Gangliosides

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Quantitative Screening of Cell-Surface Gangliosides by Nondestructive Extraction and Hydrophobic Collection

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Abstract: *A screening strategy involving designed extractors and collectors was used for the nondestructive quantitation of gangliosides on cell surfaces. The extractors were constructed by functionalizing maleimide silica bubbles with a DNA probe, which contains an endonuclease cleavage site and a boronic acid end to extract cell-surface sialic acid-containing compounds through simple centrifugation. After the extractors containing the extracted compounds were incubated with endonuclease, the released oligonucleotide-gangliosides were selectively collected by silanized collector bubbles through hydrophobic interactions. The in vitro fluorescent signals from the collectors were used for the quantitation of cell-surface gangliosides. By combining with sialidase cleavage, a protocol for the identification of ganglioside subtypes was developed. The successful monitoring of the regeneration of cell-surface gangliosides demonstrates the potential of this strategy in probing related biological processes.*

Gangliosides are a type of glycosphingolipid with one or more sialic acid (Sia) residues. They are distributed on the cell surface by embedding into the plasma membrane through two hydrocarbon chains of the ceramide moiety to mediate cell structure, cell adhesion, and signal transduction[1,2] and to act as acceptor sites of microbial toxins, bacteria, and viruses.[3,4] Aberrant pathways of gangliosides are related to certain diseases, such as hereditary disorders[5] and malignant transformations of cancer,[6,7] and overexpression of different gangliosides subtypes is related to different types and progression of tumors.[8] Thus, the screening of cell-surface gangliosides is important to reveal ganglioside-related biological processes and potential tumor progression.

The analysis of gangliosides is generally performed with mass spectrometry (MS). This technology can determine their molecular structures.[9,10] Some chromatographic and immunochemical techniques have also been developed for the detection of primary species of major gangliosides.[11,12] However, these methods need to destroy tissues or cells to separate gangliosides through complicated procedures, which leads to a loss of target information. Although FRET-based fluorescence probes have been designed to detect ganglioside-related enzyme activity on the cell surface,[13] they cannot provide quantitative information on gangliosides. Therefore, there is an urgent need for a quantitative protocol for the analysis of cell-surface gangliosides.

Based on the design of two types of functional microbubbles (diameter = 30 μm, density = 0.6 g mL^{-1}), an extractor and a collector, this work proposes a quantitative strategy for the nondestructive screening of gangliosides on the cell surface (Figure 1). The extractor was constructed by linking thiol-terminated DNA probes (HS-DNA1/2-APBA) to a maleimide bubble, which was prepared by binding succinimidylester-PEG$_2$-maleimide (NHS-PEG$_2$-Mal) as a linker on an (3-aminopropyl)triethoxysilane (APTES) silylated microbubble. The DNA probe had a 3-aminophenylboronic acid (APBA) end on DNA1 and an endonuclease cleavage site formed by hybridization of 6-carboxyfluorescein (6-FAM)-labeled DNA1 and DNA2 (Supporting Information, Figure S1). The collector was a trichloro(octadecyl)silane (OTS) silylated micro-bubble. After the Sia-containing compounds were bound to APBA on the extractors,[14] they could be

