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ORIGINAL ARTICLE

Cell surface and *in vivo* interaction of dendrimeric *N*-glycoclusters

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Abstract While many examples have been reported that glycoclusters interact with target lectins more strongly than single molecules of glycans, through multivalency effects, literature examples to support lectin interactions/modulations on cell surface and in live animals is quite rare. Our *N*-glycoclusters, which were efficiently prepared by immobilizing 16 molecules of the asparagine-linked glycans (*N*-glycans) onto a lysine-based dendron template through histidine-mediated Huisgen cycloaddition, were shown to efficiently detect platelet endothelial cell adhesion molecule (PECAM) on human umbilical vein endothelial cells (HUVEC) as a α(2-6)-sialylated oligosaccharides recognizing lectin. Furthermore, the identity of the *N*-glycans on our *N*-glycoclusters allowed control over organ-selective accumulation and serum clearance properties when intravenously injected into mice.

Keywords Asparagine-linked glycans (*N*-Glycan) · Dendrimer · Platelet endothelial cell adhesion molecule (PECAM) · Glycocluster · *In vivo* imaging · Lectin

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Introduction

Among the various types of oligosaccharide structures, asparagine-linked oligosaccharides (*N*-glycans) are the most prominent in terms of diversity and complexity. For sialic acid-containing *N*-glycans, their biological roles have been implicated in a variety of important physiological events, which includes cell-cell recognition, adhesion, signal transduction, and quality control [1]. A natural phenomenon associated with *N*-glycans is their ability to enhance their biological interactions by forming glycoclusters (termed as cluster effects and/or multivalency effects). This is exemplified by the fact that typical millimolar binding, single *N*-glycans can be enhanced by 1000 fold when formed into clusters. In addition, natural glycoclusters are often composed of several kinds of glycans (termed as glycoform), thus highly diversifying the glycobioenvironment. These heterogeneous glycoclusters are not only responsible for enhancing glycan-dependent interactions, but are also involved in regulating and/or adjusting target-molecule interactions through "pattern recognition" mechanisms [2–4]. To chemically mimic these natural *N*-glycocluster environments, literature examples have utilized proteins, polymers, dendrimers, and microarrays [5–11]. While many successful examples have dealt with various *N*-glycoclusters to enhance and/or identify target lectins interactions, *e.g.*, those imprinted on microarrays, the application in identifying or modulating interactions with cell surfaces or in live animals is rather rare. In this personal account, we wish to describe the synthesis, and *in vitro*/*in vivo* properties of lysine-based dendric *N*-glycoclusters (Fig. 1) [12]. The new *N*-glycoclusters, which contain 16 molecules of various *N*-glycan molecules, were efficiently prepared by the histidine-mediated Huisgen cycloaddition. Application of these clusters not only allowed detection of platelet endothelial cell adhesion molecule (PECAM) on human umbilical vein endothelial

