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Novel GH109 enzymes for bioconversion of group A red blood cells to the universal donor group O

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ABSTRACT

Glycoside hydrolases (GHs) have been employed for industrial and biotechnological purposes and often play an important role in new applications. The red blood cell (RBC) antigen system depends on the composition of oligosaccharides on the surface of erythrocytes, thus defining the ABO blood type classification. Incorrect blood transfusions may lead to fatal consequences, making the availability of the correct blood group critical. In this regard, it has been demonstrated that some GHs may be helpful in the conversion of groups A and B blood types to produce group O universal donor blood. GHs belonging to the GH109 family are of particular interest for this application due to their ability to convert blood from group A to group O. This work describes the biochemical characterisation of three novel GH109 enzymes (NAg68, NAg69 and NAg71) and the exploration of their ability to produce enzymatically converted RBCs (ECO-RBC). The three enzymes showed superior specificity on pNP-α-*N*-acetylgalactosamine compared to previously reported GH109 enzymes. These novel enzymes were able to act on purified antigen-A trisaccharides and produce ECO-RBC from human donor blood. NAg71 converted type A RBC to group O with increased efficiency in the presence of dextran compared to a commercially available GH109, previously used for this application.

Introduction

The worldwide demand for human blood for saving lives is constantly increasing. Over the years, several attempts have been made to stabilise the blood supply [1]. A critical shortage of blood availability for transfusion exists, especially in low- and middle-income countries, representing 85 % of the global population [2]. In a study using World Health Organization and Global Burden of Disease data, the total global blood supply was estimated to be around 272 million units in 2017, whereas the total global demand was approximately 303 million units, a shortfall of around 30 million units [3]. In addition, the COVID-19 pandemic has not spared this field either. As a result of the health emergency, a decrease in donation intentions has been observed, and strategies to remedy the resulting blood shortage are being implemented [4,5].

The discovery of enzymes capable of performing the conversion of

Abbreviations: CAZy, carbohydrate active enzymes database; ECO-RBC, enzymatic converted red blood cells; ET-RBC, enzymatic treated RBCs; FACS, fluorescence activated cell sorting; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GH, glycoside hydrolase; GlcNAc, *N*-acetylglucosamine; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detector; pNP, *p*-nitrophenol; TrBgA, blood group A trisaccharide; RBC, red blood cells; ID-card, test for typing ABO group, specifically made for the Bio-Rad HI-500 system; FITC-A, Fluorescein isothiocyanate; APC, allophycocyanin.

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blood groups has given an impetus to developing a universal blood production technology [6]. Enzymatically converted RBC (ECO-RBC) O-type from A-type and B-type is considered a valuable alternative to overcoming the low-blood supply issue. In the case of limited supplies, emergencies, and situations in which the subject's RBCs group is not known, an adequate supply of universal donor O-type (or H-type) blood is indispensable, being compatible with all blood groups [7]. Recently, in the field of organ transplantation, a strategy has been proposed using enzymes capable of modifying blood group antigens for converting A-antigens to H-antigens from donor's lungs during *ex vivo* lung perfusion [8]. Improving this enzymatic bioconversion would bring considerable benefits in the clinical field, improve transfusion safety and increase the overall blood supply [9,10].

The most common blood group classifications are the ABO and Rh systems. The ABO classification system for RBCs, is based on the presence or absence of specific antigens on the RBC surface, represented by oligosaccharides linked to the cell membrane glycolipids and glycoproteins [11]. The antigenic determinant base structure is a fucosyl galactose (Fuc-α-1,2-β-Gal) moiety bound to a *N*-acetylglucosamine (GlcNAc) which represents the antigenic core structure and characterises the O-type blood group. The addition of terminal monosaccharides forms the A-type and B-type antigens, in which the immunodominant sugar moiety is α-1,3-linked *N*-acetylgalactosamine (GalNAc) for group A and an α-1,3-linked galactose (Gal) for group B (Fig. 1) [12]. In addition, blood group A can be subdivided in blood subtypes A_1 and A_2 based on the density of A-antigens on the RBCs [13]. This difference is due to the weakest activity in the A_2 subgroup of the glycosyl transferase enzyme that adds the GalNAc to the H-antigens. The different A subgroups do not influence the immunogenic recognition of group A, however, patients with type A_2 present a lower number of H-antigen modified compared with type A_1 [10,14].

The conversion of A-type or B-type antigen to O-type via enzymatic cleavage, does not affect the Rh blood group system, which is defined by the presence or absence of the RBC surface protein RhD antigen, resulting in Rh positive (Rh^+) and Rh negative (Rh^-) respectively [15]. Accordingly, the enzymatic conversion of RBC group A or B Rh^+ would produce RBC group O Rh^+; conversely, RBCs A or B Rh^- would produce only O-type RBC Rh^- [10].

Certain glycoside hydrolases (GH) have been utilised to remove the saccharide antigen from blood groups A and B to obtain blood group O [16]. These ECO-RBCs have already been used in human transfusions and shown to be safe and efficacious [17–19]. The specific GHs able to convert RBC of group A to group O, have been identified in the family GH109 of the Carbohydrate Active enZymes classification (CAZy database, www.cazy.org) [20]. Those from GH109 do not follow the classical Koshland double displacement mechanism, but, like family GH4, they catalyse hydrolysis through an atypical NAD^+ dependent reaction mechanism. The NAD^+ cofactor is tightly bound to the enzyme, and a histidine in a conserved and flexible G-G-H-G-G motif has been identified as the catalytic acid-base conferring to the enzymes the ability to have both α-*retaining* and β-*inverting* activities [21,22].

The *N*-acetylgalactosaminidase activity, which is the only activity reported for the GH109 family, is responsible for the conversion of group A RBCs to group O. In the first study describing the GH109 family, a GH109 α-*N*-acetylgalactosaminidase and a GH110 α-galactosidase were found to be capable of removing the immunodominant monosaccharides on A-type and B-type, respectively [22]. Although GH110 converts the group B RBCs efficiently, the conversion of the A group by the GH109 required significant amounts of enzyme and particular buffer conditions (Fig. 2a) [21]. To date, the approach describing the lowest enzymatic loading to convert group A to group O is based on the use of two different enzymatic activities. A two-step bioconversion of GalNAc A-type antigen was developed in which a GalNAc deacetylase removed the *N*-acetyl group from GalNAc, followed by an α-galactosaminidase catalysed hydrolysis of the remaining galactosamine (Fig. 2b) [23].

