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SPECIAL ISSUE

Blood Group O→A Transformation by Chemical Ligation of Erythrocytes

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Agglutination of red blood cells (RBCs) remains the only practical method for routine use for ABH typing in clinical practice. However, exact mechanistic details of agglutination are not yet thoroughly studied. In this research, RBCs of blood group O were converted to blood group A through two approaches: by chemical ligation of the cells' glycocalyx with synthetic blood group A tetrasaccharide, and by insertion of synthetic glycolipid carrying the same A antigen into the cells' membranes. The O→A ligated RBCs and natural A RBCs showed comparable agglutination characteristics with antibodies. As expected, RBCs with inserted glycolipid showed lower agglutination scores. This approach could help cell biologists in site-specific and cell-friendly modification of glycocalyx by other ligands.

ABH system allo-antibodies develop in all populations in infancy and persist throughout life. Binding of these ubiquitous antibodies to red blood cells (RBCs) produces severe clinical sequelae and predetermines the outcomes of blood transfusion and organ transplantation.[1] Despite advances in molecular techniques[2] and immunoassays,[3] agglutination of human RBCs remains the only reliable and practical method for ABH typing for daily use in immunohaematology. However, the precise mechanisms of agglutination (and more generally, interactions between RBCs and antibodies) are still poorly understood. Reasons for this include the heterogeneity of A and B antigens on human RBCs and immunological presentation (accessibility) of antigens to antibodies.[4] In this work, we modified blood group O RBCs (i.e., free of A or B antigens) with A-ONSu, a synthetic tetrasaccharide identical to the natural A antigen, that bears a short linker including an *N*-hydroxysuccinimide (NHS) ester group (Scheme 1).

We selected the modification chemistry for three reasons. Firstly, it was expected that chemical ligation with A-ONSu would lead to local modification of the periphery of the red cell glycocalyx. For this, the high hydrolysis rates of NHS esters are advantageous because the material that diffuses deeper into the glycocalyx is hydrolysed during the reaction. Secondly, we can conduct the reaction under physiological conditions favourable to living cells. Thirdly, our method is a simple one-step modification. In contrast, currently fashionable methods such as natural chemical ligation[5] or click chemistry with the chemical or metabolic introduction of a "handle" into the cell[6] are multistage approaches that are more likely to interfere with the physiology of the living cell. Such handles are alien hydrophobic motifs that cause nonspecific interactions.[7] Our method adds nothing unnatural to the cell except the propyl spacer. The protein amino groups that already exist naturally provide sufficient reactivity for modification.

A-ONSu was synthesised from compound **1**, the 3-aminopropyl glycoside of blood group A (type 2) tetrasaccharide GalNAcα1–3(Fucα1–2)Galβ1–4GlcNAcβO$(CH_2)_3NH_2$,[8] by treatment with adipic acid bis-(*N*-oxysuccinimide ester) (**2**) in 86% yield. Synthetic glycolipid A-Ad-DE was obtained by conjugation of **1** with DE-Ad-ONSu (**3**)[9] in 97% yield (Scheme 1, for details and spectral data see the Supporting Information). The stoichiometry of ligation was designed in such a way that the amount of inserted tetrasaccharide corresponds with the natural content of A antigens in blood group A_1 RBCs, in which 1 mL of blood contains 5×10^9 RBCs and each cell contains $\approx5\times10^6$ epitopes.[4] To match the natural content, we took 10^{-7} mol of tetrasaccharide A-ONSu, anticipating that the reaction yield would be 25% (according to the evaluation of A-ONSu hydrolysis under the same conditions but without RBCs). A-ONSu was suspended in 0.9% buffered saline at pH 7.5, this pH representing a compromise between favourable amidation conditions at elevated pH values and accelerated hydrolysis of the activated ester at such a pH.

We further modified O RBCs with synthetic function-spacer-lipid (FSL) A-Ad-DE to produce "kodecytes".[9] A-Ad-DE is an A tetrasaccharide with a dioleoylphosphatidylethanolamine tail, which inserts into the RBC phospholipid membrane through hydrophobic interaction and contains a short adipate linker to space the antigen from the membrane in similar manner to typical natural glycolipids (Figure 1C): that is, in proximity to the membrane.[10,11]

Both cell types (A-Ad-DE kodecytes and A-ONSu cells), together with natural A_1 cells, were studied in agglutination assays with anti-A (immunoglobulin M, bioCSL, Melbourne, Australia), diluted in a doubling dilution series.

