Fluorogenic 2D Peptidosheet Unravels CD47 as a Potential Biomarker for Profiling Hepatocellular Carcinoma and Cholangiocarcinoma Tissues

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Hepatocellular carcinoma (HCC), which is the most common form of liver cancer, represents the third-leading origin of cancer-related death.[1] Similar to HCC, cholangiocarcinoma is a destructive malignancy that is difficult to diagnose and that is associated with a high mortality rate.[2] As a consequence, there is urgent need for diagnosis of HCC and cholangiocarcinoma at an early stage where effective interventions can be conducted.[3] Cancer biomarkers are objective and specific indicators of cancer-related processes, and are widely used for assessing the disease state of an individual, thereby shedding light on appropriate and timely therapeutic interventions. Over the past few decades, several biomarkers for HCC have been identified, for example alpha-fetoprotein (AFP) and glypican-3 (GPC3), which can function both as diagnostic and therapeutic targets.[4] However, the sensitivity and specificity of these HCC biomarkers remain to be improved. In particular, rare biomarkers for cholangiocarcinoma have been identified.[5] On the basis of such evidence, the identification of more effective biomarkers is required for the diagnosis and treatment of hepatobiliary cancers.

CD47 (also called integrin-associated protein) is a membrane protein that is composed of an extracellular immunoglobulin variable (IgV)-like domain, five membrane spanning segments and an alternatively spliced carboxy-terminal cytoplasmic tail ranging from 3 to 36 amino acids in length. A long-distance disulfide bond links Cys33 residue in the IgV-like domain to Cys263 residue in the last extracellular loop, and mutation of these residues could disrupt CD47 signaling. Although a high-resolution X-ray crystallographic structure of CD47 has been reported, the IgV-like domain crystallized as a dimer with G strands swapped between the two domains. Therefore, the exact function of CD47 is not fully understood. The extracellular domain of CD47 interacts with three classes of ligands: TSP1 (thrombospondin-1), the signal-regulatory protein family and integrins. The IgV-like domain is N-glycosylated and modified with an O-linked glycosaminoglycan. The five N-glycosylation sites are not necessary for SIRPα binding, but the glycosaminoglycan modification is necessary for TSP1 binding to CD47.[6]

It has been determined that the expression of CD47 is elevated in many types of cancer, such as ovarian carcinoma, multiple myelomas and T-cell acute lymphoblastic leukemia.[7–9] In comparison with cancer tissues, the expression level of CD47 is relative low in normal tissues, suggesting that CD47 could be an effective biomarker for cancer diagnosis. Further, it has been reported that CD47 expressing on cancer cells can interact with signal regulatory protein α (SIRPα) in macrophage, thus compromising macrophage-mediated phagocytosis against tumor cells.[10] Blocking of CD47-SIRPα signaling axis with antibodies or recombination proteins can retard tumor progression,[11–13] implying that CD47 may also function as a tumor immunotherapy target. However, to our knowledge, so far the protein expression pattern of CD47 in HCC and, especially in cholangiocarcinoma remains poorly understood. This is largely because of the lack of effective tools to profile membrane proteins using cells and tissue samples. Here we show that CD47 is highly expressed in both HCC and cholangiocarcinoma as unraveled by a 2D material composite.

Because of their outstanding optoelectronic properties, increasingly much effort has been devoted to the development of functional 2D graphene analogs, such as transition metal dichalcogenides and oxides (TMD/Os), by the academic and industrial communities.[14–17] Despite being at an embryonic stage, several impressive breakthroughs have already been made in the biomedical applications of 2D TMD/Os in the past few years.[18–21] On the one hand, 2D TMD/Os composites have been developed for sensing biomacromolecules such as nucleotides and proteins,[22–24] On the other hand, considering their good biocompatibility and low toxicity, these materials have also shown the promise for multimodal (e.g., photodynamic, photothermal and photacoustic) theranostics of cancer.[25–35] However, the amenability of 2D TMD/Os to imaging of clinical specimens has been rarely explored. Here we show that a 2D molybdenum disulfide (MoS2) based “peptidosheet” can be used for the fluorogenic profiling of HCC and cholangiocarcinoma tissues over healthy tissues on clinical samples (Figure 1). The supramolecular assembly of a fluorophore-tagged peptide ligand to a polyethylene glycol (PEG)-coated...
2D MoS₂ sheet forms the “peptidosheet” with quenched fluorescence (Figure 1a). Then, the fluorogenic peptidosheet can target and thus image cancer tissues that overexpress CD47 over healthy tissues with minimal CD47 expression (Figure 1b). Importantly, this research, to the best of our knowledge, unravels CD47 as a potential biomarker for HCC and, particularly, cholangiocarcinoma on the molecular level for the first time, thanks to the effectiveness of the functional 2D material developed.