As of August 2023, the CAZy database has 2169 entries for the GH109 family of which only eight members have been biochemically characterised. Among the latter, four have been tested using RBCs and have successfully produced ECO-RBC [21,24–26]. Moreover, another GH109 enzyme was active on a synthetic trisaccharide of blood group A [27]. New enzymes that catalyse the conversion of group A to group O by an efficient single step are urgently needed to lower the costs and develop a universal blood production method, to meet the demand for worldwide blood supplies. In this work three novel GHs of family GH109 were characterised and their ability was tested to bio-convert the type-A antigen on isolated oligosaccharides and on purified A-type RBC.

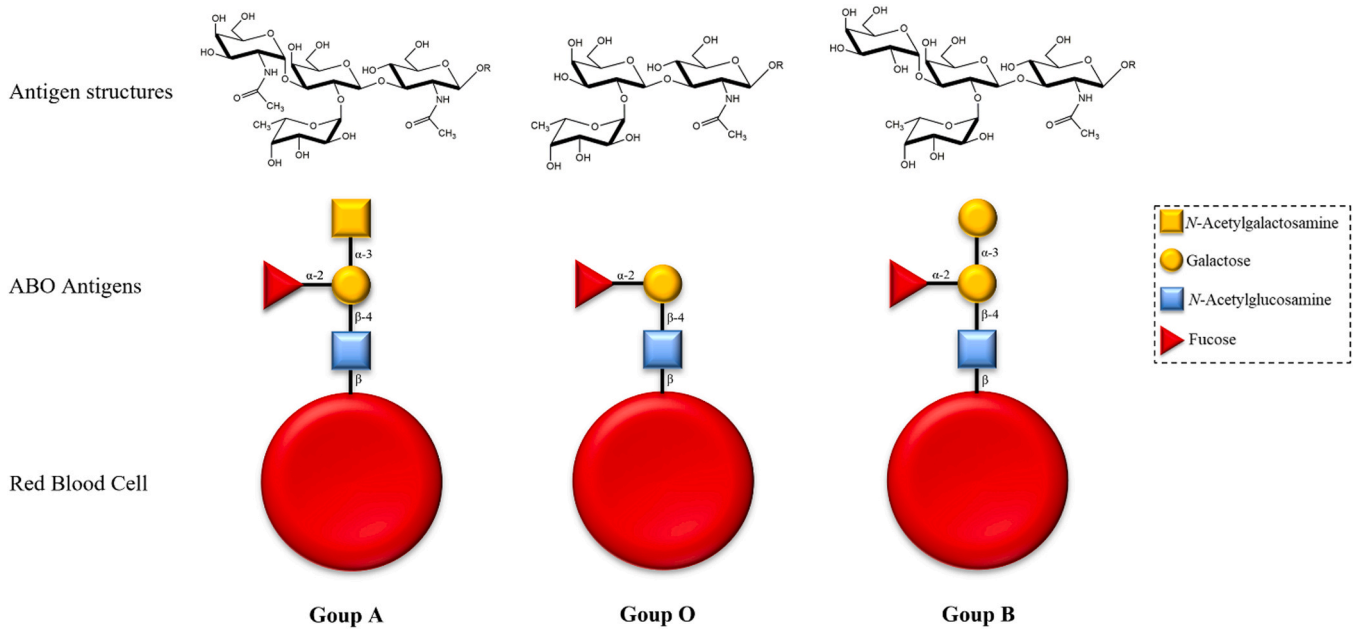

Fig. 1. Schematic representation of basic A-, H-, and B-antigens on the surface of Type A, O, and B Red Blood Cells. The fucosyl galactose H-antigens linked to a *N*-acetylglucosamine define the base structure of O-type blood. While the A and B-antigens are defined through the addition to the H-antigens of an α-linked *N*-acetylgalactosamine for A-type blood and an α-linked galactose for B-type blood.

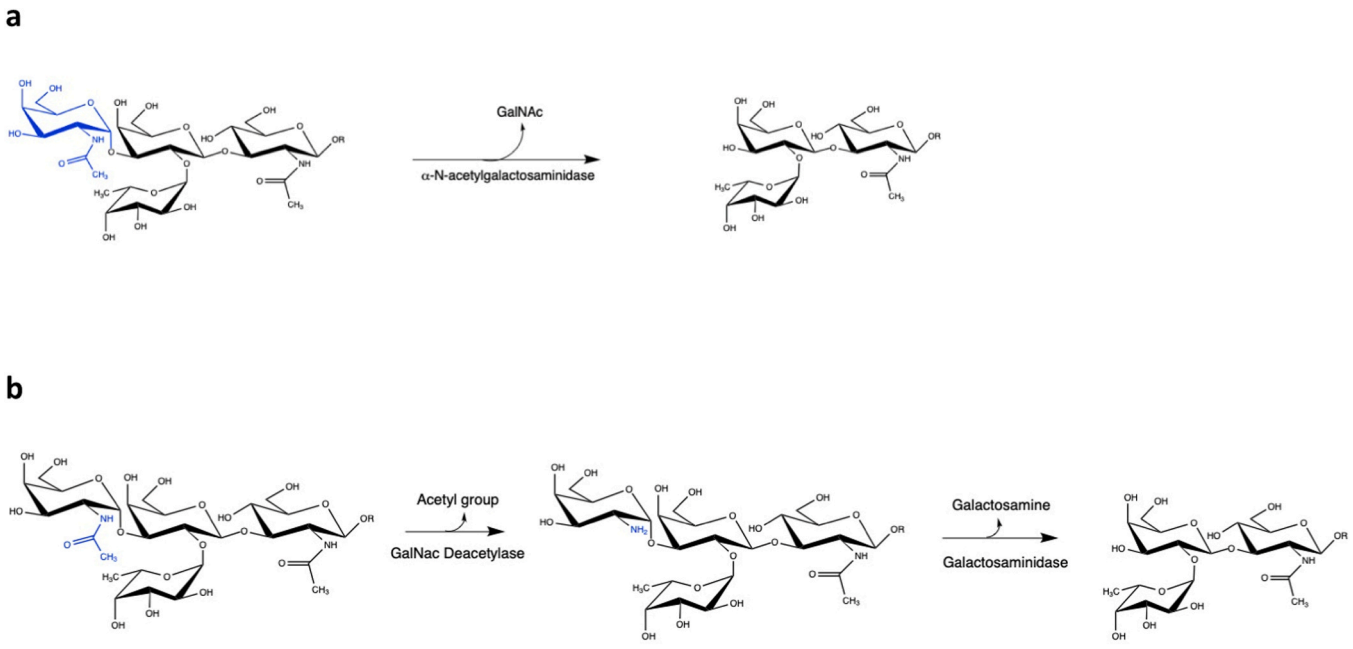

Fig. 2. Enzymatic modification of Red Blood Cells. (a) Single step A-type antigen removal by α-*N*-acetylgalactosaminidase; the removed GalNAc is shown in blue; (b) Double step A-type antigen removal by GalNAc deacetylase and Galactosaminidase. The N-acetyl group modified by the GalNAc deacetylase is shown in blue.

