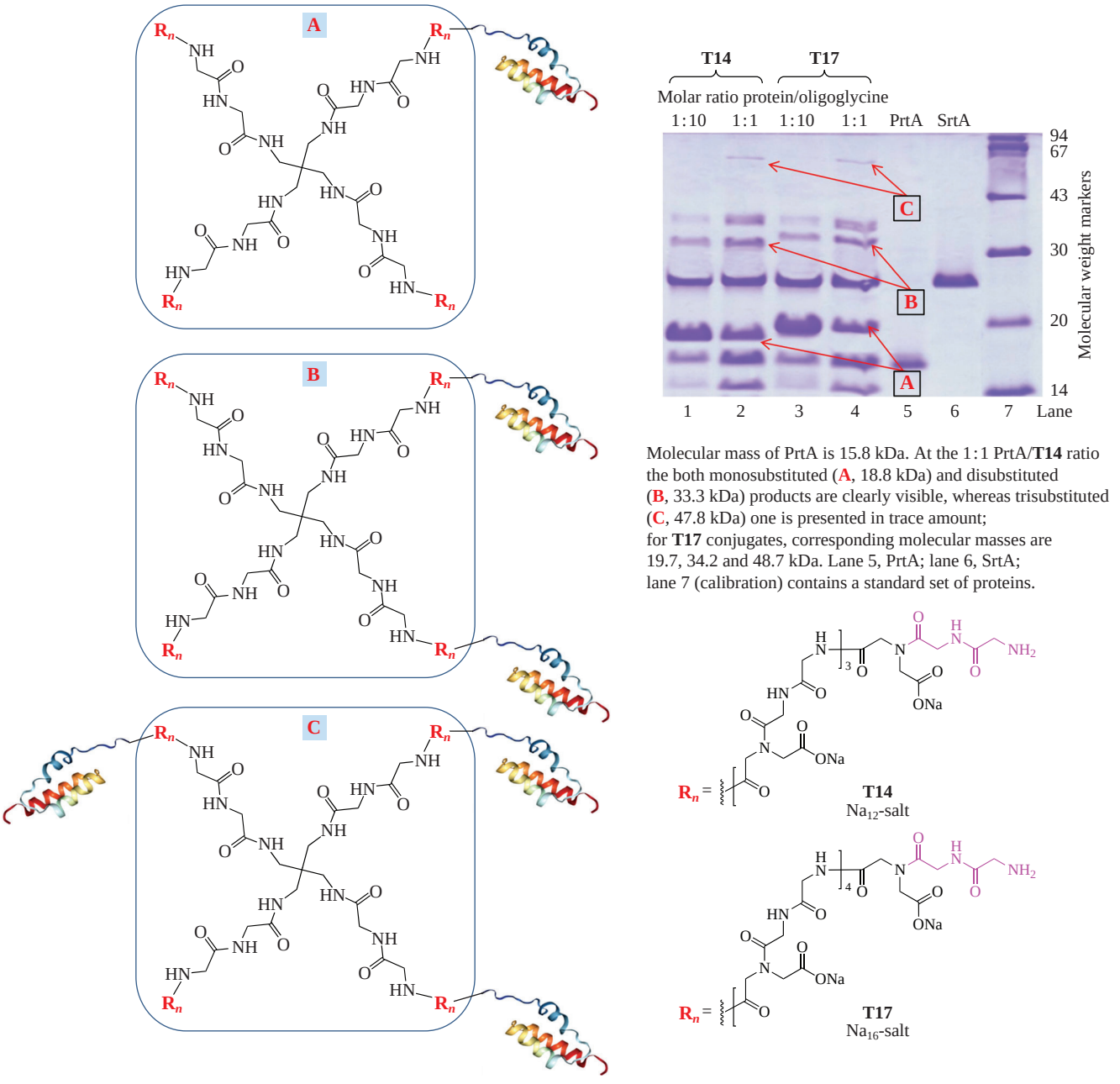


Figure 1 *Left:* Schematic representation of conjugates of **T14** and **T17** 1 : 1 (A), 1 : 2 (B) and 1 : 3 (C) with Protein A (PrtA, its structure shown schematically). *Right:* SDS gel electrophoresis data of reaction mixture for sortase A (SrtA)-catalyzed reaction of PrtA with the templates. Red arrows on the foregram, according to their position and MS data, correspond to the 1 : 1 (A), 1 : 2 (B) and 1 : 3 (C) conjugates.

Densitometry of the bands in lane 2 of the foregram (that is, at an equimolar ratio of **T14** to PrtA; here we refer to the mole of the entire molecule, not just one of its antennas) indicates that under the selected conditions, about half of the initial PrtA undergoes the conversion, and that the ratio of the resulting 1 : 1, 1 : 2 and 1 : 3 conjugates turns out to be 72 : 24 : 4. The 18.8 kDa mass of the 1:1 conjugate corresponds to the sum of the masses of PrtA and the template ($C_{791}H_{1224}N_{236}O_{293}S$, M_w = 18.760 kDa). The 33.3 kDa band corresponds to the 1 : 2 conjugate composition ($C_{1433}H_{2232}N_{412}O_{498}S_2$, M_w = 33.264 kDa), and the 47.8 kDa minor band corresponds to the conjugate in which the protein is attached to three ($C_{2075}H_{3240}N_{588}O_{703}S_3$, M_w = 47.768 kDa) of the four template antennas; the latter is clearly visible only in the case of a 1 : 1 ratio of the initial components. Note the absence of a noticeable difference in the reactivity of the **T14** and **T17** molecules (*cf*. lanes 2 *vs.* 4). With a 10-fold excess of the template relative to PrtA, the 1 : 1 conjugate dominates in the reaction mixture (lane 1, see Figure 1) both in relation to other reaction products and in relation to the starting PrtA. Similarly, in the case of **T17** we have 19.7 kDa mass of the 1 : 1 conjugate ($C_{823}H_{1268}N_{248}O_{313}S$, M_w = 19.676 kDa). The 34.2 kDa band corresponds to the 1 : 2 conjugate composition ($C_{1465}H_{2276}N_{424}O_{518}S_2$, M_w = 34.180 kDa), and the 48.7 kDa minor band corresponds to the conjugate in which the protein is attached to three ($C_{2107}H_{3285}N_{600}O_{723}S_3$, M_w = 48.685 kDa) of the four template antennas. Besides, proteomic MS analysis proves the presence of PrtA in the corresponding bands.

In conclusion, the sortase-catalyzed reaction between the tetraantennary templates **T14** and **T17** and the protein leads to mono-, di-, and tri-substitution products, and the control of the component ratio makes it possible to improve the yield of the disubstituted conjugate. Note that sub-optimal diglycine fragments are located at the end of the antenna, while tri-, tetra-, or pentaglycine analogs are known to be much better sortase substrates; we also note that the used enzyme is not the most active of the known sortases,[3] so the proposed route of enzymatic synthesis is not closed to shifting the reaction towards trisubstituted conjugates. At the same time, it is hardly possible to obtain a tetrasubstituted variant in this way, even when conjugating such small substrates as PrtA; however, we do not exclude the possibility of attaching a low molecular weight ligand to the fourth antenna. And this opens the way to the synthesis, in particular, of molecules of the [protein]$_3$-T14-lipid type, capable of inserting into the cell membrane.[7]

This article does not contain any research involving humans and animals as research objects. The authors declare they have no conflicts of interest.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.09.011.

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