TSP1 is a physiological ligand of CD47. The peptide 4N1K is derived from the C-terminal globular domain of TSP1 and contains a Val–Val–Met motif crucial for binding to CD47. The use of 4N1K can produce many biological effects, including cell adhesion, cell content release, and cell differentiation. These effects could be further diminished by blocking CD47 or down-regulation of CD47 expression. In addition, 4N1K has been used to affinity-label CD47 and as an immobilizing ligand to affinity-purify CD47. These pieces of evidence encouraged us to use the 4N1K ligand for selective CD47 binding.

TAMRA (tetramethylrhodamine)-tagged 4N1K peptide (sequence: KRFYVVMWKK, Figure 1a), DWT, was synthesized by the solid-phase method. Thin-layer 2D MoS₂ was produced by the established liquid exfoliation approach from commercial MoS₂ powder. The produced 2D material was primarily characterized by high-resolution transmission electron microscopy (HRTEM). The gradually enlarged HRTEM images shown in Figure 2a displayed objects of thin-layer sheets, which are in agreement with the morphology of 2D MoS₂ produced in previous reports. With the 2D material and peptide ligand in hand, their supramolecular assembly was carried out by mixing the peptide and 2D material in a full buffer medium (phosphate buffered saline [PBS], 0.01 m, pH 7.4), and the mixture was sonicated for 15 min and then stirred overnight. The assembly between the material and TAMRA-tagged peptide might be driven by van der Waals forces.

To characterize the 2D peptidosheet, we used a series of techniques including Raman spectroscopy, dynamic light scattering (DLS), zeta potential and fluorescence spectroscopy (Figure 2). We observed typical Raman shifts of 2D MoS₂ at 404 and 378 cm⁻¹, which are assigned to the A₁𝑔 (out-of-plane vibration of sulfur) and E₁2g (in-plane relative motion between sulfur and molybdenum) modes of the hexagonal MoS₂ crystal, respectively, with an E₁2g/A₁𝑔 ratio of 0.57 (Figure 2b). In contrast, the E₁2g/A₁𝑔 ratio of 2D peptidosheet (0.64) is larger than that of 2D MoS₂ alone (0.57), suggesting that the coating of the peptide ligand to the surface of material perturbed the in-plane motion between S and Mo atoms. Although DLS is a method typically for measuring the particle size of spherical materials, recent literature has supported the amenability of this technique to graphene-like materials. The result obtained by DLS showed that the particle size of peptidosheet increased with respect to 2D MoS₂ alone (Figure 2c), while the zeta potential of the negatively charged material also increased positively after assembly with the peptide ligand (DWT) with positive charge (Figure 2d). We also observed a concentration-dependent fluorescence quenching of DWT in the presence of increasing 2D MoS₂ (Figure 2e). This is in accordance with the quenching property of the 2D material for closely attached fluorescence species, as similarly evidenced by previous reports. These pieces of data suggest the formation of the 2D peptidosheet.

Next, we tested the fluorescence change of the peptidosheet toward purified CD47 in buffer solution. With increasing CD47, the fluorescence of the peptidosheet increased gradually (Figure 2f) with a good linearity that produces a nanomolar limit of detection (1.6 × 10⁻⁹ M) for the protein (Figure S1, Supporting Information). A subsequent competition assay by
adding 4N1K and another control peptide 4NGG (sequence: KRFYGGMWKK; without the binding ability for CD47)\(^\text{[46]}\) to the mixture of 2D peptidosheet and CD47 showed that 4N1K rather than 4NGG competitively decreased the fluorescence of the system (Figure S2, Supporting Information). The selectivity of the 2D composite was also tested with a range of other proteins. We determined that an evident fluorescence enhancement was selectively produced for CD47 over other proteins including human serum albumin, bovine serum albumin, pepsin, lysozyme, Helix aspersa agglutinin, ribonuclease, and cyt c (cytochrome c; CD47 = cluster of differentiation 47). All spectroscopic measurements were carried out in phosphate buffered saline (0.01 \(\mu\)M, pH 7.4) with excitation of 520 nm for all fluorescence measurements.