Materials and methods

Materials

GH109 enzymes (NAg68; NAg69; NAg71) were provided by Novozymes (Bagsværd, Copenhagen, Denmark). Blood samples and Anti-A serum from BIRMA-1 (BIOSCOT®) Edinburgh, Scotland, UK) were provided by the Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II" (Naples, Italy). pNP-substrates and Blood GroupA trisaccharide was purchased from Biosynth-Carbosynth® (St. Gallen, Switzerland).

Cloning, expression, and purification of GH109

The three GH109 genes (NAg68; NAg69; NAg71) were cloned and expressed in *Bacillus subtilis* with a His tag at the N-terminus as described [28]. Enzymes were purified by immobilised metal chromatography (IMAC) using Ni^{2+} as the metal ion on HisTrap Excel columns (GE Healthcare, Chicago, Illinois, USA) and eluted with imidazole following the standard procedure reported in [28]. The concentration of the enzymes was determined by absorbance at 280 nm after a buffer exchange in 50 mM HEPES, 100 mM NaCl pH7.0. The sequences of the genes encoding NAg68, NAg69 and NAg71 from *Alkalimonas sp-62417*, *Pararheinheimera soli* and *Amycolatopsis keratiniphila* respectively were deposited in GenBank with accession nos. OQ269604, OQ269605 and OQ269606.

In silico analysis

Multisequence alignment of the GH109 conserved motif was performed by Clustal Omega tools (Clustal Omega < Multiple Sequence Alignment < EMBL-EBI).

Molecular mass determination

Native molecular masses of NAg68, NAg69, and NAg71 were determined by gel filtration using AKTA prime FPLC equipped with Superdex 10/300 GL (Cytiva, Marlborough, England, UK). The chromatography was performed with the mobile phase consisting of buffer 50 mM Hepes, 100 mM NaCl, pH 8.0, with an elution flow rate at 0.7 mL/min. The Superdex column was calibrated for protein sizing by using the following protein markers: carbonic anhydrase (29,000 Da), bovine serum albumin (66,103 Da) alcohol dehydrogenase (150,000 Da), β-amylase (200,000 Da), apoferritin (443,000 Da) and thyroglobulin (669,000 Da).

Activity assay

The standard assay for GH109 enzymes (7.5 μg of NAg69, 5 μg of NAg68, and 2.5 μg of NAg71) was performed in 50 mM sodium phosphate pH 8.0 on 3 mM pNP-β-GalNAc and 2 mM pNP-α-GalNAc at 40 °C for NAg69 and 45 °C for NAg71 and NAg68, in a final volume of 400 μL. The pNP release (mM extinction coefficient determined for this assay was 13.2 mM^{-1} cm^{-1}) was monitored continuously at 420 nm by using a double beam spectrophotometer Cary 100 UV (Agilent Technologies, Santa Clara, California, USA. One unit of enzymatic activity was defined as the amount of enzyme catalysing the hydrolysis of 1 μmol of substrate in 1 min at the condition described.

Temperature and pH dependence

Optimal pH and buffer saline conditions were determined by using sodium phosphate and sodium borate buffers in the pH ranges 6.0–8.0 and 8.0–9.0, respectively. The effect of temperature on enzyme activity was evaluated in the range of 20–50 °C in sodium phosphate buffer pH 8.0 on 3 mM of pNP-β-GalNAc as substrate.

Effect of additives

Assays were carried out in the enzyme's standard conditions reported above by adding 2 mM of each additive (NAD^+, EDTA, $CuCl_2$, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $FeCl_2$) in the reaction mixture. Control refers to the assay in standard condition without additives.

Kinetic parameter determination

Steady-state kinetic constants of the enzymes were measured in the standard conditions on various concentrations of pNP-α- and pNP-

β-GalNAc. NAg69: 0.01–2 mM of pNP-α-GalNAc and 0.05–5 mM pNP-β-GalNAc. NAg68: 0.01–2 mM of pNP-α-GalNAc and 0.05–5 mM of pNP-β-GalNAc. NAg71: 0.01–2 mM of pNP-α-GalNAc and 0.25–2 mM of pNP-β-GalNAc. The data were analysed with GraphPad Prism 8 program, by nonlinear regression using the Michaelis-Menten equation and linear regression using Lineweaver-Burk equation for kinetic parameters of NAg71 on pNP-β-GalNAc.

Thermal stability

The thermal stability of NAg68, NAg69 and NAg71 was evaluated by testing their activities in their standard conditions after incubation of the enzyme solution at 37 °C for different lengths of time.

Activity on blood group A trisaccharide

The activity of the NAg68, NAg69 and NAg71 on A-type RBC antigen trisaccharide as substrate was evaluated at 37 °C, by using 300 μg of substrate and 50 μg of enzymes in sodium phosphate buffer pH 8.0. An assay mixture without enzymes was used as control. After incubation of 10 min, the reactions were stopped by freezing in dry ice for 1 min. The reaction products were evaluated and quantified by high performance anion exchange chromatography with pulsed amperometric detector (HAEPC-PAD).

HPAEC-PAD analysis

The analysis was performed by using an HPAEC-PAD Dionex system (ICS 3000, Waltham, MA, USA) equipped with PA-100 column (Dionex, Sunnyvale, CA, USA) in 11 mM NaOH. GalNAc was used as standard to calibrate the retention time and to build a calibration line at concentrations of 0.5 nmol 25 μL^{-1}, 1.0 nmol 25 μL^{-1}, and 2.0 nmol 25 μL^{-1} with fucose as internal standard at concentration of 1 nmol 25 μL^{-1}. 25 μL of diluted stopped reactions supplemented with 1 nmol of fucose were injected for HAEPC-PAD analysis. Moreover, to confirm the substrate and GalNAc signals in the HPAEC-PAD profile, 2 nmol of substrate or 5 nmol GalNAc were added to different aliquot of the reactions and analysed separately.