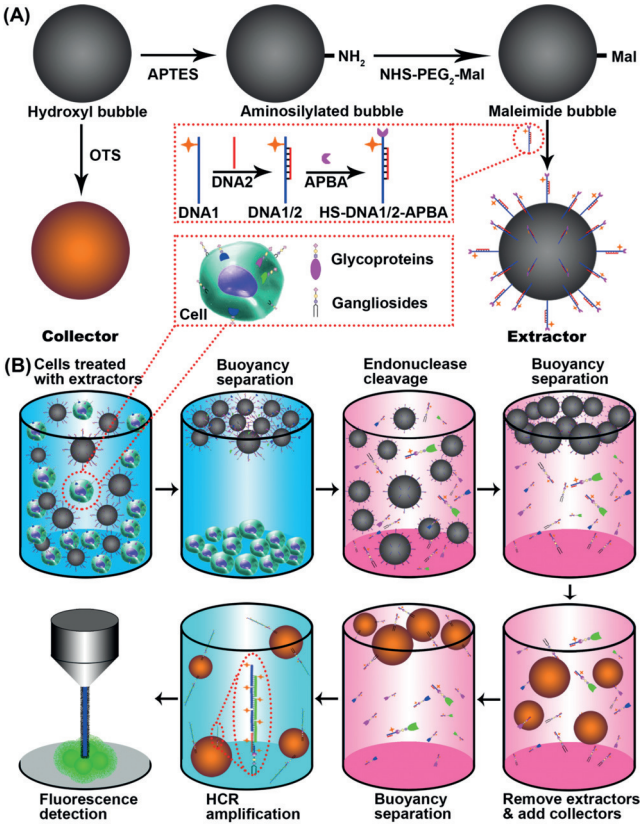

Figure 1. Schematic of A) the preparation of the extractor and collector and B) quantification of cell-surface gangliosides.

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extracted from the cell surface through buoyancy and then released into solution by endonuclease cleavage. Oligonucleotide-gangliosides could be selectively collected by collectors through hydrophobic interactions between the octadecyl moieties and gangliosides, which could be sensitively detected with the hybridization chain reaction (HCR) technique.[15] Moreover, the profile of ganglioside subtypes could be achieved through sialidase cleavage of Sia and subsequent recognition of fluorescein-labeled lectins. The regeneration of gangliosides on the cell surface could also be conveniently monitored.

The characterization of the functional micro-bubbles was demonstrated by contact angle measurements, gel electrophoresis, and fluorescence spectroscopy and imaging. The contact angles of unmodified micro-bubbles (61.8°), collectors (135.6°), and extractors (15.7°) confirmed the hydrophobic collector and hydrophilic extractor surfaces (Supporting Information, Figure S2). Gel electrophoresis verified the function of the designed DNA probe (Supporting Information, Figure S3), which was formed through the efficient hybridization of DNA1 and DNA2 (lane 3) and could be cleaved by endonuclease (lane 4) to hybridize with the HCR primer (lane 8) to trigger the HCR amplification (lane 9). The conjugation of APBA to the carboxyl on DNA1 was confirmed by the molecular ion peaks (Supporting Information, Figure S4). After linking the HS-DNA1/2-APBA to maleimide bubbles, the amount of HS-DNA1/2-APBA conjugated to each bubble was calculated to be 2.3×10^6 molecules with the fluorescence decrease of the mixture supernatant compared to the initial HS-DNA1/2-APBA solution (Supporting Information, Figure S5). After the extractors were incubated with endonuclease, about 97% of HS-DNA1/2-APBA on the extractors were cleaved and released into the supernatant. The fluorescence images also showed similar results (Supporting Information, Figure S6), further confirming the successful construction of the extractors and the release of cleaved product from the extractors.

To verify the collecting ability of the collectors, fluorescein-labeled 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine (fluorescein-DSPE) with a similar hydrophobic structure to the gangliosides was synthesized (Supporting Information, Figure S7). The collectors themselves were nonfluorescent (Figure S6). After they were incubated with 5-carboxyfluorescein (5-FAM), fluorescein-DSPE, and fluorescein-labeled EpCAM antibody, only the fluorescein-DSPE-treated collectors exhibited obvious fluorescence (Figure S6), indicating that the hydrophilic molecules and proteins could not adsorb onto the collectors, while the hydrophobic molecules could be collected by the collectors through hydrophobic interactions.

Breast cancer MCF-7 cells with abundant gangliosides[16,17] were used to examine the extracting ability of the extractors. As shown in Figure 2A, the cells were first incubated with tetraacetylated N-azidoacetyl-D-mannosamine (ManNAz) to metabolically form an azide on the Sia residues,[18–20] which further conjugated dibenzocyclooctyne-Cy3 (DBCO-Cy3)[21–23] to obtain Cy3-conjugated cells (Cy3-cells). The labeling procedure did not affect the conjugation of APBA to Sia because of the different labeling[23] and

