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We have developed a supramolecular imaging probe based on thin-layer manganese dioxide functionalized with a fluorescent, multivalent glyco-poly-cyclodextrin for the targeted, stimulus-responsive bioimaging of cancer cells.

This paper describes the supramolecular construction of a biocompatible thin-layer manganese dioxide-based probe for the targeted, biothiol-responsive fluorescence imaging of liver and triple-negative breast cancer cells. Target-specific imaging is crucial for improving the precision of diagnostics. Specificity can be achieved through the simultaneous targeting of a membrane receptor and the stimulus-responsive signal production upon interacting with an intracellular biomarker. However, the majority of the current small-molecular probes or polymeric materials for sensing the overexpression of an intracellular disease biomarker lack the ability to be actively internalized by living cells. To address this issue, recent literature has seen an active movement towards the introduction of a targeting agent such as aptamers, peptides and carbohydrates to the imaging material, increasing the accumulation, and thus the stimulus-responsiveness of the probes in a target cell or tissue.  

There are two key issues that need to be carefully considered to achieve targeted bioimaging. First, multivalent display of the targeting ligands on a material scaffold is crucial for significantly increasing the binding avidity for cell-surface receptors; this is particularly important for carbohydrate-based materials. On a second front, the materials used should be biocompatible in order to achieve real-world applications. Despite the extensive development of oligo- and polymeric architectures to realize multivalency through covalent bonding chemistries, simple yet effective functional imaging probes formed through the non-covalent supramolecular approaches remain much less explored.

Here we show the construction of an imaging probe based on 2D manganese dioxide (2D probe) using two supramolecular...
approaches. Host-guest interaction between an adamantane-grafted fluorescent glycoprobe and poly-β-cyclodextrins (poly-CD) with different CD-grafting ratios (Fig. 1a, where m and n are the equivalent of CD and that of acrylamide, respectively) forms a multivalent, fluorescence-enhanced glycodot, which can be subsequently self-assembled with a biocompatible 2D material (manganese dioxide – MnO₂), producing the stimulus-responsive, fluorogenic 2D probe (Fig. 1b) capable of target-specific imaging of liver cancer and triple-negative breast cancer cells (Fig. 1c).

![Diagram of concentration-dependent fluorescence enhancement](image)

Fig. 2 Concentration-dependent fluorescence enhancement of (a) Man-probe (1 μM) with increasing poly-CD₆₂₀ (0-90 μg mL⁻¹; interval: 15 μg mL⁻¹) and (d) Gal-probe (1 μM) with increasing poly-CD₆₂₀ (0-90 μg mL⁻¹; interval: 15 μg mL⁻¹). Concentration-dependent fluorescence quenching of (b) Man-dot (Man-probe/poly-CD₆₂₀ = 1 μM/80 μg mL⁻¹) and (e) Gal-dot (Gal-probe/poly-CD₆₂₀ = 1 μM/80 μg mL⁻¹) with increasing 2D MnO₂ (0-5 μg mL⁻¹; interval: 1 μg mL⁻¹). Concentration-dependent fluorescence recovery of (c) 2D Man-probe (Man-probe/poly-CD₆₂₀/2D MnO₂ = 1 μM/80 μg mL⁻¹/5 μg mL⁻¹) with increasing GSH and concanavalin A (Con A, a mannose selective lectin) and (f) 2D Gal-probe (Gal-probe/poly-CD₆₂₀/2D MnO₂ = 1 μM/80 μg mL⁻¹/5 μg mL⁻¹) with increasing GSH and peanut agglutinin (PNA, a galactose selective lectin). The fluorescence spectra for glycoprobos and glycodots were obtained in phosphate buffered saline (PBS, 0.01 M, pH 7.4). The fluorescence spectra for 2D probes were obtained in Tris-HCl (0.01 M, pH 7.4). Excitation wavelength: 450 nm; slit widths ex = 5 nm and em = 5 nm.

Cyclodextrins (CDs) have been widely used as a macrocyclic host molecule by the supramolecular inclusion of hydrophobic guest compounds in aqueous solution. The supramolecular “host-guest” chemistry of CDs plays an important role in photochemistry, analytical science, materials science and chemical biology. They have also been used extensively for enhancing the drug-delivering efficacy in vivo.⁶⁻⁸ With the rapid progress in the discovery of advanced new materials, a myriad of 2D materials have been synthesized and employed for biosensing and bioimaging.⁹ The 2D materials developed, thin-layer MnO₂ has proven to be rapidly degradable in the presence of reducing agents¹⁰ such as the physiologically important glutathione (GSH) that exists at higher levels in cancer cells.¹¹⁻¹³ However, efforts to incorporate the biocompatible poly-CD with 2D MnO₂ producing functional material architectures for biomedical applications remain rare. To the best of our knowledge, we describe the first supramolecular ensemble formed by functionalized poly-CDs and 2D MnO₂ for targeted, biothiol-responsive fluorescence imaging. We began with the design and synthesis of a naphthalimide glycoprobe (Fig. 1a and Scheme S1). In previous studies, we have shown that the introduction of carbohydrate targeting agents to naphthalimide based probes significantly enhanced their cell-targeting ability and imaging of intracellular species with a lowered cytotoxicity. We synthesized two such glyco-naphthalimide probes with galactose and mannose as the targeting ligand for the asialoglycoprotein receptor (ASGPR) and mannose receptor (MR) that exist on the surface of different cancer cells, respectively. An additional adamantane group was introduced to the probes for host-guest assembly with CDs,¹⁵⁻¹⁶ producing the Man-probe and Gal-probe (Fig. 1a). The CD monomer (6-α-cyclamido-β-CD) was synthesized according to the literature,¹⁷ and a subsequent radical binary copolymerization between acrylamide and the CD-monomer with different loading concentrations afforded the poly-CDs with different CD-grafting ratios (poly-CD₆₂₀/poly-CD₁₀₀ and poly-CD₃₀₀, where the numbers refer to the equivalent of acrylamide in the co-polymer).