Activity on RBCs

Purified RBCs from blood donations were centrifuged at 1000 × *g* for 5 min at room temperature (RT), washed three times in PBS buffer (150 mM NaCl, 50 mM sodium phosphate) pH 7.4 and resuspended at 10 % haematocrit in the same buffer. In this way, the cells were not subjected to osmotic stress or pH changes, ensuring their integrity during enzymatic tests. For the enzymatic assay, RBCs were resuspended at 10 % haematocrit in PBS pH 7.4, supplemented with 300 mg mL^{-1} of dextran 40 kDa. In a final volume of 200 μL, various enzyme amounts (2, 5, 10, and 20 μg) were added to the resuspended RBCs. The reaction mixtures were incubated at 37 °C for 1 h under shaking. The reaction was stopped by cooling in ice and centrifuging at 1000 × *g* for 40 min to separate RBCs from the supernatant (containing the enzymes). Enzyme-treated RBCs were washed three times as reported above and resuspended at 67 % haematocrit for HI-500 system analysis.

FACS analysis

Enzyme-treated RBCs were washed twice with PBS and resuspended at 1 % haematocrit in 3 % BSA/PBS staining solution. All stainings and washes were performed at RT in staining solution, unless otherwise indicated. Enzymatically converted RBCs were firstly stained with 1:100 anti-blood group H (mouse IgM, Abcam, cat. ab24213) for 30 min. Excess Ab was removed by washing RBCs three times, then cells were stained with 1:200 AlexaFluor®488 conjugated anti-Mouse IgM (μ chain) (goat IgG, Immunological Sciences, cat. IS20840) for 60 min in the dark. After three additional washes, RBCs were finally stained with 1:100 AlexaFluor®647 conjugated anti-blood group A (mouse IgG, BD Pharmingen, cat. 565384) for 30 min in the dark. At the end of this incubation, cells were washed three times. Events acquisition was performed, after resuspension of the RBCs in PBS at 0.5 % haematocrit, on a FACS Canto II flow cytometer using FACS Diva software (v 9.0). The data were refined by FlowJo (v 10). RBCs were identified based on morphological parameters (FSC and SSC) and a gate was applied to select single cells.

Results and discussion

Enzyme characterisation

The enzymes analysed in this work, NAg68, NAg69 and NAg71 are obtained from bacteria of the genera *Alkalimonas*, *Pararheinheimera,* and *Amycolatopsis*, respectively. Multi sequence alignments with other characterised GH109 revealed that these enzymes share the consensus motif G-G-H-G-G containing the catalytic acid-base histidine (Fig. 3).

NAg68, NAg69 and NAg71 had a native molecular mass of 71.7 kDa, 74.2 kDa and 56.02 kDa, respectively (GenBank numbers in Materials and Methods). All the enzymes were optimally active in sodium phosphate buffer at pH 8.0. NAg68 showed the highest activity at 45 °C (Fig. 4) and exhibited a specific activity in standard conditions of 11.6 U mg^{-1} and 7 U mg^{-1} for pNP-α- and β-GalNAc, respectively. NAg69 showed an optimal activity at temperature of 40 °C and the measured specific activities were of 12 U mg^{-1} on pNP-α-GalNAc and 2.7 U mg^{-1} on the β-anomer. NAg71, was optimally active at 45 °C, like NAg68 (Fig. 4), and had a specific activity of 11 U mg^{-1} on pNP-α-GalNAc and 7.2 U mg^{-1} on the β-anomer.

The three enzymes did not require exogenous addition of NAD^+ and their activity in the presence of 2 mM of cofactor was similar to the standard assays in its absence (Fig. 4). These data agreed with GH109 enzymes previously described in which the cofactor is tightly bound to the active site of the enzymes purified in recombinant form [21,22]. The presence of NAD^+ in the structure of GH109 enzymes has been experimentally demonstrated for the first characterised GH109 from *Elizabethkingia meningoseptica* (NagA) and two other enzymes from *Akkermansia muciniphila* [21,22]. EDTA had no negative effect on the enzymes and exogenous metal ions did not enhance activity, indicating that they did not require metal ions for catalysis. EDTA had a small activating effect on NAg68, while all metal ions had negative effects on NAg69 and NAg71 (Fig. 4). The three GH109s were tested on different

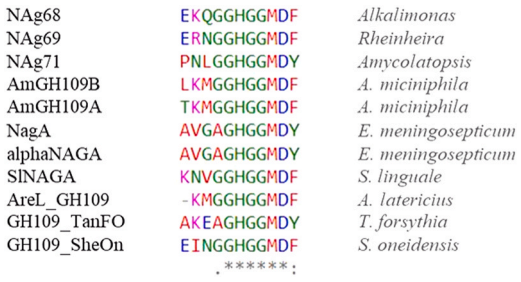

Fig. 3. Multialignment of acid-base catalytic residue regions of NAg68, NAg69 and NAg70 versus characterised GH109. Asterisk (*): single, fully conserved residue. Colon (:): conservation between residues groups of strongly similar properties. Period (.): conservation between residues groups of weakly similar properties. Residues colours according to Clustal Omega colour code (Help Clustal Omega FAQ - Tools Help & Documentation - EMBL-EBI). GenBank ID: *A. muciniphila* ACD04752.1 (AmGH109A) [21]; *A. muciniphila* ACD03864.1 (AmGH109B) [21]; *E. meningoseptica* CAJ01375.1 (alphaNAGA) [23]; *Arenibacter latericius* ADM26828.1 (Arel_GH109) [24]; *Tannerella forsythia* CAJ01380.1 (GH109_TanFO) [22]; *Shewanella oneidensis* CAJ01377.1 (GH109_SheOn) [22]; *E. meningoseptica* CAJ01376.1 (NagA) [22]; *Spirosoma linguale* ADB42594.1 (SlNAGA) [25].

