Artificial Enzymes

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Design of Artificial Glycosidases: Metallopeptides that Remove H Antigen from Human Erythrocytes

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Abstract: *Catalysts that promote carbohydrate degradation have a wide range of potential applications, but the use of either enzyme glycosidases or small-molecule catalysts in biological systems raises significant challenges. Herein, we demonstrate a novel strategy for the design of synthetic agents that mimic natural glycosidases and address current problems for biological use. This strategy is illustrated by application to the development of potential blood substitutes for the rare Bombay blood type that is characterized by a deficiency of H2 antigen. Metallopeptides with 16 to 20 amino acids were constructed as artificial fucosidases that exhibit selective carbohydrate cleavage reactivity toward* L*-fucose over* D*-glucose. Selective fucose cleavage from the H2-antigen saccharide enables efficient removal of H2 antigen from erythrocytes and thereby accomplishes the conversion of regular human type-O blood into a potential blood substitute for the rare Bombay blood type.*

The development of tools for the catalytic degradation of carbohydrates has a broad range of potential applications.[1] Glycosidases are enzymes responsible for the selective degradation of carbohydrates, however, their selectivity toward specific carbohydrate substrates can be disadvantageous in the context of biotechnology application.[2] The presence of certain residues adjacent to the target carbohydrate can severely influence or even suppress enzyme activity.[3] For example, an α-fucosidase from *bacteroides thetaiotaomicron* can hydrolyze pNP-fucoside, but exhibits no activity towards other fucose-containing disaccharides or trisaccharides.[4] Additional problems for protein-based glycosidases include limited options in terms of natural protein families, many of which can cause immune responses in vivo, and a requirement for non-neutral pH.[5] As an alternative, both small-molecule catalysts and heterogeneous catalysts have been developed to promote the hydrolysis of carbohydrates, however, harsh conditions that involve non-neutral pH, the use of organic solvents, and negligible discrimination between different carbohydrate substrates undermine their potential application in biological systems.[6] Herein, we demonstrate a strategy to design synthetic agents as selective artificial glycosidases that degrade carbohydrates in a controlled and targeted manner. The design of our artificial glycosidases includes a targeting moiety that directs binding toward the substrate of interest, and an independent carbohydrate-degrading moiety that mediates oxidative chemistry at the substrates under physiological conditions. Isolation of the catalytic domain from the binding domain ensures greater flexibility for the catalytic apparatus and improved selectivity of the artificial glycosidases toward a broader range of substrates containing the target sugar.

By applying this strategy, we designed artificial fucosidases to produce a potential blood substitute for a rare blood type. While recent studies have described protein-based glycosidases that convert type-A blood and type-B blood into universal type-O blood, there are few efforts to develop blood substitutes for rare blood types owing to a lack of economic incentives.[7] Blood transfusions for patients with rare blood groups are perceived to be a significant challenge in transfusion medicine owing to the scarce resource of donated blood from rare blood groups.[8] Type-2 H antigen (H2, or CD173) is the terminal polysaccharide residue of glycolipids or glycoproteins on erythrocytes (red blood cells) with the sequence Fucα1-2Galβ1-4GlcNAcβ.[9] Deficiency of H2 antigen on the erythrocyte surface is known as the Bombay phenotype (also Oh, or h/h).[9,10] The prevalence of this phenotype is low (1 of 10000 in India, and 1 per million in Europe).[10,11] Serum in patients carrying the Bombay phenotype contains antibodies against the H2 antigen, therefore, transfusion of regular type-O blood to patients with the Bombay phenotype can lead to a severe and fatal transfusion reaction.[12] Herein, synthetic metallopeptides were designed as artificial fucosidases to remove fucose from H2 antigens on human erythrocytes and thereby produce a substitute for the Bombay blood type.