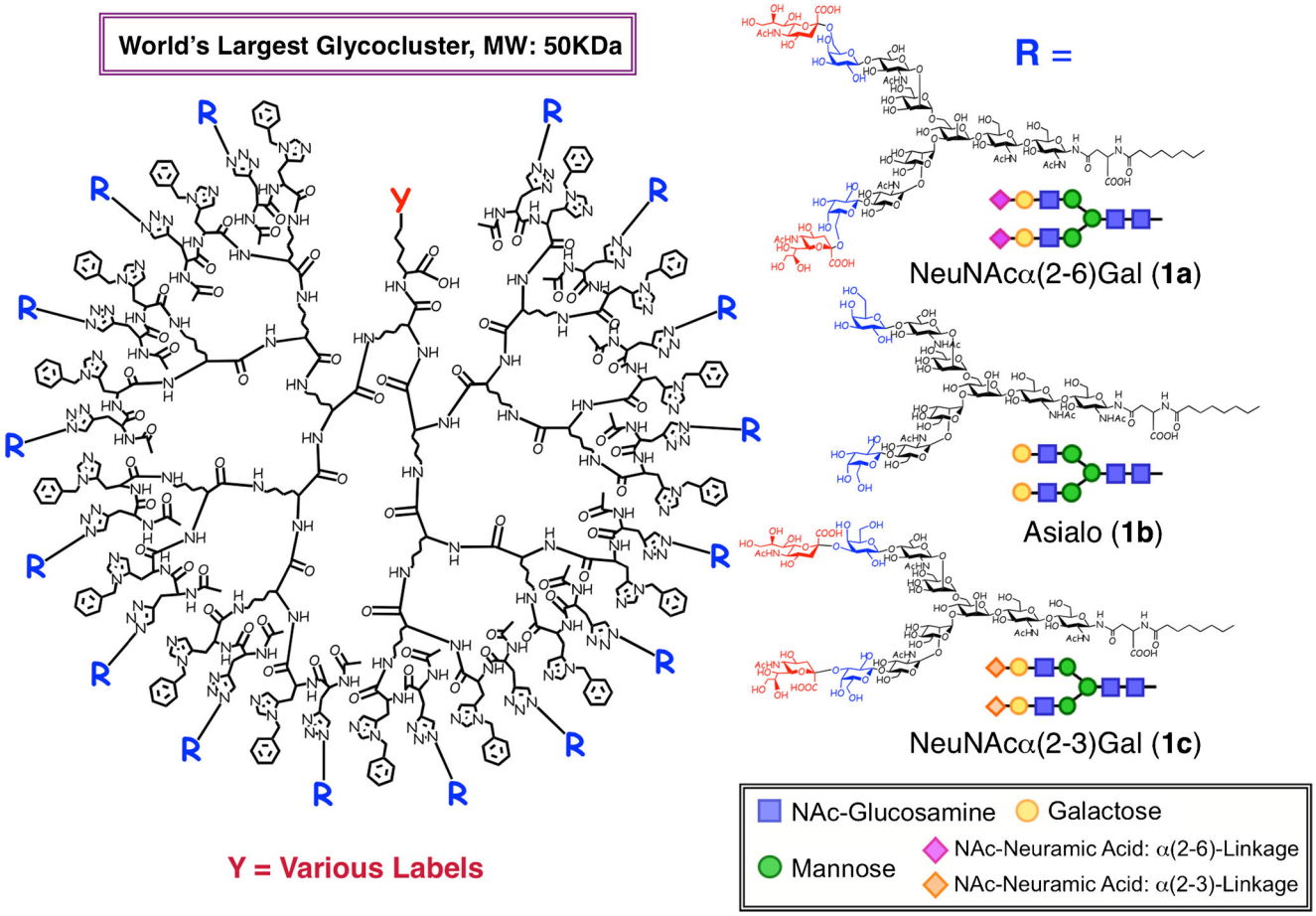

Fig. 1 Structures of dendrimeric *N*-glycoclusters [12, 13]

cells (HUVEC) as a α(2-6)-sialylated oligosaccharides recognizing lectin [13], but when intravenously injected into mice, was also shown to control organ-selective accumulation and possess differential serum clearance properties based on *N*-glycan structures [12].

Synthesis of lysine-based dendrimeric *N*-glycoclusters

In order to mimic the natural glycobioenvironment and to explore cluster (multivalency) effects, we designed and prepared polylysine-based dendrimer-type glycoclusters (Figs. 1 and 2) [12]. These clusters are structurally characterized by 16 molecules of varying *N*-glycan derivatives (**1a**–**c**), as well as a terminal lysine ε-amino group for incorporation of either a fluorescent label or positron emission tomography (PET) radiolabel (ie/ ^{68}Ga-1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (DOTA). In order for selective lysine labeling in the presence of numerous glycan hydroxyl groups, a 6π-azaelectrocyclization protocol previously developed in our group was used under mild conditions (Fig. 2) [14, 15]. The synthesis of the polylysine-based dendrimer core with terminal histidine and propargyl glycine residues followed a standard solid-support protocol. *N*-glycans were subsequently introduced by Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition (reaction between acetylene on the polylysine template and azide on the *N*-glycans, Fig. 2). Nevertheless, the Sharpless/Meldal click reaction was not readily performed on the peptide-based substrates because the copper ion can coordinate strongly to the peptide backbone, resulting in the deactivation of the reaction. Introducing several molecules of *N*-glycans with large and complex structures, *i.e.*, many hydroxyls and molecular weight of ca. 1500, to the polylysine dendrimer template, make Huisgen 1,3-dipolar cycloaddition more difficult. To overcome these problems, we introduced a *N*-benzyl histidine next to the terminal acetylene functions on the polylysine template (Fig. 2) to influence the "self-activating" Huisgen 1,3-dipolar cycloaddition [16]. The *N*-benzyl histidine coordinates to the copper ion, thereby significantly activating the reaction. An example of this process is illustrated by the smooth reaction between the terminal acetylene of polylysine-based dendrimer **1** and its azide-partner **a**, bis-NeuNAcα(2-6)Gal containing complex-type *N*-glycan (Fig. 2), in the presence of equimolar amounts of copper sulfate and diisopropylethylamine, as well as 3 equivalents of

