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COMMUNICATION

Water-soluble AIE-active polyvalent glycocluster: design, synthesis and the studies on carbohydrate-lectin interactions for visualization of Siglecs distributions on living cell membranes

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Guang-jian Liu,[a] Yuan Zhang,[a] Lingyun Zhou,[b] Li-yan Jia,[a] Guohua Jiang,[c] Guo-wen Xing*[a] and Shu Wang[b]

In this work, we designed and synthesized an aggregation-induced emission (AIE)-active tetraphenylethene-decorated *pseudo*-trisialic acid (TPE3S) and validated its high affinity for Siglecs using microscale thermophoresis techniques. TPE3S was a unique *binding-on* fluorescent trivalent sialocluster which was successfully utilized for the visualization of Siglecs expressed on the surface of mammalian cells.

Sialic acid binding immunoglobulin-like lectins (Siglecs) which contain sialoside binding *N*-terminal domains are an important family of lectins predominantly present on the surface of tumor cells, hematopoietic cells and immune cells.[1,2] All Siglecs are single-pass transmembrane proteins and are heavily involved in immunoregulation and other physiological functions by recognizing and reversibly binding sialosaccharides.[3,4] A wide range of potential Siglecs-targeting drug candidates, such as epratuzumab, inotuzumab ozogamycin, and gemtuzumab ozogamicin etc., have provided opportunities for innovative therapeutic strategies for many kinds of immunological disorders or cancers.[5-8] Based on the previous research,[9-13] rational modifications of sialic acid glycoside framework and preparations of sialic acid glycoclusters are two effective ways to lead to the discovery of specific ligands for Siglecs.

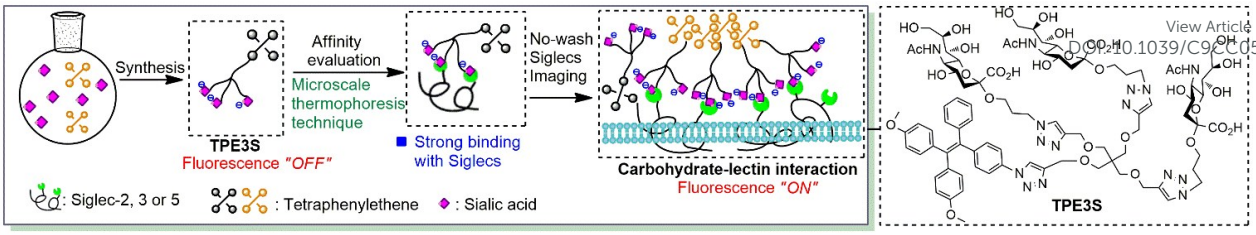
Until now, there are some molecular biology tools such as fluorescent antibodies, dye-tagged glycoprobes, biotinylated probes, to locate Siglecs on living cell membranes.[14-15] However, all these dye-labeled molecular tools belongs to the "*always-on*" fluorescent probes with high background signals, which not only result in the low sensitivity but also need the washing steps before imaging (Scheme S1a). Developing facile and effective "*binding-on*" probes to replace previous "*always-on*" probes for detecting and visualizing the glycan-binding receptors on the cell surface is of great value but has not been investigated.

Considering that the aggregation-induced emission (AIE) materials have been illustrated to possess many advantages, such as facile functionalization and strong photobleaching resistance in the sensing field and the AIE mechanism is the restriction of intramolecular motions,[16,17] we envisioned that non-fluorescent and water-soluble AIEgens should act as the "*binding-on*" probes upon binding with Siglecs to restrict intramolecular motions and to realize the visualization of Siglecs distribution pattern on cell membranes with low background signals. Besides, since high affinity and high specificity of lectins binding could be achieved as a result of the polyvalency of the interactions,[10,13] we designed a trivalent sialocluster (TPE3S) decorated by tetraphenylethene, which is a typical AIE-active fluorophore (Scheme 1). The binding of TPE3S to available recombinant human Siglecs in the form of Siglec–Fc chimeras (Fc = fragment crystallizable region of an antibody), like Siglec-2, -3, -5, was assessed by microscale thermophoresis (MST) which provides the means for interaction analyses with very low consumption of samples.[18]