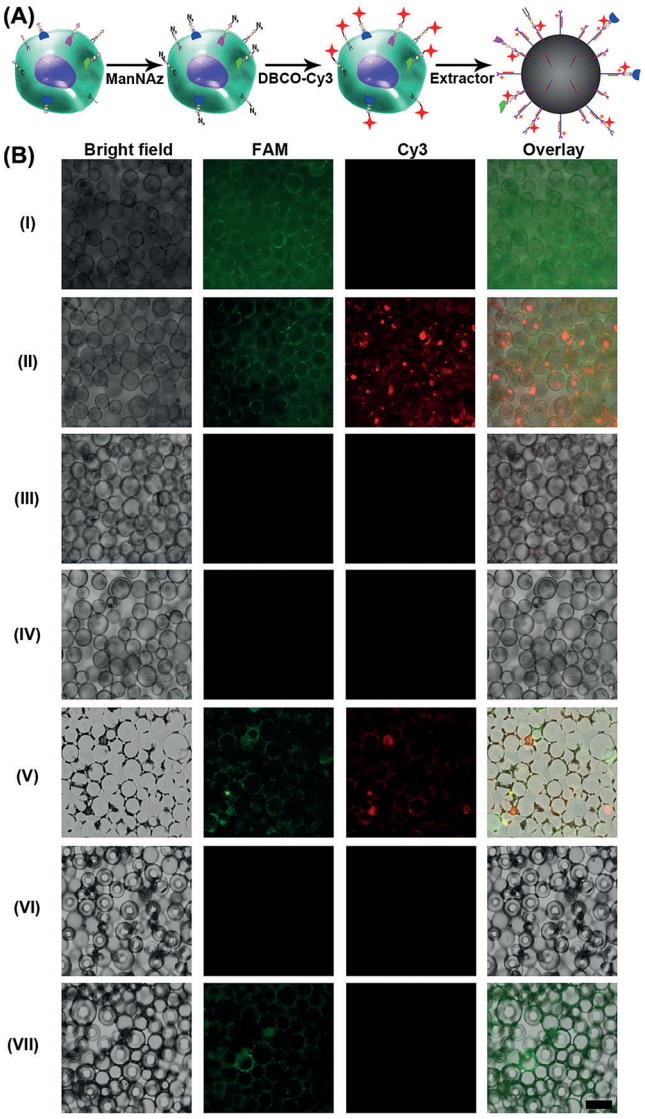

Figure 2. A) Schematic of the preparation of Cy3-cell-treated extractors. B) Bright field, fluorescence (FAM and Cy3 channels), and overlay images of (I) extractors, (II) Cy3-cell-treated extractors, (III) Cy3-cell-treated extractors after endonuclease cleavage, (IV) collectors, (V–VII) collectors incubated with released extracts from (V) Cy3-cell-treated extractors, (VI) extractors, and (VII) GD1a-treated extractors during endonuclease cleavage. Scale bar = 60 μm.

binding[24] sites (Supporting Information, Figure S8). Thus, APBA could bind all Sia-containing compounds on Cy3-cells. After the extractors were incubated with Cy3-cells and then separated by centrifugation, they showed the obvious fluorescence from Cy3 and the initial FAM (Figure 2B, II). Meanwhile, Cy3 fluorescence on Cy3-cells exhibited a corresponding decrease after treatment with the extractors (Figure S8), which was also observed by flow cytometry. Interestingly, the Cy3 fluorescence was not observed for the no-APBA extractors and did not change on Cy3-cells after the same incubation process (Supporting Information, Figures S9 and S10). These phenomena confirmed the APBA-mediated specific extraction of the Sia-containing compounds from the

cell surface. After the Cy3-cell-treated extractors were incubated with endonuclease, the fluorescence from both FAM and Cy3 disappeared (Figure 2B, III), and the mass spectrum of the released extracts showed the peaks of oligonucleotide-gangliosides (Supporting Information, Figure S11), indicating the release of Sia-containing compounds from the extractors.

After incubating the collectors with the released extracts from Cy3-cell-treated extractors, both FAM and Cy3 fluorescence was observed on the collectors (Figure 2B, V), and the peaks of the oligonucleotide-gangliosides disappeared from the mass spectrum of the released extracts (Figure S11). The FAM fluorescence was also observed on the collectors after incubation with the released extract from GD1a (a commercially available ganglioside)-treated extractors (Figure 2B, VII). In the absence of the hydrophobic structure, the incubation of the collectors did not show the FAM fluorescence (Figure 2B, VI). Thus, the collectors could not collect the oligonucleotide fragments themselves. On the cell surface, the Sia-containing compounds are mainly gangliosides, glycoproteins, and free carbohydrates.[25] Considering that the collectors could not absorb hydrophilic molecules and proteins (Figure S6), these results demonstrated the hydrophobic collection of oligonucleotide-gangliosides in the released extracts from both Cy3-cell- and GD1a-treated extractors.