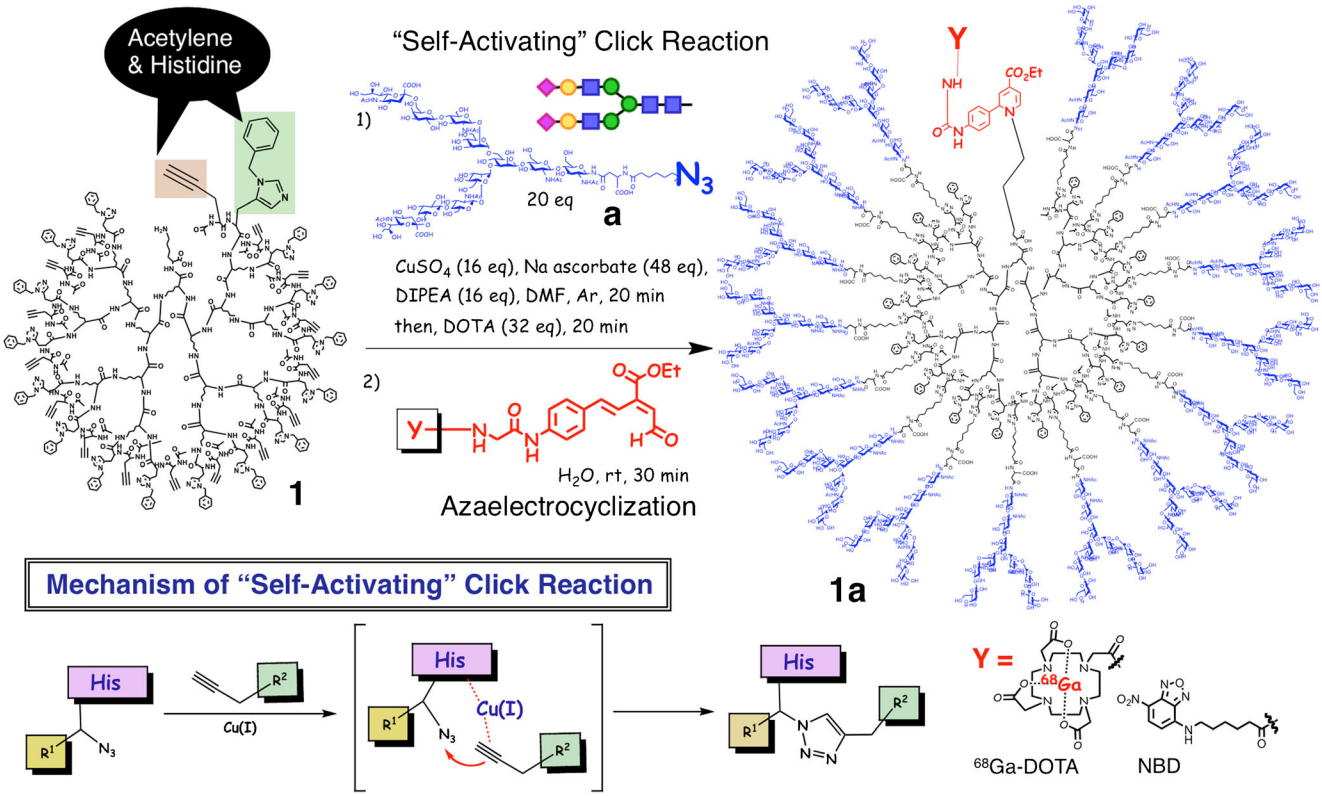

Fig. 2 Preparation of dendrimeric *N*-glycoclusters through histidine-accelerated Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition and labeling by 6π-azaelectrocyclization [12]

sodium ascorbate (all relative to the *N*-glycan molecules) at room temperature for 20 min. Residual copper ions were removed by DOTA chelation and size-partitioning centrifugal filtration. Subsequent HPLC purification afforded hexadecaglycoclusters **1a**–**c** with a molecular weight of ca. 50 kDa in almost quantitative yields (Figs. 1, 2). These clusters were subsequently labeled by ^{68}Ga-DOTA or a NBD fluorophore through rapid 6π-azaelectrocyclization.

