A New Helicase Assay Based on Graphene Oxide for Anti-Viral Drug Development

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Recently, graphene oxide (GO), one of the carbon nanomaterials, has received much attention due to its unique physical and chemical properties and high potential in many research areas, including applications as a biosensor and drug delivery vehicle. Various GO-based biosensors have been developed, largely based on its surface adsorption properties for detecting biomolecules, such as nucleotides and peptides, and real-time monitoring of enzymatic reactions. In this review, we discuss recent advances in GO-based biosensors focusing on a novel assay platform for helicase activity, which was also employed in high-throughput screening to discover selective helicase inhibitors.

INTRODUCTION

Graphene, a one-atom-thick nanomaterial with sp2 carbons arranged in a two-dimensional honeycomb structure, has received much attention recently as an emerging material with a variety of potential applications based on its unique mechanical, electrical, optical, and biological properties (Zhu et al., 2010). The oxidized form of graphene, or graphene oxide (GO, Fig. 1), has been extensively explored in both basic and biomedical research largely because of its good biocompatibility, colloidal dispersibility in aqueous solution, flexible surface chemistry, amphiphilicity, and superior fluorescence quenching capability (Loh et al., 2010; Morales-Narvaez et al., 2012). Studies have demonstrated that the GO surface may interact through pi-pi stacking and hydrogen bonding interactions (Park et al., 2013) with various biomolecules, including thrombin (Chang et al., 2010), dopamine (Wang et al., 2009), nucleic acids (Lu et al., 2009), peptides (Wang et al., 2011a), proteins (Mu et al., 2012; Zhang et al., 2012), and lipids (Frost et al., 2012).

These interactions are key to the use of GO for biological applications and enable the loading and release of various drug candidates (e.g., oligonucleotides and small molecules) and sensing probes. Additionally, the fluorescence quenching property of GO leads to a wide range of active research and development of fluorescence resonance energy transfer (FRET) biosensors (Wang et al., 2010). The nano-sized GO (nGO, 50-300 nm) has been engineered, often by tuning GO preparation conditions, for use in intracellular delivery (Luo et al., 2010; Pan et al., 2011). The mechanism underlying GO cellular uptake remains to be determined; however, endocytosis appears to be involved in the process. Versatile covalent functionalization can be achieved through hydroxyl and carboxylic acid groups present on the GO surface to enhance its physiochemical, electrochemical, or biological properties depending on the application (Huang et al., 2011; Zhang et al., 2011).

GO-BASED HELICASE BIOSENSOR

Various GO-based enzymatic activity assay systems have been developed to target nucleases (Lee and Min, 2012), methyltransferases (Lee et al., 2011), and caspases (Wang et al., 2011b). Here, we focus on the first reported GO-based enzyme activity assay, namely the GO-based helicase activity assay (GOHA), and its use in identifying helicase nsp13 from the severe acute respiratory syndrome coronavirus (SARS CoV, SCV) (Jang et al., 2010). SARS is a viral respiratory disease in humans characterized by flu-like symptoms and high mortality rates. SCV helicases have been recognized as a primary target for direct-acting antiviral agents against SARS (Huang et al., 2008). The GOHA platform relies on the preferential binding of GO to single-stranded DNA (ssDNA) over double-stranded DNA (dsDNA) and the quenching of DNA-conjugated fluorescent dyes when the GO and dyes are present in close proximity. Strong adsorption of single-stranded nucleotides to GO is mediated by pi-pi stacking interactions between the aromatic rings in the exposed bases that constitute single-stranded nucleotides and the sp2 hybridized hexagonal structure of GO (Liu et al., 2008; Varghese et al., 2009). Contrary to ssDNA, dsDNA cannot interact with GO because the bases of dsDNA are located inside the double helix within the negatively charged phosphate backbone (He et al., 2010). Unwinding of dsDNA is initiated by addition of SCV helicases to a mixture of fluorescence-labeled substrate dsDNA and GO. As the helicase reaction proceeds, the fluorescence intensity decreases due to the energy transfer-mediated quenching that occurs upon binding.
Helicase Assay Based on Graphene for Viral Drug Discovery
Hongje Jang et al.

of unwound ssDNA to the GO surface (Fig. 1). The GOHA platform is a good example of a GO-based enzymatic biosensor due to its highly selective adsorption to ssDNA and exceptionally high fluorescence quenching ability. Further application of GOHA for more pathophysiological relevance has been extensively studied including a robust and cost-effective drug discovery platform.