Agglutination of RBCs with anti-A reagent demonstrated comparable agglutination of A-ONSu-ligated RBCs and natural

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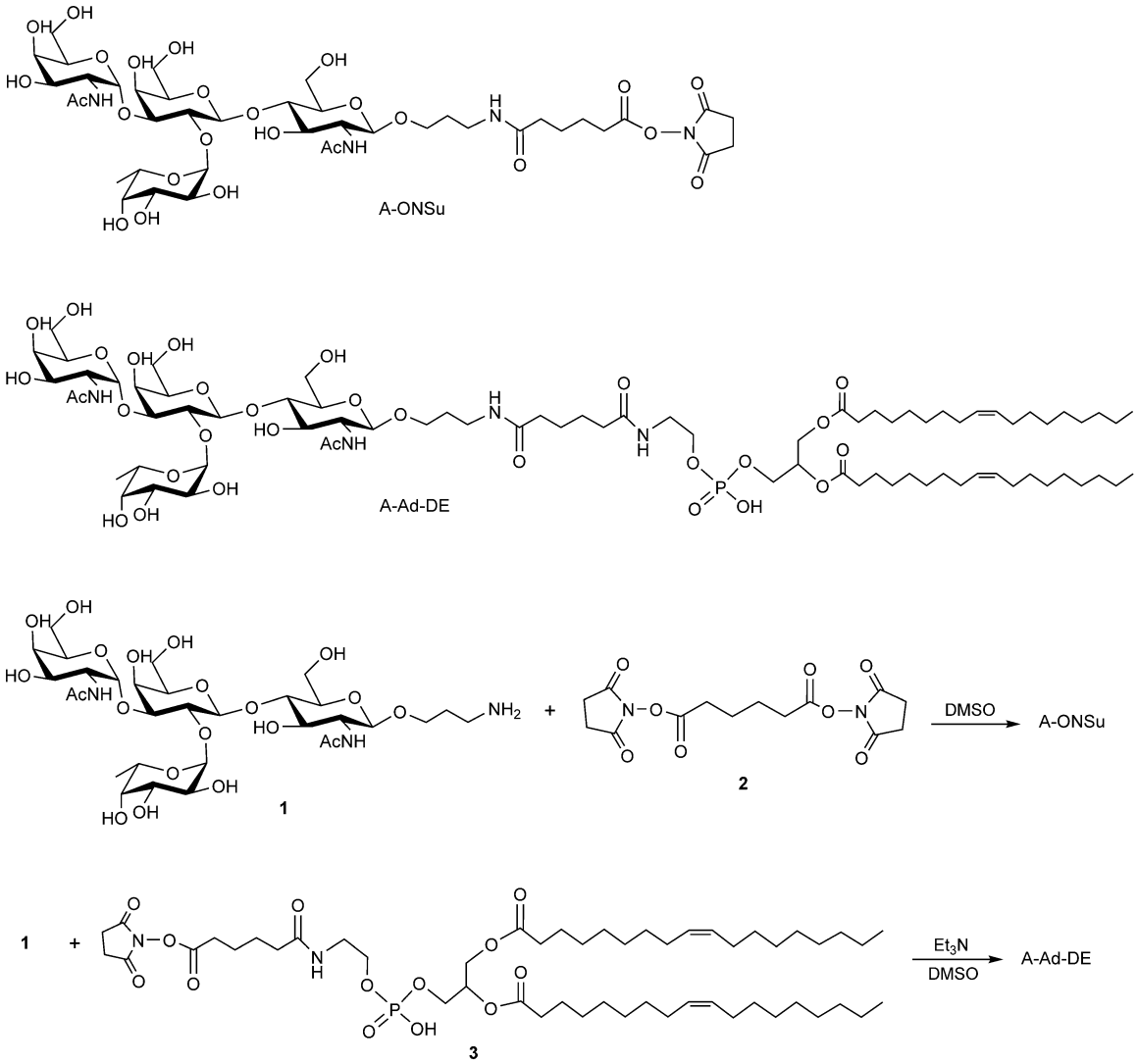

Scheme 1. Structure of bioconjugates used in this work and scheme of syntheses. DE: dioleoylphosphatidylethanolamine. Ad: adipoyl.

blood group A_1 cells (Figure 1 A). (Note that one dilution difference in an agglutination series with doubling dilutions of antibody is not considered significant). These results confirm that the density and presentation of A antigens in A-ONSu-ligated RBCs are close to those of natural A_1 RBCs, and the conditions for chemical ligation were selected appropriately.