**Figure 2.** a) High-resolution transmission electron microscopy of 2D MoS\(_2\) (scale bars: field 1 = 500 nm; field 2 = 40 nm; field 3 = 20 nm). b) Raman spectroscopy of 2D MoS\(_2\) (35 \(\mu\)g mL\(^{-1}\)) and peptidosheet (DWT/2D MoS\(_2\) = 0.25 \(\times\) 10\(^{-6}\) w/35 \(\mu\)g mL\(^{-1}\)). c) Dynamic light scattering of 2D MoS\(_2\) (35 \(\mu\)g mL\(^{-1}\)) and peptidosheet (DWT/2D MoS\(_2\) = 0.25 \(\times\) 10\(^{-6}\) w/35 \(\mu\)g mL\(^{-1}\)). d) Zeta potential of 2D MoS\(_2\) (35 \(\mu\)g mL\(^{-1}\)), DWT (0.25 \(\times\) 10\(^{-6}\) w) and peptidosheet (DWT/2D MoS\(_2\) = 0.25 \(\times\) 10\(^{-6}\) w/35 \(\mu\)g mL\(^{-1}\)). e) Fluorescence titration of DWT (0.25 \(\times\) 10\(^{-6}\) w) in the presence of increasing 2D MoS\(_2\) (0–35 \(\mu\)g mL\(^{-1}\)). f) Fluorescence titration of peptidosheet (DWT/2D MoS\(_2\) = 0.25 \(\times\) 10\(^{-6}\) w/35 \(\mu\)g mL\(^{-1}\)) in the presence of increasing CD47 (0–0.8 \(\mu\)g mL\(^{-1}\)). g) Fluorescence change of peptidosheet (DWT/2D MoS\(_2\) = 0.25 \(\times\) 10\(^{-6}\) w/35 \(\mu\)g mL\(^{-1}\)) in the presence of different proteins (1 \(\times\) 10\(^{-6}\) w), where \(I_0\), \(I_1\), and \(I_2\) are the fluorescence intensity of DWT, peptidosheet, and peptidosheet with protein, respectively (abbreviations: HSA = human serum albumin; BSA = bovine serum albumin; Pep = pepsin; Lys = lysozyme; Aβ42 = amyloid \(\beta\) peptide[0–42]; HAA = Helix aspersa agglutinin; RNase = ribonuclease; Cyt c = cytochrome c; CD47 = cluster of differentiation 47). All spectroscopic measurements were carried out in phosphate buffered saline (0.01 \(\mu\)M, pH 7.4) with excitation of 520 nm for all fluorescence measurements.

We then tested the fluorescence imaging ability of the 2D peptidosheet for HCC and cholangiocarcinoma cell lines. A hepatoma cell line (HCC-LM3) was primarily used. The cell line stably overexpressing CD47 with Flag-tag (CD47-FlagOV) was generated, and that with a reduced CD47 expression level (sh-CD47) was established, both by lentivirus infection. Real-time quantitative polymerase chain reaction (RT-qPCR) verified that, comparing to HCC-LM3, the mRNA level of CD47 was largely enhanced in CD47-FlagOV but suppressed in shCD47 (Figure S4a, Supporting Information). We then incubated the cells with the 2D peptidosheet, and the fluorescence images were taken by confocal laser scanning microscopy (Figure 3). We observed that the fluorescence of the peptide ligand (DWT) was produced in control HCC-LM3 cells (CD47-NC). In the meanwhile, a fluorescence intensity increase was observed for CD47-FlagOV, whereas the intensity was decreased in shCD47 (Figure 3a). This suggests that the 2D peptidosheet could be used for CD47-targeting imaging of HCC-LM3 cells. In addition, by incubation of the 2D peptidosheet with cocultured CD47-FlagOV and sh-CD47 cells we determined that a stronger fluorescence was produced for the former with a higher CD47 expression level than the latter (Figure S5, Supporting Information). We also determined that the material did not cause cell death of HCC-LM3 over a long incubation time (Figure S6, Supporting Information).
express CD47 by both RT-qPCR (Figure S4b, Supporting Information) and flow cytometry (Figure S7, Supporting Information). The level of CD47 mRNA of these cells was determined to be higher than that of the hepatoma cell line used (Figure S4b, Supporting Information). Subsequently, we observed an evident fluorescence production in all of the cholangiocarcinoma cells with CD47 expression after treatment with the 2D peptidosheet. Considering its minimal background fluorescence owing to the presence of 2D MoS₂, we carried out a wash-free imaging for HCC-LM3 using both the 2D peptidosheet and DWT alone. The result showed that while a strong background fluorescence was generated in the cells by the peptide ligand alone, the use of 2D peptidosheet largely suppressed the background interference even without washing (Figure S8a, Supporting Information). This suggests that the peptide ligand would only fluoresce extensively through detachment from the 2D MoS₂ surface upon conjugation with CD47 that is expressed by the cells (this is in accordance with the result obtained by fluorescence spectroscopy shown in Figure 2f), thereby largely improving the contrast of imaging. This unique feature of the 2D composite sets a basis for imaging of tissues where a mixture of different types of cells exists.