The cleavage domain, composed of an amino terminal Cu^{II}- and Ni^{II}-binding (ATCUN) motif (GGH) was incorporated into a fucose-selective binding domain derived from odorranalectin (OL: YASPKCFRYPNGVLACT) or its truncated form (tOL: KCFRYPNGVLACT).[13] In addition, two C-terminal-amidated analogues were also designed, since reduction of the negative charge may enhance the interaction between the peptides and the negatively-charged surface of erythrocytes.[14] The L-fucose binding affinity of the designed peptides was studied by isothermal titration calorimetry (ITC). CuGGH-tOL-NH_2 displays significant binding affinity for L-fucose, with a K_D of 57.8 μM (Figure S3), which is consistent with the reported K_D (54.7 μM) of odorranalectin.[13] Interestingly, the binding of the copper-free GGH-tOL-NH_2 to L-fucose exhibits a negative ΔH in contrast to the endothermic binding pattern of its copper-bound analogue (Figure S3). This change in ΔH can be ascribed to the

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potential coordination to Cu^{II} by hydroxy groups from fucose, with binding reflecting the entropic loss of water molecules.[15] Nevertheless, titration of L-fucose into other peptides, either in copper-bound form or copper-free form, resulted in a negligible enthalpy response that precluded calorimetric evaluation of binding affinity. Surface plasmon resonance was also used to measure the fucose binding affinity of all of the peptides (Table 1 and Figure S4 in the Supporting Information). In fact, removal of the first four residues (YASP) from full-length odorranalectin improves the fucose-binding affinity of peptides by around 18–26%, while amidation of the C terminus improves the binding affinity by around 60–71%. Therefore, the fucose-binding affinity mainly arises from the domain K5–T17 of odorranalectin, while the N-terminal YASP residues and the C-terminal carboxylate contribute little to carbohydrate binding affinity.

Table 1: Binding affinity of the metallopeptides with L-fucose.

	CuGGH-tOL-NH2	CuGGH-tOL-OH	CuGGH-OL-NH2	CuGGH-OL-OH
K_D [μM][a]	61.3 ± 7.5	105 ± 17	77.6 ± 6.9	124 ± 7

[a] Dissociation constants were measured by surface plasmon resonance.

The carbohydrate-cleavage reactivity of the metallopeptides was evaluated by using chromogenic substrates linked to *p*-nitrophenolate (pNP). A physiologically relevant co-reagent (ascorbate or hydrogen peroxide) is required to stimulate the redox chemistry of the Cu-ATCUN motif and promote the formation of metal-bound oxygen species.[16] The latter abstract hydrogen from the carbohydrate ring, which eventually leads to glycosidic bond cleavage.[17] All of the metallopeptides with a fucose-targeting domain exhibit a strong preference for L-fucose cleavage over D-glucose (Table 2 and Figure S5), which is abundant in blood and is commonly used for blood preservation.[18] The lower K_M for the fucose substrate relative to glucose demonstrates selectivity arising from the fucose-binding domain. The larger k_{cat} for the metallopeptides with truncated odorranalectin CuGGH-tOL-NH_2 and CuGGH-tOL-OH can be ascribed to the shorter distance between the copper center and the fucose-binding site relative to the analogues CuGGH-OL-NH_2 and CuGGH-OL-OH.

CuGGH, which lacks the fucose-targeting domain, exhibits no discrimination between L-fucose and D-glucose. These results demonstrate that the targeting domain is a prerequisite for the selective L-fucose cleavage activity of these artificial fucosidases.