Interaction with HUVEC cell surface: Identification of PECAM as α(2-6)-sialylated oligosaccharides-recognizing lectin

PECAM, a member of the immunoglobulin superfamily (IgSF), is expressed in endothelial cells and limited types of hematopoietic cells [17], where its plays multiple roles in cell adhesion, mechanical stress sensing, anti-apoptosis, and angiogenesis. Although the extracellular domain of PECAM is critical for homo- and heterophilic interactions [18], studies have shown that PECAM primarily acts through homophilic interactions in the signal transduction of anti-apoptosis for endothelial cells [19, 20]. By virtue of α(2-6)-sialic acid-deficient cells, we have previously reported that the homophilic PECAM interaction is α(2-6)-sialic acid dependent, critical for cell surface PECAM retention and PECAM-dependent cell signaling [21]. Our subsequent studies demonstrated that α(2-6)-sialylated oligosaccharides have significant *in vitro* inhibitory activity against homophilic PECAM-PECAM interactions [13]. Interestingly, research has also shown PECAM to possess α(2-6)-sialylated *N*-glycans.

Although we failed to detect binding of PECAM to monovalent α(2-6)-sialylated oligosaccharides on cell surfaces, we envisioned that our dendrimeric *N*-glycocluster probes **1a**–**c** may allow us to prove that PECAM possesses specific lectin activity. In our experiments, we used NBD-labeled dendrimer-type glycocluster probes **1a**–**c** in a binding assay with human vein endothelial cells (HUVEC). We found that the bis-NeuNAcα(2-6)Gal cluster (**1a**) bound more readily to the surface of the cells than the other types of clusters probes, asialo-type (**1b**) and bis-NeuNAcα(2-3)Gal (**1c**) (Fig. 3). As a result, this demonstrates for the first time where PECAM displays lectin-like properties in preferentially binding to α(2-6)-sialic acid containing glycans [13].

Interactions in live animals: *N*-glycan-dependent regulation of *in vivo* kinetics

Given that our *N*-glycoclusters can interact with cell surface lectins depending on the *N*-glycan constituents, our next concern was the *in vivo* kinetics. In general, a single molecule of

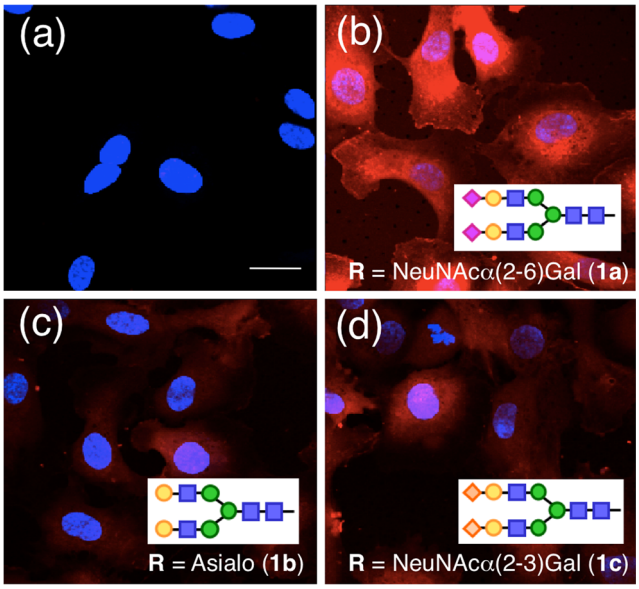

Fig. 3 Interaction of PECAM on HUVEC with dendrimeric *N*-glycoclusters, **1a-c** [13]. Prefixed HUVEC were incubated with (**a**) buffer as the control, (**b**) NBD-labeled **1a**, (**c**) **1b**, and (**d**) **1c**, each 10 μM at 25 °C for 45 min and observed by fluorescence microscopy. Scale bar, 20 μm

the glycans (monomeric glycan) is not suitable as an imaging probe due to its small size and weak interactions with sugar binding proteins *in vivo* [22, 23]. As a consequence, they will not accumulate in specific organs, but be rapidly excreted from the kidney *via* biofiltration.