In addition, we determined that, comparing to the free peptide (DWT), the 2D peptidosheet produced a stronger level of fluorescence with HCC-LM3 (Figure S9, Supporting Information), suggesting that the presence of 2D MoS₂ might enhance the binding between the ligand with CD47. The photostability of the free peptide ligand and 2D peptidosheet in the presence of CD47 and CD47-expressing HCC-LM3 cells was also tested. The results shown in Figure S10 in the Supporting Information suggest that the fluorescence produced by both DWT and the 2D peptidosheet is stable over 2 h (whereas it only requires less than 1 h to accomplish the imaging), even with light irradiation (365 nm) every 10 min, at both the molecular and cellular levels.

Eventually, we used clinical tissue specimens collected from patients that had been pathologically confirmed with hepatocarcinoma or cholangiocarcinoma to test the imaging ability of the material for realistic samples. We first determined that an evident TAMRA fluorescence was produced on two independent HCC tissues after treatment with the 2D peptidosheet (Figure 4a). Subsequently, the wash-free protocol performed showed that the 2D material produced a much better imaging contrast than using the peptide ligand alone (Figure S8b, Supporting Information). Then, several other clinical specimens containing both hepatocarcinoma and paracarcinoma tissues were used. Interestingly, we observed that the 2D peptidosheet could clearly distinguish cancer tissue from paracarcinoma tissues by the selective production of the peptide probe fluorescence (Figure 4b). Similar imaging results were also observed with two cholangiocarcinoma tissue specimens. With the peptide probe fluorescence, the cancerous region of bile ducts was clearly profiled (Figure 4c). These results suggest the promise of the fluorogenic 2D peptidosheet for imaging human cancer tissues that overexpress CD47.

To conclude, we have demonstrated the possibility of using a 2D biocomposite formed by the supramolecular assembly between a peptide ligand and a 2D material for the imaging of cancerous tissues. The 2D peptidosheet constructed has proven to be suitable for the solution-based selective detection of a protein biomarker (CD47) and the CD47-targeting imaging of cancer cells. Importantly, the fact that the 2D material can be
used to effectively profile HCC and cholangiocarcinoma tissues over paracarcinoma tissues on clinical specimens paves the way for the development of a new generation of 2D biocomposites based on the vast variety of graphene analogs toward biomarker identification, clinical diagnosis and fluorescence imaging-guided surgery. This research also unravels CD47 as a potential biomarker for hepatobiliary cancers, particularly cholangiocarcinoma for which rare biomarkers have been available clinically. We also envision the use of these 2D peptidosheets for CD47-targeting theranostics given the therapeutic properties of 2D MoS2.[25–35]

The currently used method for the detection of CD47 is the anti-CD47 antibody based immunostaining assay.[50] In comparison with this conventional technique, the 2D peptidosheet developed here has several advantages in terms of the following points: (1) The staining process is more convenient and time-saving since samples can be stained with 2D peptidosheet in less than 60 min, while antibody-based staining may cost one day according to the manual. (2) The 2D peptidosheet has good photostability, which may be more suitable for clinical samples and repeated tests. (3) The cost of the 2D peptidosheet comprising a short peptide ligand (produced by a quick solid-phase synthetic procedure) and 2D MoS2 (produced by a simple liquid-exfoliation method) is much lower than that of antibodies. An important issue to address in the future, however, would be the production of structurally more unified 2D MoS2 sheets in order to further improve the reproducibility of imaging. Furthermore, the 2D peptidosheet can be easily extended to the detection and identification of other biomarkers by the simple alternation of the peptide probe to other ligand molecules with specificity for other protein receptors.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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