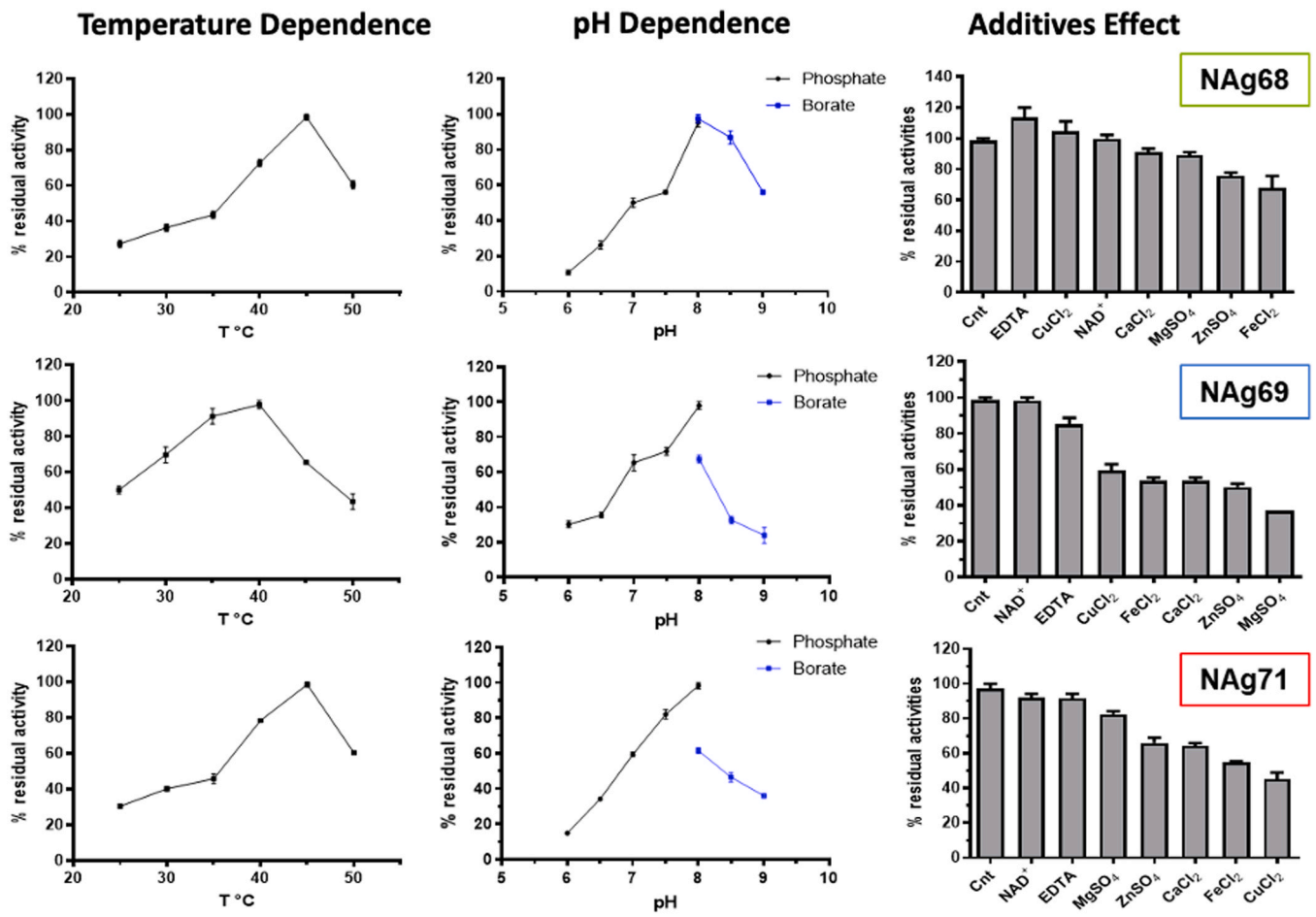

Fig. 4. Characterisation of GH109 enzymes: Left panel: Temperature dependence determined in sodium phosphate buffer pH 8.0 on 3 mM of pNP-β-GalNAc as substrate. Central panel: pH dependence evaluated by using sodium phosphate (in black) and sodium borate (in blue) buffers in the pH ranges 6.0–8.0 and 8.0–9.0, respectively. Right panel: Effect of additives on the enzymes evaluated 50 mM sodium phosphate pH 8.0 on 3 mM pNP-β-GalNAc and 2 mM pNP-α-GalNAc at 40 °C for NAg69 and 45 °C for NAg71 and NAg68; Cnt: control with no additives.

aryl substrates (pNP-α/β-glucopyranoside, galactopyranoside, and -GalNAc) showing higher and measurable activity only on α/β-GalNAc derivatives, on which the kinetic constants have been determined.

Table 1 shows that pNP-α-GalNAc was the preferred substrate for all three enzymes. NAg68 had k_{cat} values for the two substrates within the same order of magnitude (8.5 and 13.9 s^{-1} for pNP-β- and -α-GalNAc, respectively). It showed a K_M of 0.05 mM for pNP-α-GalNAc, almost 5-fold lower than that of the other anomer (0.23 mM), resulting in a

Table 1
Steady-state kinetic constant comparison of characterised GH109 on pNP-α and -β-GalNAc.

Enzyme	Microorganism	Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	Assay conditions
AmGH109A [22]	*Akkermansia muciniphila*	α	2.6 ± 0.2	1.1 ± 0.1	2.4	HEPES buffer 50 mM pH 6.6, 25 °C
		β	1.10 ± 0.03	0.15 ± 0.01	7.3	
AmGH109B [22]	*Akkermansia muciniphila*	α	16.5 ± 0.3	0.39 ± 0.02	42.3	HEPES buffer 50 mM pH 6.6, 25 °C
		β	0.90 ± 0.01	0.38 ± 0.02	2.4	
NagA [21]	*Elizabethkingia meningoseptica*	α	9.84 ± 0.16	0.077 ± 6 × 10^{-3}	127.8	Sodium phosphate buffer 100 mM pH 6.8, NaCl 50 mM, 26 °C
		β	0.015 ± 0.001	0.23 ± 0.01	0.065	
αNAGA [23]	*Elizabethkingia meningoseptica*	α	7.47	0.063	118.6	Sodium phosphate buffer 50 mM pH 6.8, NaCl 50 mM, 25 °C
SlNAGA [25]	*Spirosoma linguale*	α	128	0.63 ± 0.05	203.0	Sodium phosphate buffer 20 mM pH 7.0, 37 °C
BvGH109_1 [26]	*Bacteroides vulgatus*	α	0.25 ± 0.01	0.011 ± 3 × 10^{-4}	22.7	HEPES buffer 50 mM pH 7.0, 37 °C
NAg68[1]	*Alkalimonas sp.*	α	13.9 ± 0.2	0.05 ± 4 × 10^{-3}	278.0	Sodium phosphate buffer 50 mM pH 8.0, 45 °C
		β	8.5 ± 0.3	0.23 ± 0.05	36.7	
NAg69[1]	*Pararheinheimera sp.*	α	15.0 ± 0.6	0.01 ± 1 × 10^{-3}	1500	Sodium phosphate buffer 50 mM pH 8.0, 40 °C
		β	3.30 ± 0.03	0.11 ± 0.02	30.0	
NAg71[1]	*Amycolatopsis sp.*	α	20.6 ± 0.2	0.03 ± 4 × 10^{-3}	686.7	Sodium phosphate buffer 50 mM pH 8.0, 45 °C
		β	14.10 ± 0.03	0.31 ± 0.06	45.5	