The HCR amplification was performed by incubating the oligonucleotide-ganglioside-adsorbed collectors with the mixture of HCR primer and hairpin H1 and H2 in hybridization buffer. Clearly, the HCR enhanced the fluorescence signal from the collectors (Supporting Information, Figure S12), which led to sensitive in vitro detection of cell-surface gangliosides. The concentration ratio of the extractors to cells was optimized to be 8:1, and the extracting time was optimized to be 4 h, after which the complete extraction of cell-surface Sia-containing compounds could be achieved (Supporting Information, Figure S13). The optimal concentration ratio of the collectors to cells and collecting time were 0.15:1 and 90 min, respectively (Supporting Information, Figure S14).

Under the optimal conditions, a quantitative method for detection of cell-surface gangliosides was designed with GD1a as the standard target. The fluorescence intensity (I) of FAM on GD1a-relased-extract-treated collectors was linear with the GD1a concentration in the range from 0.5 to 2.5 nM (Supporting Information, Figure S15A,C). The total amount of gangliosides on the cell surface could be obtained from the fluorescence intensity of FAM on cell-released-extract-treated collectors. Using MCF-7 cells as a model, the plot of I versus cell concentration was linear in the range from 2.0×10^4 to 6.0×10^4 cells mL^{-1} (Figure S15B,D). Given that the difference in affinity of APBA to different gangliosides was negligible, the average number of ganglioside molecules on each MCF-7 cell was calculated to be 1.0×10^7.

Gangliosides have diverse structures. These structures may be classified into three subtypes (GX1, GX2, and GX3; Supporting Information, Figure S16), which can be analyzed by recognition of different lectins after cleaving all Sia groups, leading to a method for subtype screening of cell-surface gangliosides (Figure 3A). As a proof-of-concept, the collectors treated with MCF-7 cell released extracts were incubated with sialidase to remove all Sia groups, which was demonstrated through the disappearance of FAM fluorescence (Figure 3B). The Sia-cleaved collectors were then incubated with fluorescein-labeled lectins, F-Jacalin,[26] F-*Ricinus communis* agglutinin I (F-RCA I)[27], and F-*Vicia villosa* lectin(F-VVL)[28] for recognition of galactosyl (β-1,3) N-acetylgalactosamine (Galβ3GalNAc), galactose (Gal), and N-acetylgalactosamine (GalNAc), respectively. After incubation for 1 h, these lectin-treated collectors showed different fluorescence intensities (Figure 3B,C). Thus, the relative quantities of GX1, GX2, and GX3 on collectors or the cell surface were calculated to be 48%, 37%, and 15% (Figure 3D).

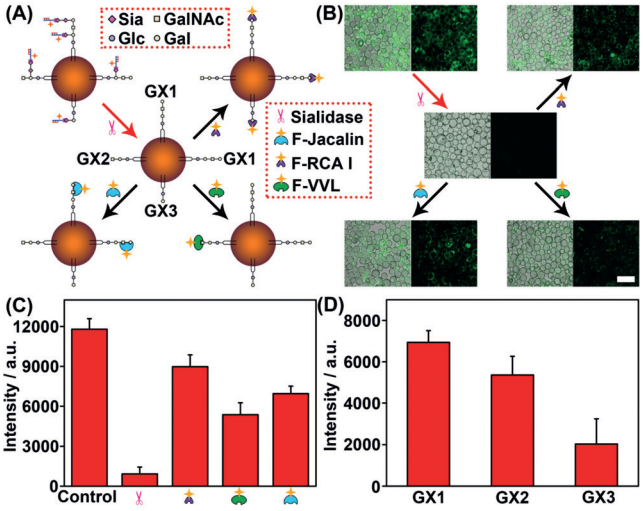

Figure 3. A) Schematic of subtype screening of gangliosides. B) Fluorescence and overlay images of collectors incubated with released extracts from cell-treated extractors during endonuclease cleavage, and then sialidase, and treated with F-Jacalin, F-RCA I and F-VVL, respectively. Scale bar = 100 μm. C) Fluorescence intensities in (B) from green channel. D) Fluorescence intensities from (C) for subtype GX1, GX2, and GX3.