The recent successful non-invasive imaging and biodistribution study of glycans and/or glycoconjugates, including both *N*- and *O*-glycans as well as monosaccharides, dealt with natural- and neo-glycoproteins, liposomes, and nanoparticles [24–26]. For example, biodistribution study of ^{125}I-labeled neoglycoproteins composed of albumin and biantennary *N*-glycans in mice indicated the increased presence of α(2-3)-sialylated or α(2-6)-sialylated neoglycoproteins in serum. The uptake, especially of the α(2-3)-sialylated neoglycoprotein was clearly elevated in mice for kidneys and Ehrlich tumors [27]. Biodistribution study of ^{125}I-labeled neoglycoproteins also revealed that core-fucosylation significantly affected to protein kinetics *in vivo*; the fucosylated α(2-6)-sialylated neoglycoproteins showed a reduced serum half-life in mice relative to the fucosylated α(2-3)-sialylated isomer and the non-fucosylated congeners [28]. Fluorescent imaging of inflammation and tumor regions was realized by using Sialyl Lewis X coated liposome [29]. *In vivo* targeting of B-cell lymphoma was achieved by using doxorubicin-loaded liposomal nanoparticles displaying high-affinity glycan ligands of CD22, which recognizes α(2-6)-sialylated glycans [30]. A novel drug delivery system using oligomannose-coated liposomes (OMLs) was developed by the use of the intraperitoneal macrophages as cellular vehicles for OMLs containing antitumor agent. Co-administered OMLs containing 5-fluorouracil or magnetic nanoparticles into the peritoneal cavity were effectively captured by macrophages, which effectively trafficked to metastases in the omentum. Control release of 5-fluorouracil was achieved by *in vivo* hyperthermia produced from magnetic nanoparticles by treatment with an alternating magnetic field to successfully control the tumor development [31]. Magnetic resonance imaging (MRI) of glyconanoparticles that display glycan ligand of E- and P-selectins effectively visualized inflammation in cerebral endothelium [32, 33]. PEGylated quantum dots (QDs) capped with D-mannose, D-galactose, and D-galactosamine have been synthesized to study specific carbohydrate-protein interactions *in vitro* and *in vivo* [34].

Out of these precedent examples, nevertheless, *in vivo* imaging studies of *N*-glycans are those of neoglycoproteins described above [27, 28]. Although these studies constitute a pioneering research field of glycoconjugate *in vivo* kinetics, notable biodistribution, serum stability, or tumor accumulation, which are precisely regulated by the *N*-glycan structures, could not be observed, since only a few molecules of *N*-glycan were introduced on the protein. Therefore, we investigated the multivalency effects *in vivo* using our glycoclusters. Following the injection of our ^{68}Ga-DOTA-labeled glycoclusters **1a**–**c** into BALB/c nude mice, we utilized positron emission tomography (PET) for examination [12]. As a control, smaller glycoclusters (see structures and images in Fig. 4a–b) were rapidly cleared first through the kidney, then through the urinary bladder over a 1 h timeframe. In contrast, when the 16-mer **1a** was administered, its presence was still retained in the body after 4 h (Fig. 4c), and was slowly excreted from the kidney, urinary bladder, and the gallbladder (intestinal excretion pathway). Biodistribution studies of the dissected tissues after 4 h detected the ^{68}Ga-radioactivity of **1a** mostly in the liver, gallbladder, and blood. These results clearly show the significance that cluster and/or multivalency effects has on influencing *in vivo* kinetics.