[a] This study.

higher specificity constant for the α-substrate (Table 1). NAg69 showed different kinetic parameters for the tested substrates. The K_M for the α-anomer is 10-fold lower compared to that on pNP-β-GalNAc (0.01 and 0.11 mM, respectively), and the k_{cat} for the same substrate is 4.5-fold higher (15 s^{-1}) (Table 1). Therefore, the enzyme exhibited a very high specificity constant of 1500 s^{-1} mM^{-1} for pNP-α-GalNAc. NAg71, like NAg69, showed a K_M for the pNP-α-GalNAc ten-fold lower than for the β-substrate (0.03 and 0.31 mM, respectively) and exhibited a k_{cat} of the same order of magnitude for the two substrates. As a consequence, the specificity constant for the pNP-α-GalNAc was much higher, as shown in Table 1. All the novel GH109s and NagA displayed similar specific activities on pNP-α-GalNAc (about 11 U mg^{-1}) [21]. However, the specificity constant values (k_{cat}/K_M) of NAg68, NAg69, and NAg71 were 2.2-fold, 11.7-fold, and 5.4-fold, respectively, higher than that of NagA for this substrate (127.8 s^{-1} mM^{-1}). Moreover, compared to the specificity constants available for the other characterised enzymes of the family GH109, the value of k_{cat}/K_M of the enzymes described in this work are the highest reported so far (Table 1). On the basis of their increased specificity for the hydrolysis of pNP-α-GalNAc substrate analogues, these three novel GH109 were further characterised to test the possible application on RBC conversion.

Activity on blood group A trisaccharide

To evaluate the activity of the three enzymes on a more complex substrate and their potential application in the conversion of A-type RBC, the blood group A trisaccharide (TrBgA) was used as substrate. This sugar is the antigenic determinant of type-A RBCs and consists of a α-1,3-linked *N*-acetyl-galactosamine bound to the antigenic base structure of group O the Fuc-α-1,2-Gal-β (Fig. 1). The assays were performed at 37 °C in sodium phosphate buffer pH 8.0, as described in Materials and Methods. Before incubating the enzymes with the TrBgA substrate, their thermal stability at 37 °C was evaluated: after 16 h of incubation, NAg68 and NAg71 maintained almost 80 % of activity, while NAg69 maintained 70 % of activity (Supplementary Fig. S1). The hydrolysis of TrBgA was tested after 10 min of incubation at 37 °C on 0.56 μmol of substrate, and the products were separated and quantified by HPAEC-PAD chromatography.

The three enzymes hydrolysed TrBgA with the release of GalNAc. The HPAEC-PAD chromatographic profiles of the three GH109 reactions were very similar (Fig. 5). Taking the NAg71 panel in Fig. 5 as a model, in the elution profile of the mix without enzyme (chromatogram Blank), the substrate peak (**S**) had a retention time of 12.6 min. After the enzymatic reaction (chromatogram R), this peak was no longer present. Instead, two reaction products were observed eluting at 13.4 and 15.2 min (**1** and **2**, respectively), the latter with the same retention time as the standard sugar GalNAc (chromatogram Std). To confirm these data, TrBgA substrate and GalNAc were added to the stopped reactions and analysed separately. After the addition of TrBgA (chromatogram R + TrBgA), despite a small delay in the elution of the analytes, which might be ascribable to the presence of the enzyme, compared to elution profiles of R and Blank, the reappearance of the peak related to the substrate is clearly appreciable. On the other hand, adding the GalNAc standard to the stopped reaction produced a higher signal of reaction product **2**. These results confirmed that after the action of the three enzymes on the trisaccharide, the substrate was no longer detectable and GalNAc was released. The reaction product **1** should be related to the disaccharide α-*L*-Fuc-(1,2)-Gal, being present only after incubation of the enzymes with the substrate, and the chromatographic profiles of the enzymes alone were missing this peak (data not show).

The μmol amounts of GalNAc released are 0.64 ± 0.02, 0.56 ± 0.02, and 0.61 ± 0.01 μmol by 1.39 μM, 1.34 μM and 1.78 μM of NAg68,