Some protein-based fucosidases have been discovered to induce cleavage of pNP-fucose, while lower enzyme activity was observed with polysaccharides as a result of their substrate selectivity.[4,19] To confirm the fucose cleavage reactivity of our artificial fucosidases towards polysaccharides, a trisaccharide Fuca1-2Galb1-4GlcNAc (H-trisaccharide, exact mass = 529.2) was used as a model to represent the H2 antigens of human erythrocyte (Figure 1a). The cleavage products were separated from the intact trisaccharide by using an amino (NH_2) column and then analyzed by mass spectrometry (MS). CuGGH-tOL-NH_2 exhibits efficient cleavage of fucose from H-trisaccharide in the presence of ascorbate and peroxide, where the diminishing MS response of H-trisaccharide indicates its disappearance (Figure 1b). In contrast to protein-based fucosidases, our artificial fucosidases can display cleavage reactivity towards a more diverse group of fucose substrates by virtue of their smaller size and distinct mode of action. Specifically, they can overcome the incomplete-cleavage issue that is often encountered with protein-based fucosidases.[20] Following removal of fucose from H-trisaccharide, a disaccharide product (Galβ1-4GlcNAc, exact mass = 383.1) was identified by MS that corresponds to the saccharide antigen of the Bombay phenotype (Figure 1c). A shorter retention time (ca. 14.5 min) confirms the existence of this disaccharide, since the amino column exhibits weaker affinity for disaccharides than trisaccharides. Consistent with the results of pNP-fucose cleavage, both CuGGH-tOL-NH_2 and CuGGH-tOL-OH, each with a shorter distance between the fucose-binding domain and copper center, exhibit more robust cleavage reactivity towards H-trisaccharide relative to the longer CuGGH-OL-NH_2 and CuGGH-OL-OH analogues (Figure 1d). CuGGH also displays cleavage reactivity, likely as a result of nonselective cleavage, but it is significantly less reactive than the metallopeptides containing a fucose-binding domain.

H2 antigen on the cell surface was quantitated by using a FITC-labelled antibody (Figure 2a). In the presence of ascorbate, each of the artificial fucosidases significantly reduced the FITC intensity, thus indicating removal of H2 antigen from the erythrocytes. We propose that copper redox chemistry mediated by the metallopeptides promotes selective fucose cleavage from the H2 antigens and induces regression of the H2 antigen to the precursor saccharides corresponding to the Bombay blood type. The H2 antigens remain intact in the absence of either copper or ascorbate, thus confirming that copper redox chemistry is the origin of antigen removal (Figure 2b). All of the artificial fucosidases with a requisite targeting domain were observed to effectively remove H2 antigen, with EC_{50} values of approximately 8–11 μM (Figure 2c,d and Figure S6). The metallopeptides with an amidated C terminus display improved

Table 2: Michaelis–Menten parameters for carbohydrate cleavage.

Peptide sequence	pNP-α-L-fucoside[a]			pNP-β-D-glucoside[a]		
	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$M^{-1} min^{-1}$]	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$M^{-1} min^{-1}$]
CuGGH-tOL-NH_2	0.36 ± 0.08	1.20 ± 0.17	3360 ± 310	2.25 ± 0.62	0.51 ± 0.08	245 ± 34
CuGGH-tOL-OH	0.51 ± 0.07	0.84 ± 0.03	1680 ± 240	2.94 ± 0.31	0.93 ± 0.28	376 ± 25
CuGGH-OL-NH_2	0.35 ± 0.02	0.56 ± 0.08	1630 ± 310	2.16 ± 0.08	1.07 ± 0.13	514 ± 37
CuGGH-OL-OH	0.42 ± 0.06	0.44 ± 0.05	1380 ± 300	2.12 ± 0.17	0.32 ± 0.08	150 ± 31
CuGGH	1.17 ± 0.13	1.49 ± 0.12	1270 ± 36	0.72 ± 0.18	1.40 ± 0.34	1930 ± 120

[a] Reactions were performed with 5 μM metallopeptides, 1 mM ascorbate, and 1 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.4) at 37°C.