We subsequently examined the *in vivo* kinetics and biodistribution of asialo-glycan **1b** and bis-NeuNAcα(2-3)Gal glycan **1c** with the 16-mer template (Fig. 4d, e). Unlike bis-NeuNAcα(2-6)Gal **1a** (Fig. 4c), asialo-glycan cluster **1b** was rapidly cleared through the kidney to the bladder (Fig. 4d); although some accumulation was observed in the liver due to its high asialoglycoprotein receptor expression [35]. In the case of the α 3-linked galactose containing glycocluster **1c**, which also contains sialic acid, excretion was readily observed through the kidney/urinary bladder, as shown in Fig. 4e. Given that the only structural difference between **1a** and **1c** is either an α 3-linked or an α 6-linked galactose, respectively, it can thus be suggested that specific sialoside linkages to galactose play an important role in the circulatory residence of *N*-glycans. In addition, this specific sialoside linkage markedly differentiates the excretion mechanism from those of the asialo- and Neuα(2-3)Gal cases,

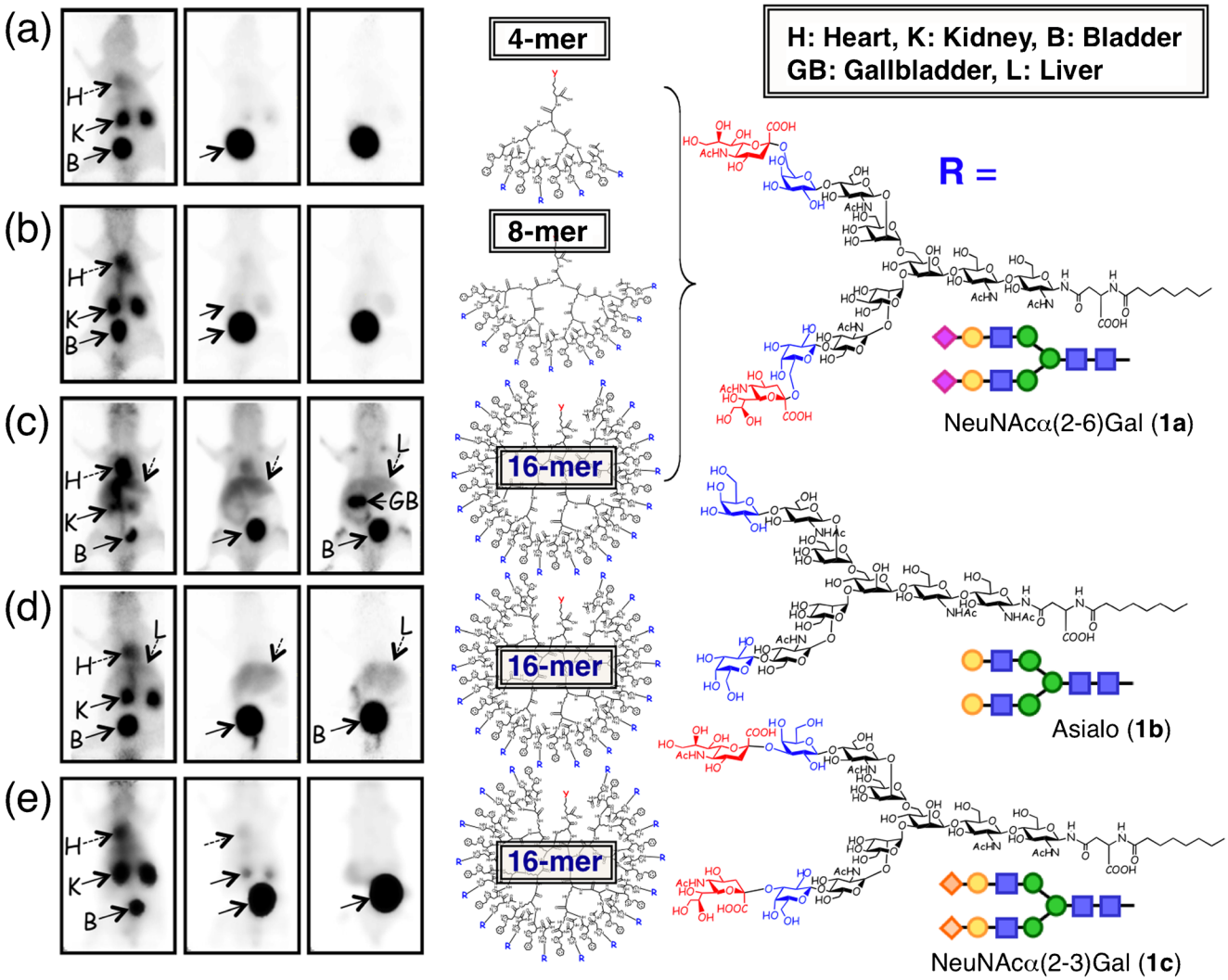

Fig. 4 Dynamic PET imaging of glycoclusters **1a-c** in normal BALB/c nude mice [12]. ^{68}Ga-DOTA-Labeled glycoclusters (10 MBq) were administrated from the tail vein of the mice (n=3, 500 pmol, 100 μL/mouse) and the whole body was scanned by a small animal PET scanner, microPET Focus 220 (Siemens Medical Solutions Inc., Knoxville, TN, USA), over 0–4 h after injection; H: heart; K: kidney; L: liver; B; urinary bladder; GB: gallbladder. (**a**) 4-mer glycocluster; (**b**) 8-mer glycocluster; (**c**) NeuNAcα(2-6)Gal-glycocluster **1a**; (**d**) Asialo-glycocluster **1b**; (**e**) NeuNAcα(2-3)Gal-glycocluster **1c**

which proceeds *via* a bio-filtration pathway through the kidney.

The prolonged *in vivo* life-time for the sialic acid-containing glycoclusters is in accordance to the well-known hypotheses that the clearance of asialoglycoproteins proceeds through the asialoglycoprotein receptors [35, 36]. However, the notable difference in the serum stability due to the sialoside bond linkages to galactose, *i.e.*, the α(2-6)- *vs.* α(2-3)-linkages, is an intriguing observation. Our dynamic PET images of the *N*-glycoclusters therefore suggests a new receptor-mediated excretion mechanism for Neuα(2-3)Gal-containing glycans. Namely, Neuα(2-3)Gal-cluster **1c**, which usually cannot be found in serum, is probably recognized as a foreign antigen and smoothly excreted by vascular endothelial cells, erythrocytes, leucocytes, or *via* phagocytosis