With the compounds and polymers ready, the self-assembly was carried out in a phosphate buffered saline (PBS, 0.01 M, pH 7.4) as solvent. We determined that the fluorescence of the two glycoprobos enhanced gradually with added poly-CDs (Fig. 2a and Fig. 2d for Man-probe with poly-CD₆₂₀ and Gal-probe with poly-CD₆₂₀, respectively; for fluorescence spectra of the glycoprobos with all the poly-CDs, see Fig. S1a), suggesting that the host-guest insertion of adamantane to the CD cavities might enhance the hydrophobic environment and thus enhance the fluorescence emission.¹⁸⁻¹⁹ We then observed that the enhanced fluorescence intensity of the probes was proportional to the CD density of the polymers (poly-CD₆₂₀/poly-CD₁₀₀/poly-CD₃₀₀) (Fig. S1b). This suggests that the polymers with a higher CD density can host more guest molecules to better enhance the overall fluorescence emission. To corroborate that the fluorescence enhancement was the result of host-guest interactions, we used 1-bromonaphthalene as a competing molecule. The results indicated that the presence of 1-bromonaphthalene decreased the fluorescence of the glycoprobe/poly-CD ensembles (glycodots), suggesting the interruption of the adamantane-CD binding (Fig. S2).

Having developed the glycodots, we set out to further construct the 2D probe. The glycodots were mixed with an aqueous dispersion of 2D MnO₂ (with a size distribution in the nanometer range – Fig. S3) produced using an established method.²⁰ Then, the assembly was measured by fluorescence spectroscopy. We observed a concentration-dependent fluorescence decrease of the glycodots with 2D MnO₂ (Fig. 2b and Fig. 2e for a Man-dot and a Gal-dot, respectively), which is in agreement with the fluorescence quenching property of the 2D material for surface-bound fluorophores.²¹,²² The quenching plateau was reached with 5 μg mL⁻¹ of the 2D material. The 2D probes were shown to be thermally stable over a temperature range of 0-200 °C as determined by thermogravimetric analysis. (Fig. S4).

Next, we tested the stability of the 2D probes with both lectins (proteins that selectively recognize carbohydrates) and GSH. Interestingly, whereas the presence of selective lectins (concanavalin A and peanut agglutinin for mannose- and galactose-based glycodots, respectively) caused minimal
fluorescence recovery, the addition of GSH completely restored the fluorescence of the glycoconjugates (Figs. 2c and 2f for 2D Man-probe and 2D Gal-probe, respectively; for original fluorescence spectra, see Fig. S5). This implies that the poly-CD probes are stably immobilized on the surface of 2D MnO₂, whereas the degradation of the substrate material by GSH release the fluorescent polymers, thus producing a stimulus-responsive, fluorogenic 2D probe. The stability over carbohydrate receptors is meaningful since we envision that the glycoconjugates on the material surface would remain integrated upon receptor endocytosis at the cellular level but become activated only when a target intracellular marker for material disruption exists, thereby improving the imaging accuracy.

With these promising solution-based results in hand, we evaluated the imaging ability of the 2D probes. We first used the glycoconjugates consisting of different polymer backbones and glycoprobe alone (8 μM) or glycoconjugates (glycoprobe/poly-CD = 8 μM/640 μg mL⁻¹) for imaging solutions. The choice of the density in CD fraction of the poly-CDs had a significant impact on the imaging efficiency. The poly-CD₄₀₀ and poly-CD₉₆ scaffold produced the highest imaging brightness for MDA-MB-231 and Hep-G2 cells, respectively, among other control poly-CDs (Fig. S6). This suggests the sharp difference in preferred multivalent glycoconjugates of ASGP₄ and MR probably because of the different membrane-bound architectures of the receptors.₂₆-²₈

Encouraged by these results, we used the thin-layer MnO₂ base 2D probes for GSH-responsive imaging of the target cancer cells. Owing to the presence of a high level of endogenous GSH in cancer cells, the cells were pre-treated with GSH (a GSH quencher) followed by incubation of exogenous GSH to examine the sensitivity of the probes. We observed that the fluorescence of the 2D probes produced in untreated MDA-MB-231 was almost completely suppressed in the cells pre-treated with NEM (N-ethylmaleimide) (Fig. 4). However, the subsequent addition of exogenous GSH further enhanced the fluorescence. These results indicate that 1) the 2D probe is GSH-responsive probably through the intracellular degradation of 2D MnO₂ by GSH found in the cells, and that 2) the 2D probe does not produce the imaging signal without intracellular GSH, suggesting good stability of the supramolecular architecture. The second point is particularly important for imaging probes since false-positive signals produced by the non-specific interaction within complex cellular environments could be minimized.

To conclude, we have developed a 2D probe for the targeted, fluorogenic imaging of cancer cells through both host-guest interaction.
interactions and self-assembly between fluorescent polymers and 2D materials. The simplicity in material construction yet effectiveness of the probes for stimulus-responsive fluorogenic imaging makes it possible to extend these systems for an even wider diversity of supramolecular, biocompatible imaging probes for functional bioimaging.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Notes and references