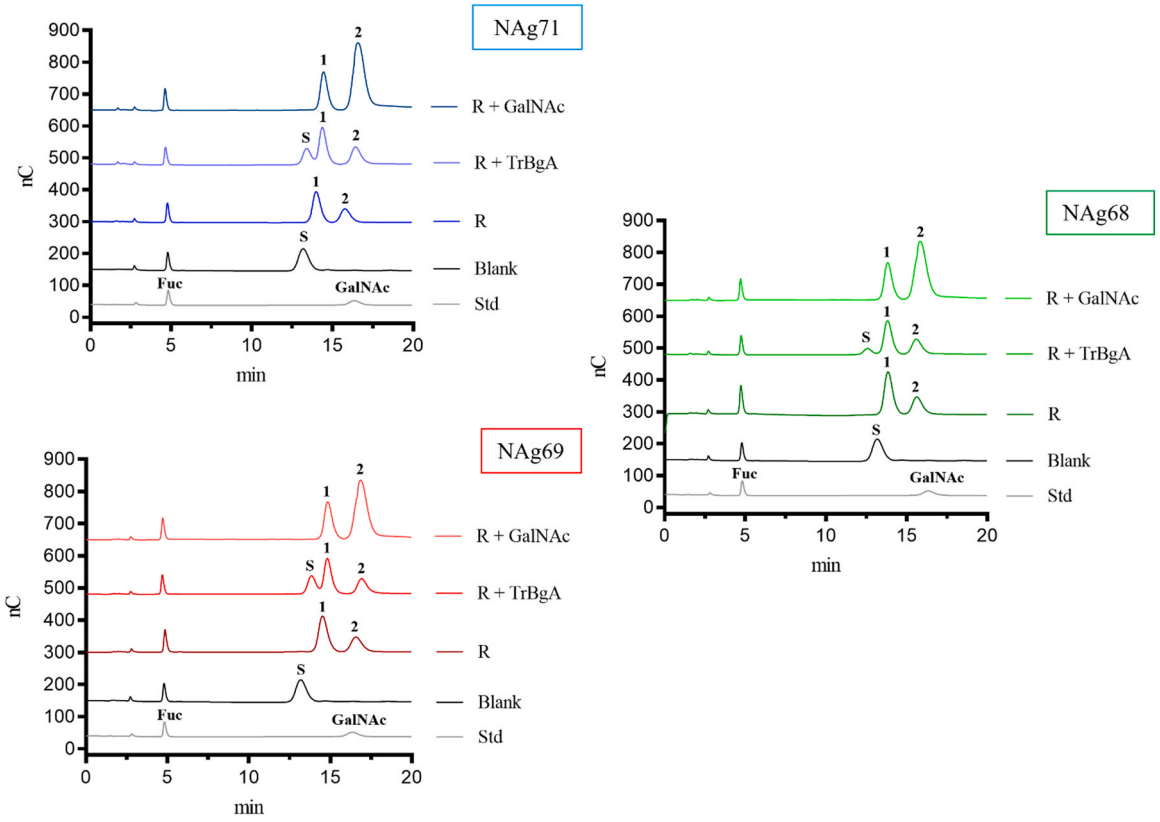

Fig. 5. HPAEC PAD chromatograms. Panels NAg71, NAg68, and NAg69 represent the chromatographic profile after incubation of the three GH109 enzymes on TrBgA and the respective controls. Blank: reaction mixture without enzyme. Std: Standard sugars fucose and N-Acetylgalactosamine. R: elution profiles of the reaction stopped after 10 min of incubation with the enzyme. R + TrBgA: stopped reaction implemented with TrBgA. R + GalNAc: stopped reaction implemented with GalNAc. Labels on the peak indicate the TrBgA substrate (S), the first eluted (1), and the second eluted (2) reaction products.

NAg69, and NAg71, respectively, showing that after 10 min of incubation, the enzymes can completely hydrolyse 0.56 μmol of TrBgA. These activities confirm the potential of GH109 to release GalNAc on the whole RBC, converting an A- to an O-antigen.

A-type RBC conversion

To test if the enzymes were effective on RBCs, purified A-type RBCs from blood donations were resuspended in a PBS solution at physiological pH 7.4. To increase the association of the enzymes onto the cell surface, the enzymatic reactions were performed in the presence of dextran 40 kDa as a macromolecular crowder [29]; in these preliminary assays on resuspended RBCs at 10 % haematocrit from Type-A donor, different enzyme concentrations were used. The detection to evaluate the conversion was performed through an IH-500 System (Bio-Rad, USA) equipped with the kit/test for typing ABO group (ID-card), specifically made for HI-500 system of Bio-Rad.

In Table 2, no agglutination (-) or agglutination (+) would be expected by effective *N*-Acetyl-galactosaminases or untreated RBC, respectively. NAg71 and NAg68 converted the RBCs at concentrations as low as 5 and 10 μg mL^{-1}, respectively, while NAg69 and NagA were unable to efficiently convert RBCs at the conditions tested. Therefore, NAg71 was further tested on five different blood donors (Table 3).

The minimum enzymatic concentration (MEC) required to obtain absence of agglutination was different in all samples, and depended on the RBC donors. For example, to convert RBCs of donors 1 and 5, a higher concentration of enzyme was required, whereas for donors 2–4, 5 μg mL^{-1} of NAg71 were sufficient to fully convert type A RBCs. This difference is most likely due to the subgroups of A-type RBCs, A_1 and A_2. Indeed, RBC with A_1 structure displayed a higher number of antigens on the surface than A_2-type [14]. These data were consistent with those reported by the other GH109 enzymes active on RBCs, which required different MECs to convert A_1- and A_2-type [22,24,26]. Based on this observation, we refer to the MEC needed to convert RBCs to the enzyme concentration where the enzymatically treated RBCs (ET-RBC) do not exhibit agglutination in all samples. Accordingly, the MEC for NAg71 was 10 μg mL^{-1}.

In order to fully confirm and quantify the effectiveness of the enzymes to produce ECO-RBC, the ET-RBC by NAg71 of donor 5 were analysed by fluorescence-activated cell sorting (FACS), using anti-A and anti-H antibodies. Fig. 6 shows the FACS analysis of ET-RBC with anti-A antibody labelled with allophycocyanin (APC) and anti-H antibody labelled with Fluorescein isothiocyanate (FITC-A). The negative controls of non-treated A and O RBCs, and the reference NagA are shown in the upper panels while the lower panels report the result of the incubation of group A RBCs with NAg71 (Figs. 6a–c and 6d and 6e, respectively). It is clear that the signal in the negative control is rather similar to that obtained after incubation with NagA (compare Figs. 6a and 6c). In fact, after reaction with 15 μg mL^{-1} of NagA, more than 40 % of the population was still labelled by anti-A (area Q3 in Fig. 6c), suggesting an incomplete conversion. Instead, the incubation with NAg71, at all the concentrations tested, produced a cell population more similar to the positive control (compare Figs. 6d–e and 6b). After treatment of RBCs with 2 μg mL^{-1} of NAg71 (Fig. 6d) more than 2 % of double labelled populations (anti A + H) were detected (area Q2 in Fig. 6d), consistent with the agglutination test in the ID card system (Table 3, donor 5). Remarkably, after treatment with 10 μg mL^{-1} NAg71 (Figs. 6e), 1.3 % of double stained cells (area Q2: anti A + H) and only 0.44 % labelled by Anti-A alone (area Q3) were detected, suggesting an apparently complete conversion, as also confirmed by the absence of agglutination (Table 3, donor 5).

It is shown here that, in the presence of dextran, NAg68 and NAg71 were able to efficiently remove GalNAc, as revealed by the absence of agglutination (Table 3). Moreover, NAg71 was demonstrated to catalyse the complete conversion of whole A- type RBCs at a concentration of 10 μg mL^{-1} confirmed by IH500 and FACS analysis (Table 3 and Fig. 6). As a comparison, NagA, previously used to convert RBCs [21], was not able to convert blood group A at concentrations 15 μg mL^{-1} under the same conditions used for NAg71. Thus, NAg71 represents an excellent biocatalyst capable of improving the process of blood conversion in a single step.

Table 2
Qualitative analysis of the effect of the enzymatic treatment of red-blood cells. Score of ID-Card resulted after reaction with one of the enzymes. According to the manufacturer, the score means (-) No agglutination; (+) Most agglutinated RBCs concentrated at the top of the gel or upper half of the gel column; na, not assayed. NagA was used as reference.

Enzyme	2 μg mL^{-1}	5 μg mL^{-1}	10 μg mL^{-1}	15 μg mL^{-1}	20 μg mL^{-1}
NAg68	+	+	-	na	-
NAg69	na	na	+	na	+
NAg71	+	-	-	na	na
NagA	na	na	na	+	na

Table 3
Analysis of the effect of NAg71 on different blood donors. Score of ID-Card resulted after reaction with NagA (control) and NAg71 on five different donors. According to the manufacturer, the score means (-) No agglutination; (+) most agglutinated RBCs concentrated at the top of the gel or upper half of the gel column; (DP, double population) agglutinated RBCs as a line at the top of the gel or dispersed in upper part of the gel and non-agglutinated RBCs forming a pellet at the bottom of the well.