Agglutination of A-Ad-DE kodecytes was only seen with lower dilutions of anti-A. This is not unexpected, because these kodecytes are deliberately created to have fewer copies of the A antigen than natural A_1 RBCs.[10,11]

Agglutination of natural A_1 RBCs and of RBCs with inserted A-Ad-DE (kodecytes) was enhanced when these cells were pre-treated with papain protease. In the case of the kodecytes, this effect was much more pronounced (Figure 1 B). Both in natural A_1 RBCs and in kodecytes, the enhancement is attributable to partial degradation of the glycocalyx, thereby reducing steric hindrance and easing access of anti-A antibodies to the A glycolipids. Because A-Ad-DE labels the RBC at the lower "storey" of the glycocalyx, reduction in steric hindrance produces a high degree of enhancement. In natural cells, the glycans are distributed throughout the glycocalyx, so the enhancement of A antigen expression due to reduction in steric hindrance is not as great. Because A antigens on natural cells are carried on both glycolipid and glycoprotein chains, papain partially degrades the A antigen on glycoproteins, so there is also some loss of A antigen in papain-treated natural A_1 RBCs. In contrast to the enhanced agglutination observed with natural A_1 RBCs and kodecytes, A-ONSu cells produced the same amount of agglutination when cells were tested with and without pre-treatment with papain. This confirms that A-ONSu is labelling the periphery of the glycocalyx, where steric hindrance is not a factor. We confirmed that A-ONSu labelling does not interfere with papain degradation of the glycocalyx by testing ABO-independent blood group antigens known to be susceptible to papain reduction (results not shown).

In contrast with the natural variability of human A cells,[4] both chemically ligated and koded A cells add known quantities of A antigen to natural O cells. Therefore, technique

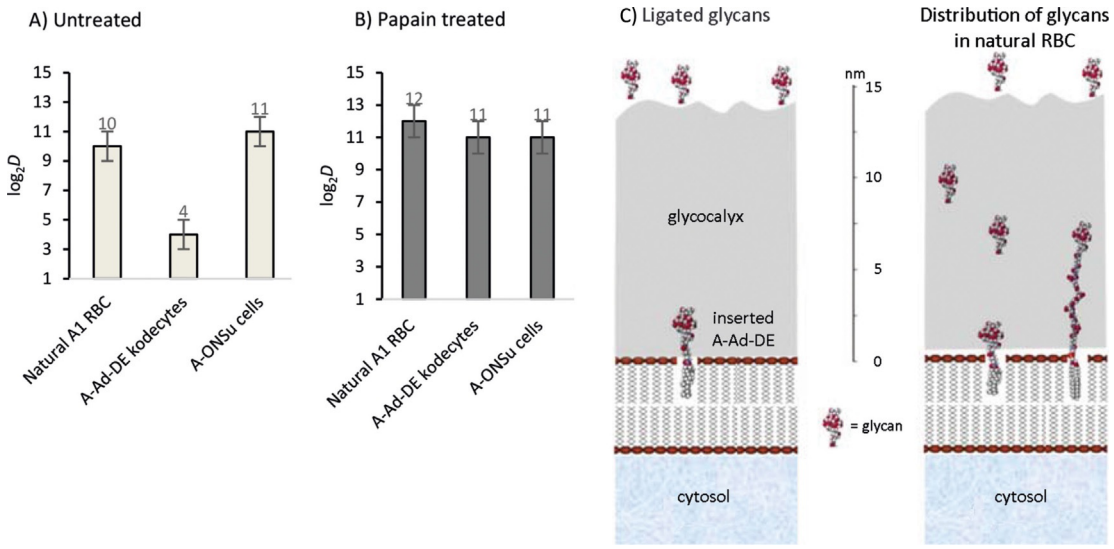

Figure 1. A), B) Titre steps showing agglutination in a doubling-dilution series, with chemically ligated O RBCs (A-ONSu), O RBCs with inserted glycolipid A-Ad-DE, and natural A_1 RBCs in A) untreated, and B) papain-treated states (D: dilution). Commercially available immunoglobulin preparation (bioCSL) was used as a source of anti-A immunoglobulin M. C) Dispositions of A glycans in glycocalyx of erythrocytes. Left: chemically (with A-ONSu) ligated in the peripheral glycocalyx and predominantly modified at the membrane surface with A-Ad-DE. Right: natural A RBCs.

control and inter-assay reproducibility are improved. Synthetic lipids containing different ligands (glycans, peptides, dyes, biotin, etc.) can be inserted into a variety of cells.[9,12] The methodology described here, consisting of chemical ligation and lipid insertion at different "storeys" of a cell covering (in this case the RBC glycocalyx), could help resolve numerous tasks of cell biology by using the methods of chemical biology.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: ABH antigens · carbohydrates · erythrocytes · glycocalyx · glycolipids

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