by a macrophage; smaller sized degradation products may be further filtered in the kidney. Alternatively, the excretion-escaping mechanism by stimulating the immunosuppressive signals through the immunoreceptor tyrosine-based inhibitory motif (ITIM) molecules *via* Siglec families [37, 38], may account for the higher stability of Neuα(2-6)Gal-glycan. It is reported that the Neuα(2-6)Gal-containing BSA reduces but does not prevent binding to the asialoglycoprotein receptor, while the Neuα(2-3)Gal-congener abolishes the binding [39]. Therefore, the prolonged half-life coupled to uptake by the asialoglycoprotein receptor account for the high accumulation of **1a** in the liver (Fig. 4c). This study for the first time demonstrated the marked difference in the *in vivo* kinetics and biodistributions between α(2-6) or α(2-3)sialosides, and evidence for the role of multivalent effects in high selectivity and affinity for ligand-protein interactions *in vivo* [5–11].

Prospective

Since our dendrimer-based *N*-glycoclusters can easily be prepared *via* histidine-accelerated "self-activating" Huisgen 1,3-dipolar cycloaddition and efficiently labeled by imaging agents through the 6π-azaelectrocyclization protocol, they have potential in the development of glycodendrimer-based imaging probes and tracers. As discussed in the introduction, natural glycans on proteins and cell surfaces are often constituted of several different kinds of glycans, *i.e.*, glycoform, thus highly diversifying the glycobioenvironment. In order to attain enhanced selectivity, it will be necessary to synthesize and mimic such heterogeneous glycoclusters. One approach would be to construct various heterogeneous glycoclusters in a combinatorial fashion, which may lead to a promising strategy for developing not only neoglycoprotein-based imaging and diagnostic probes, but also vaccine adjuvants or virus-neutralizing pharmaceuticals. In order to evaluate in details the heterogeneity in natural glycoclusters and then mimic them to develop useful glycan-based imaging tracers, a challenge that synthetic chemists must consider is how structurally defined heterogeneous glycoclusters can be prepared efficiently and rapidly, *i.e.*, by the combinatorial approach. In other words, these issues will largely depend on the progress and development in the field of bioorthogonal synthetic chemistry to identify more organic reactions that are amenable to functional groups found on oligosaccharides.

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