Donor	NAg71			NagA
	2 μg mL^{-1}	5 μg mL^{-1}	10 μg mL^{-1}	15 μg mL^{-1}
1	DP	DP	-	+
2	-	-	-	+
3	-	-	-	+
4	+	-	-	+
5	DP	DP	-	+

Conclusion

In this work, three new GH109 enzymes showing kinetic properties on pNP-α-GalNAc superior to the others from this family have been identified and characterised. Their catalytic efficiency prompted investigation of their capability to convert A-type blood to O-type blood group. All three enzymes acted on erythrocyte A antigen trisaccharide. In addition, NAg71 was able to catalyse the complete conversion of purified A-type erythrocytes at a concentration of 10 μg mL^{-1}. It is noteworthy that NAg71 catalysed the blood type A conversion more efficiently than the commercially available NagA, previously used to convert RBCs.

The data demonstrates that novel CAZymes are powerful tools for design and improvement of biomedical applications and demonstrates the importance of detailed characterisation of the enzymes to exploit their potential as biocatalysts. Members of the GH109 family represent remarkable and underexplored candidates for enhancing ECO-RBC production technology.

CRediT authorship contribution statement

NC, RI, AS, LG, and MM were involved in Conceptualization. NC, RI, DRS, MC, and AS performed Investigation. BC-P, AL, LG, and MM were involved in Resources. NC, RI, MC, and AS performed Data curation. BC-P, AL, LG, and MM performed Supervision. MM was involved in Funding acquisition. NC, RI, AS, and MM Writing – original draft. DRS, MC, BC-P, AL, and LG were involved in Writing – review & editing. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally. Nicola Curci and Roberta Iacono.

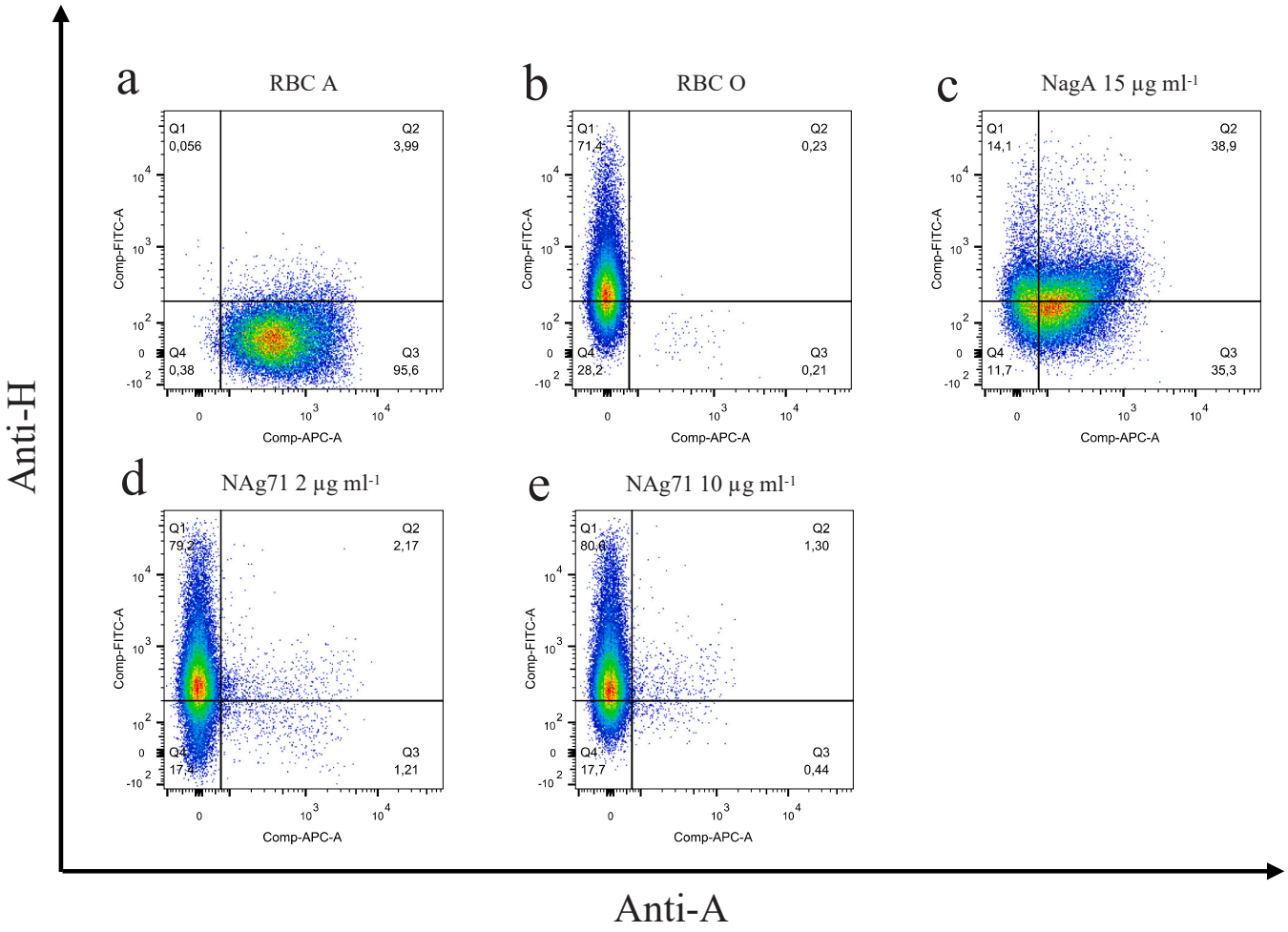

Fig. 6. FACS analysis of RBCs A treated with different concentrations of NAg71. The % of cells that reacted to the antigens H, A + H, A are shown in the areas Q1, Q2, and Q3, respectively; area Q4 contains RBC samples. RBCs A without enzymatic treatment (blank) a); RBCs O b); RBCs A after enzymatic conversion with 15 µg mL^{-1} of NagA c); 2 µg mL^{-1} of NAg71 d); 10 µg mL^{-1} of NAg71. Anti-A antibody were labelled with allophycocyanin (APC) and anti-H antibody were labelled with Fluorescein isothiocyanate (FITC-A).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.08.002.

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