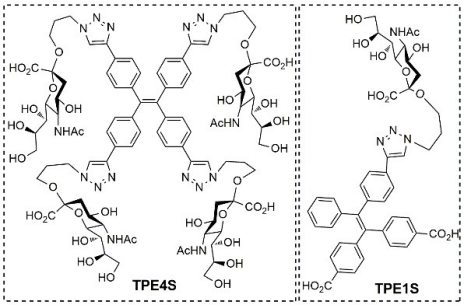
Contrastive experiments carried out with chemically modified sialic acid glycocluster mimetics (TPE1S and TPE4S), demonstrating that the high Siglec-binding affinity of TPE3S was highly dependent on the multivalency of sialic acid moiety and the flexible structure in TPE3S (Scheme 1 and 2), which may trigger tremendous conformational polymorphism to fit into Siglec topography better. In addition, TPE3S was successfully used to bind to an array of (secreted) Siglecs for the concomitant assessment of Siglec distribution on PC-12 cell and HeLa cell surfaces. The tetravalent sialic acids-coated molecule TPE4S which had lower affinity for Siglecs but high sensitivity for sialidases, was used for the imaging of cellular sialidase activity (Scheme S1). Our current work provided a *"binding-on"* strategy to visualize Siglecs without involving the use of antibodies that are relatively expensive and unstable, and would also be benefit to maximize the signal-to-noise ratio.

Initially, to develop fluorescence-based glycoprobes with AIE characteristics to visualize the Siglecs distribution pattern on living cell membranes, the concept of modular design was used to construct Siglec-specific trisaccharide mimic of sialic acid (TPE3S). TPE3S includes three parts: i) the hydrophobic tetraphenylethylene (TPE) fluorophore, which not only permits TPE3S to be detected when it interacts with Siglecs, but is also conducive to increase the interactions of TPE3S and cell membrane by its hydrophobic characteristics;[19] ii) the multiple

[a] College of Chemistry, Beijing Normal University, Beijing, 100875, China.
[b] Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China.
[c] Analysis & Testing Center, Beijing Normal University, Beijing, 100875, China.
Electronic Supplementary Information (ESI) available: Experimental procedures for TPE3S and TPE1S; structural characterization data for TPE3S, TPE1S and intermediates; supplementary fluorescent spectra and cells images. See DOI: 10.1039/x0xx00000x



Scheme 1 Schematic of tetraphenylethene-decorated sialic acid derivative TPE3S structure, measuring and imaging.



Scheme 2 Structure of TPE4S and TPE1S.

sialic acid moieties which play crucial roles in carbohydrate-lectin binding interactions; iii) the connecting backbone, pentaerythritol with flexible structure, which gives multiple sialic acid moieties adequate flexibility to tune their spatial arrangement to exactly target different Siglecs. The synthesis of TPE3S was based on a versatile, converging, three-stage modular synthetic protocol shown as in Scheme S2. Firstly, azide-annexed sialic acid derivative Sia–N_3 was obtained using the preactivation strategy with (*p*-Tol)$_2$SO/Tf_2O as glycosylation promoters.[20,21] Then, azide-functionalized tetraphenylethene fluorophore was synthesized from 4,4'-dimethylbenzophenone and 4-bromobenzophenone in two steps, followed by coupling with perpropargylated pentaerythritol to yield TPE3 in moderate yields. TPE3S-Ac was prepared using the copper-catalyzed azide-alkyne Huisgen cycloaddition in the presence of $CuSO_4$ and sodium ascorbate. Finally, treatment of peracyl protected probe TPE3S-Ac with sodium methoxide in methanol followed by saponification of the methyl ester groups using LiOH afforded the fully deprotected product TPE3S in 70% yield. The structure of TPE3S was confirmed by NMR spectroscopy and HRMS spectra. In addition, the contrastive compound TPE1S was synthesized similarly (Scheme S3).

As shown in Fig. S1, sugar-bearing TPE3S exhibited greatly quenched fluorescence in aqueous solution due to its good solubility in PBS buffer. Whereas, an increased fluorescence of TPE3S with large Stokes shift value in the mixture of PBS and glycerin was observed since higher liquid viscosity restricted the rotation of the phenyl rings of TPE3S molecules, indicating that the maintenance of AIE activity of TPE fluorophore after incorporating multiple sialic acids into it. The good solubility of TPE3S in aqueous solution and the typical AIE activity of TPE3S should endow the probe with the advantages of low background noise and wash-free property over other analytical methods when used for visualizing Siglecs *in vivo*.