Cell-surface gangliosides are dynamically synthesized and degraded, and the regeneration rates of different gangliosides subtypes are related to different biological processes.[29] To monitor the regeneration of cell-surface gangliosides, MCF-7 cells were treated with extractors at the extractors-to-cells ratios of 2:1, 4:1, 6:1, or 8:1, and then collected to re-culture for different times (Figure 4A). Their growth curves indicated that cells with greater losses of Sia-containing compounds exhibited lower growth rates (Figure 4B), which was possibly due to the decreased expression of the growth factor receptors induced by a low level of gangliosides on the cell surface.[30,31] However, cells with complete extraction of Sia-containing compounds showed cell viabilities of 75.5% and 94 or 88%, corresponding to extractors-to-cells ratios of 2:1 or 4:1 (Supporting Information, Figure S17), indicating the mechanical extraction process did not endogenously influence the vitality of the cells. The amounts of residual

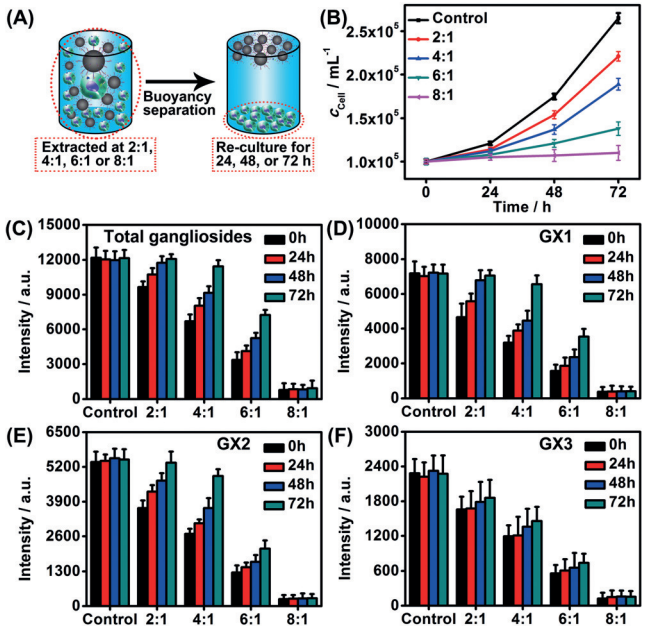

Figure 4. A) Schematic of re-culture of cells for different incubation times after surface gangliosides were extracted at different ratios of extractors to cells. B) Growth curves of MCF-7 cells after treatment with different amounts of extractors. Intensities from green fluorescence channel for C) total gangliosides, D) GX1, E) GX2, and F) GX3 on MCF-cells treated with different amounts of extractors and then re-cultured for different times.

gangliosides on the extracted cells and the regenerated gangliosides were detected by the designed method. The cells with low extraction and high viability showed full regeneration of the gangliosides, while the cells with complete extraction did not show the regeneration of the gangliosides (Figure 4C). At the extractors-to-cells ratio of 6:1, only 28.0% of gangliosides were left on cell surface, which could regenerate to 59.5% of the initial amount after re-culture for 72 h. Thus the regenerative ability of gangliosides depended on the residual Sia-containing compounds on the cell surface, indicating a self-feedback signaling pathway for the regeneration of gangliosides to maintain a certain expression level.[32] The subtype of gangliosides was further screened, which indicated that the regenerative ability of different subtypes followed the sequence: GX1 > GX2 > GX3 (Figure 4D–F). Thus, the overall regenerative ability resulted from the diverse structure and proportion of the subtypes.

In conclusion, this work proposes a versatile technique for quantitative screening of cell-surface gangliosides with two newly designed functional micro-bubbles, the extractor and the collector, which have the capability of nondestructive specific extraction and hydrophobic collection of gangliosides, respectively. Combining with in vitro signal amplification and sialidase cleavage, the quantitation, subtype screening, and monitoring of regeneration of gangliosides on the cell surface have been achieved. Three subtypes of gangliosides on MCF-7 cells have been classified, and the amounts of these subtypes have successfully been monitored during the treatment and regenerative processes. The designed method can be easily extended to other biological molecules on the cell surface by changing the corresponding extracting and collecting functional groups, demonstrating its practicality for the detection of cell-surface molecules and probing the related biological processes.

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Conflict of interest

The authors declare no conflict of interest.

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