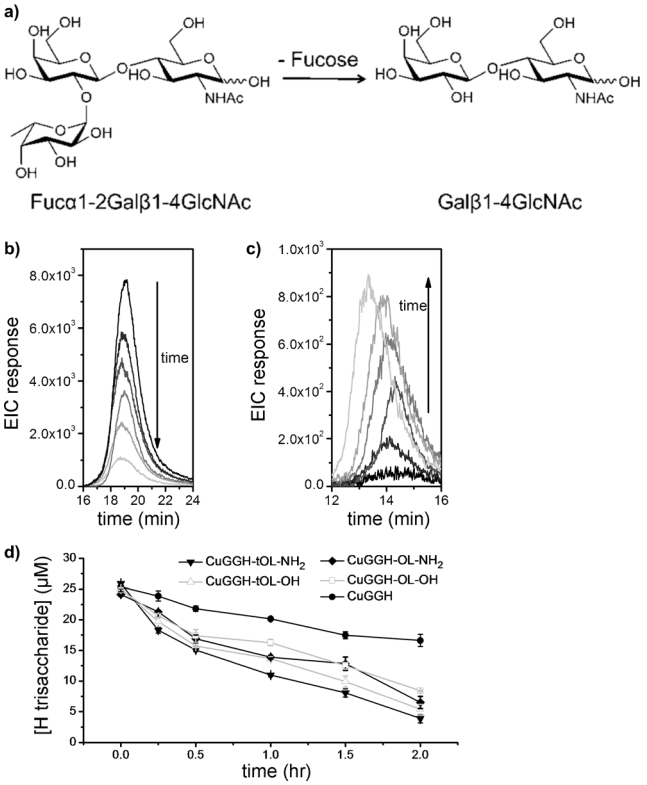

Figure 1. Cleavage of the H2 antigen trisaccharide (H-trisaccharide), as monitored by LC-ESI-MS. a) Removal of fucose from H-trisaccharide (Fucα1-2Galβ1-4GlcNAc) forms the disaccharide Galβ1-4GlcNAc. b, c) A 25 μM solution of H-trisaccharide was incubated with 50 μM CuGGH-tOL-NH_2, 1 mM ascorbate, and 1 mM H_2O_2 at 37 °C for 0, 15, 30, 60, 90, and 120 min. Extracted ion chromatogram (EIC) are shown for $m/z = 530.2 \pm 0.1$ (b) and $m/z = 384.1 \pm 0.1$ (c). d) Time-dependent cleavage of 25 μM H-trisaccharide by 50 μM metallopeptides, 1 mM ascorbate, and 1 mM H_2O_2 at 37 °C.

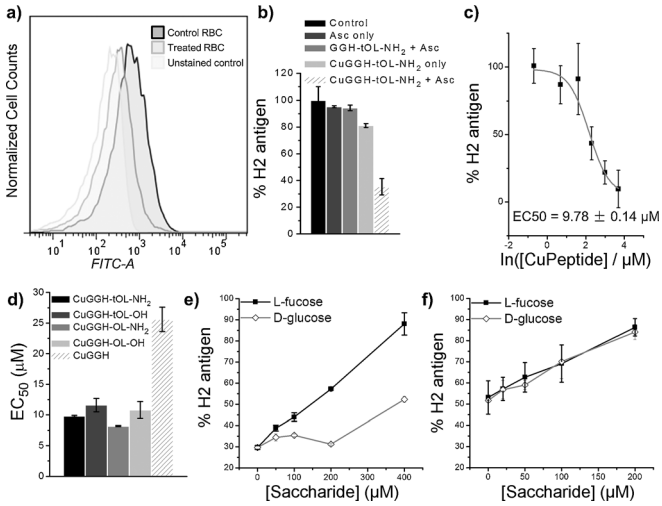

Figure 2. Removal of the H2 antigen from erythrocytes. a) Flow cytometry analysis of H2 antigen on the surface of human erythrocytes (red blood cells, RBCs). After the erythrocytes were incubated with 20 μM CuGGH-tOL-NH_2 and 200 μM ascorbate for 4 h, immunofluorescent staining was performed with a FITC-labelled anti-H2 antibody. b) Relative amount of H2 antigen on erythrocytes after incubation with or without peptide or ascorbate. Solutions containing 200 μM ascorbate and 20 μM CuGGH-tOL-NH_2 were used. c) Relative amounts of H2 antigen on erythrocytes after incubation with various amount of CuGGH-tOL-NH_2. d) The half-maximal effective concentration (EC_{50}) for H2-antigen removal by artificial fucosidases in the presence of 200 μM ascorbate. e, f) Inhibitory effect of saccharides. Human erythrocytes were incubated with 20 μM CuGGH-tOL-NH_2 (e), or CuGGH (f), with the indicated concentration of saccharides and 200 μM ascorbate.