For analyzing the binding behavior between tetraphenylethene-decorated sialic acid glycocluster TPE3S and Siglecs, MST assays[18] were performed at different concentration ratios of the binding partners. Firstly, Siglec-2 was fluorescently labelled and kept at a constant concentration and TPE3S was titrated until a saturation of all binding sites were obtained. From the obtained data in Fig. 1a, the equilibrium dissociation constant (K_d) was determined to be about 14.2 μM, which was much smaller than that between natural sialosides and Siglecs (0.1–3 mM).[1,22] Absolute binding affinity between TPE3S and Siglec-3 was also determined using MST measurements. Fig. 1b indicated that the K_d was determined to be ~96.2 μM. Similarly, we then quantified the dissociation constant of TPE3S with Siglec-5 in PBS buffer and an affinity of 10.6 ± 3.3 μM was obtained (Fig. 1c). These results showed that TPE3S with flexible structures could bind directly with the CD33-related Siglecs (Siglec-3, Siglec-5) and the conserved Siglecs (Siglec-2) successfully.

To determine whether the measured MST response represented specific binding, bovine serum albumin (BSA) and mannoside-binding protein Concanavalin A (Con A), were chosen to evaluate the binding with TPE3S. The titration results show that no significant binding response could be observed with BSA or Con A upon addition of TPE3S (Fig. S2 and 1d), and non-specific binding of TPE3S with Siglecs was excluded.

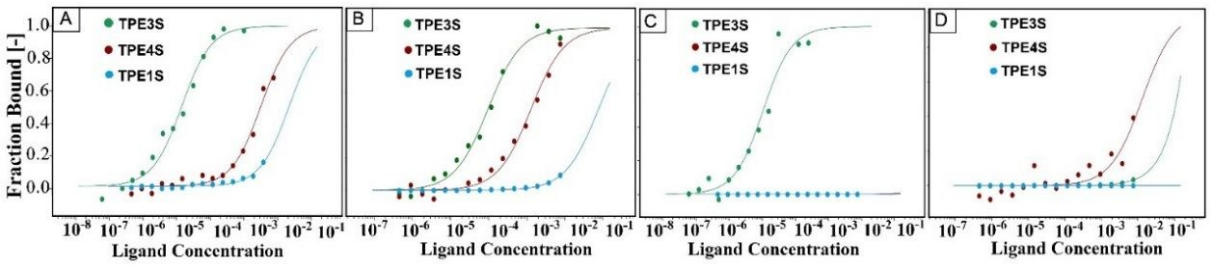


Fig. 1 Thermophoretic analysis of interactions between lectins and ligands in PBS. To determine the affinities of binding reactions, a titration series of concentrations of sialic acid bearing ligands partners were performed respectively while the fluorescent binding protein partners were kept at a constant concentration. The interactions of (a) Siglec-2, (b) Siglec-3, (c) Siglec-5, and (d) Con A with TPE3S (green spots), TPE4S (deep red spots) and TPE1S (blue spots) were measured as described in the supporting information.

Next, to further verify whether the carboxyl groups in TPE3S molecule could lead to false results in binding assays, contrastive compound TPE1S functionalized with an equal numbers of carboxyl groups with TPE3S was used to exclude non-specific contributions by electrostatic interactions. Compared with TPE3S, no signal changes were obtained using TPE1S in the MST titrations with Siglec-2, Siglec-3 and Siglec-5, respectively (Fig. 1a-c). We further analyzed the interactions between TPE1S and BSA and Con A with MST respectively, and found no binding for BSA and Con A (Fig. 1d and S2). These results substantiated the propositions that the presence of multiple sialic acid moieties, rather than carboxyl groups, enhanced the observed affinity of small-molecule ligand for Siglecs. Also, we can conclude that the weakness of single sialic acid-Siglec interactions may require the attachment of multiple sialic acid moieties to ligand for an efficient complex formation, *i.e.* multivalence glycocluster effect may trigger downstream responses that can be not achievable with single sialic acid.

To gain a better understanding of the interaction mechanisms between sialic acid binding membrane-anchored lectins and potential ligands, similar experiments were also performed with tetrasialic acids-coated tetraphenylethene (TPE4S),[26] which has relatively rigid structure features compared with TPE3S. To our surprise, the dissociation coefficient of TPE4S with Siglec-2 was determined to be K_d = 3.17 ± 0.8 mM, which is 223-fold higher than that between TPE3S and Siglec-2 (Fig. 1a and Table S1). A binding affinity of 1.29 ± 0.22 mM for Siglec-3 and no binding for Siglec-5 were further afforded from the MST experiments, which were also higher than that of TPE3S under the same conditions (Fig. 1).

The binding affinities of three sialic acid-hinged molecules with different lectins are summarized in Table S1. The data illustrated that although the ligand affinity for Siglecs was low at the level of a monovalent carbohydrate–Siglec binding interaction (like TPE1S), glycocluster effect indeed resulted in a significant higher affinity for Siglecs (like TPE3S). More importantly, it appeared unlikely that more sialic acid moieties could lead to higher affinity between low-molecular-weight ligands and Siglecs. The structure of sialylated molecules should have great influence on the affinities for Siglecs. We reasoned that TPE3S could deduce three sialic acid "*paws*" to undergo topographical changes to better mimic natural sialosides to act as potential ligand to recognize Siglecs, while the space distribution of four sialic acid moieties in TPE4S has relatively "*fixed*" positions. Moreover, since TPE3S can bind with Siglecs more tightly than natural sialosides (0.1–3 mM),[1,22] it should make TPE3S a powerful tool for Siglecs biosensing.

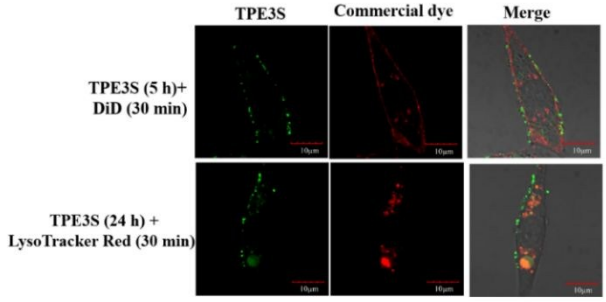


Fig. 2 Fluorescence confocal images of PC-12 cells incubated with TPE3S and commercial dyes DiD or LysoTracker Red (DiD is a commercially available membrane probe, LysoTracker Red is a commercially available lysosomal probe). Left panel is the confocal image from TPE3S on channel 1 (λ_{ex} = 405 nm, λ_{em} = 445-545 nm); Middle panel is the confocal image from DiD (λ_{ex} = 635 nm, λ_{em} = 655-755 nm) or LysoTracker Red (λ_{ex} = 559 nm, λ_{em} = 570-620 nm) on channel 2. Scale bars = 10 μm.

We then utilized TPE3S as a novel probe to study the Siglecs distribution patterns on cells based on the fluorescence techniques. After TPE3S was incubated with PC-12 cells for 5 hours, a bright fluorescence mainly on the surface of PC-12 cells was observed by confocal microscopy, whereas no obvious fluorescence could be observed in the culture medium and in cells (Fig. 2). Colocalization experiments with commercial organic fluorogen (DiD) available for cell membrane monitoring demonstrated that TPE3S mainly accumulated on cell surface in a patch-wise distribution. Even after incubation PC-12 cells with TPE3S for 24 hours, we found that most TPE3S molecules were still impermeable to cell membranes and accumulated on cell surface discretely (Fig. 2). These results indicated that TPE3S could specifically interact with Siglecs on the cytoplasmic membrane of PC-12 cells to restrict its intramolecular motions and trigger an efficient "turn-on" fluorescence.

Compared with sialocluster TPE3S, which had relatively higher binding affinities for Siglecs, TPE4S featured binding affinities in the millimolar range. To examine the ability of TPE4S to image Siglecs, confocal fluorescent imaging was also performed in cultured PC-12 cells. Fig. S3 shows that TPE4S was not prone to accumulate on cell surface and the intracellular fluorescence was detected. Meanwhile, no fluorescence was observed in the culture media without washing process before imaging. As TPE4S was an excellent probe for sialidase activity determination,[23] the light-up fluorescence signals in cells strongly suggested that TPE4S was cell membrane permeable and should be hydrolyzed by the intracellular sialidases specifically to break the glycosidic bond to form hydrophobic hydrolytic product. Further colocalization studies (Fig. S4) with LysoTracker Red as standard lysosomal markers demonstrated that the fluorescent molecules was mainly localized in lysosomes. These results were well consistent with the previous research results, *i.e.* sialidases Neu1 and Neu4 expressed in mammalian cells mainly existed in lysosomes.[24] Therefore, for the first time, TPE4S was considered as a potential tool with a visual sensitivity for identification and in situ imaging of cellular sialidases under physiological conditions.