reactivity toward H2 antigen, with an enhancement factor around 18–31%, relative to analogues with a C-terminal carboxylate. Amidation of the C terminus may reduce repulsive interactions between the peptides and the negatively-charged surface of the erythrocytes, however, the role of the YASP residues remains unclear, because removal of the YASP residues did not improve reactivity against H2 antigen. In fact, the microenvironment on the erythrocyte surface and interactions between the YASP residues and glycoproteins/glycolipids may also influence the reactivity of the designed metallopeptides. Removal of the H2 antigen by CuGGH was also observed, although with lower efficiency ($EC_{50} \approx 25$ μM) as a result of the lack of a targeting domain (Figure 2d and Figure S6). Accordingly, the selective fucose cleavage reactivity of our artificial fucosidases is most likely the origin of H2 antigen removal. The addition of L-fucose inhibits the binding of artificial fucosidases to cell-surface H2 antigen, thereby suppressing cleavage (Figure 2e). The addition of D-glucose also impairs the fucosidase activity through non-selective binding, but weaker inhibitory effects were observed relative to L-fucose. When saccharides were applied to CuGGH, no difference between the inhibitory effects of L-fucose and D-glucose was observed, which confirms that removal of the H2 antigen by CuGGH arises from non-selective cleavage (Figure 2f).

In conclusion, we have established a convenient strategy to design relatively short metallopeptides (16 to 20 amino acids) as chimeric fucosidases. All of the metallopeptides we designed exhibit selective carbohydrate cleavage of L-fucose over D-glucose. Fucose cleavage from human blood H2 antigen trisaccharides results in regression to a disaccharide product corresponding to the antigen of the Bombay blood type. We expect that this strategy could be adopted to develop glycosidases against other saccharide substrates of interest, and that the artificial glycosidases developed with our strategy could offer diverse applications complimentary to conventional protein-based glycosidases. Fucose cleavage of blood H2 antigen by artificial fucosidases leads to deletion of the H2 antigen from the erythrocyte surface. The treated erythrocytes could then potentially be applied as a substitute for the rare Bombay blood type, and so our results represent a first step to potentially addressing this problem.

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Conflict of interest

The authors declare no conflict of interest.

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Communications

Artificial Enzymes

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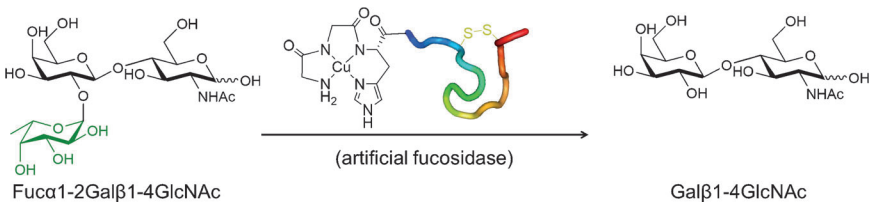
Design of Artificial Glycosidases: Metallopeptides that Remove H Antigen from Human Erythrocytes



There will be blood: Metallopeptides were designed as artificial fucosidases that can induce selective cleavage of L-fucose over D-glucose. Selective cleavage of fucose (Fuc) from the H2 antigen trisaccharide (Fucα1-2Galβ1-4GlcNAcβ) enables removal of the H2 antigen from human erythrocytes, which could potentially convert type-O blood into a substitute for the rare Bombay blood type.