Furthermore, as a negative control, TPE1S can not bind with Siglecs and there was only around a 1.5-fold increase in the fluorescence intensity after immersion of TPE1S with siliadase (Fig. S5). We found that after treatment of PC-12 cells with TPE1S, fluorescence images showed much weaker than those after incubation with TPE3S or TPE4S (Fig. S6), which in turn reflected the low affinity of TPE1S with Siglecs and the poor capability of TPE1S for sialidase detection.

Collectively, in some sense, the cellular imaging experiments explicitly reflected the consistency of the above affinity studies carried out on MST. Better than TPE4S and TPE1S, TPE3S selectively recognized and interacted with Siglecs on the cell surface due to its higher affinity to sialolectins, resulting in the

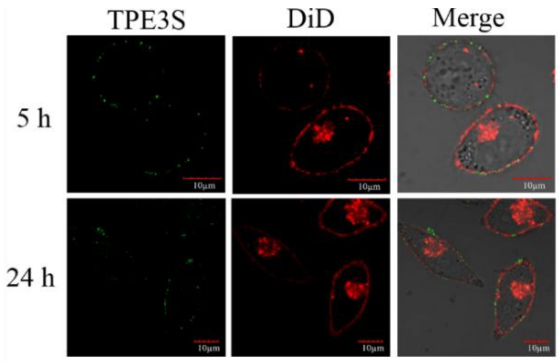


Fig. 3 Fluorescence confocal images of HeLa cells incubated with TPE3S (50 μM) and commercial dye DiD (DiD is a membrane probe). Left panel is the confocal image from TPE3S on channel 1 (λ_{ex} = 405 nm, λ_{em} = 440-540 nm); Middle panel is the confocal image from DiD on channel 2 (λ_{ex} = 635 nm, λ_{em} = 650-750 nm). Scale bars = 10 μm.

bright fluorescence images of Siglecs due to the AIE characteristics. Aimed to build a novel platform that could sensitively image Siglecs and sialidases using TPE3S and TPE4S respectively, the biocompatibility and toxicity of TPE3S and TPE4S were evaluated by MTT method. The data indicated that there were no significant differences in cell viability between the initial and that after incubation for 24 h, suggesting the low-toxicity of the sialyl derivatives used in this study (Fig. S7).

To further validate the efficiency of TPE3S for the detection of Siglecs expressed on the surface of mammalian cells, similar experiments were subsequently conducted using cervical cancer HeLa cells. Interestingly, colocalization experiments performed using DiD as control revealed that TPE3S principally enriched on the membrane of HeLa cells discretely after incubation for 5 hours (Fig. 3). Even after incubation with TPE3S for 24 hours, TPE3S still "shine" on the DiD membrane probe. The observed intense emission of TPE3S presented on HeLa cells further indicated that the specific binding between TPE3S and Siglecs. Therefore, TPE3S could be used to target Siglec-expressing cells and to visualize Siglecs efficiently.

In conclusion, we described an AIE-active *binding-on* fluorescent Siglec-targeting sialocluster (TPE3S) consisting of multivalent sialic acid moieties. TPE3S was equipped *via* click coupling reactions of alkynyl terminated pentaerythritol, azide-annexed TPE and sialic acids. The specificity of TPE3S toward Siglec-2, -3 and -5 was determined using MST technique which was suitable for the analysis of various binders. The contrast experiments with TPE1S and TPE4S suggested that both the number of sialic acid moieties and their spatial arrangement were indispensable for the promising capability of TPE3S toward Siglecs. Furthermore, TPE3S was used for the visualization of Siglecs expressed on the surface of PC-12 and HeLa cells successfully. However, TPE4S with lower affinity for Siglecs but being high sensitive for sialidase has been shown to be useful in imaging cellular sialidase with satisfactory biocompatibility. In addition, in comparison with the antibodies against Siglecs, TPE3S was a broad-spectrum ligand for most Siglecs with relatively low specificity and affinity. Further development of high-affinity ligands for individual Siglec members by chemical modifications of the sialic acid backbone is in progress.

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Conflicts of interest

There are no conflicts to declare.

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