HEPATITIS C VIRUS NS3 HELICASE AND mGOHA FOR DRUG DISCOVERY

Hepatitis C is an infectious liver disease that affects more than 170 million people worldwide. Chronic infection with hepatitis C virus (HCV) leads to severe liver disease, including cirrhosis and hepatocarcinoma (Choo et al., 1989; Francesco et al., 2005). At present, a combination of PEG-conjugated interferon-α and ribavirin is routinely prescribed for treating hepatitis C infection. Interferon-α is an immune booster and ribavirin is a nucleoside-mimicking derivative that causes lethal mutations in the virus during RNA replication and indirectly contributes to sustaining the immune response against HCV genotypes 1, 2, and 3. However, this pharmacological intervention has showed only limited efficacy with significant side effects, including hemolytic anemia, depression, and fatigue. Frequent genetic mutations in HCV and drug resistance also significantly limit the effectiveness of current hepatitis treatments. For higher therapeutic efficacy, new drugs have been anticipated to target more genetically conserved proteins of HCV such as proteases. Direct-acting antiviral agents targeting the non-structural protein 3-4A serine protease (NS3-4A) (Lee et al., 2012) and non-structural protein 5B RNA-dependent RNA polymerase (NS5B RdRp) (Farnik and Zeuzem, 2012) in the viral replication cycle have been reported to be more effective than conventional therapy.

The HCV NS3 protein contains a C-terminal RNA helicase domain that hydrolyzes ATP to catalyze the unwinding of dsDNA or dsRNA into single-stranded forms. The N-terminal serine protease domain cleaves the HCV polyprotein and, consequently, releases mature proteins. The HCV NS3 helicase is one of the essential enzymes of HCV along with NS3-4A serine protease and NS5B RdRp, which are required for processing HCV proteins and viral replication. The HCV NS3 helicase has long been considered another important target for the development of hepatitis C therapeutics (Borowski et al., 2002; Frick, 2007); however, identification of potent inhibitors using chemical library screening has been challenging because the available helicase assay systems are incompatible with high-throughput formats.

Numerous technologies have been developed to measure helicase activity in vitro, including fluorescence and radiolabeled gel electrophoresis (Kwong and Risano, 1998), scintillation proximity assay (SPA) (Kyono et al., 1998), FlashPlate (Hi-cham Alaoui-Ismaili et al., 2000), electrochemiluminescence (ECL) (Zhang et al., 2001), enzyme-linked immunosorbent assay (ELISA) (Hsu et al., 1998), FRET (Porter et al., 1998), time-resolving resonance energy transfer (TR-RET) (Earmshaw et al., 1999), and molecular beacon helicase assay (MBHA) (Belen and Frick, 2008) (Table 1). Some assay methods have contributed significantly to unveiling the helicase reaction mechanism at the molecular level. However, according to the NIH screen data (Mukherjee et al., 2012), some antiviral candidates identified by currently available helicase assays exhibited poor activity in cell-based analysis and even turned out to interfere with the assay itself and produced false positives.

Recently, our laboratory reported a multiplexed helicase assay platform based on GO (called mGOHA) that exhibits robust sensibility and high-throughput compatibility (Jang et al., 2013) (Fig. 2). Briefly, mGOHA employs SCV helicase and HCV NS3 helicase in a single mixed solution with DNA substrates labeled with two distinct fluorescent dyes, Cy3 and Cy5, respectively. The substrates were designed to selectively recognize each...
Table 1. Currently available HCV NS3 helicase activity assay platforms

| Assay  | Detection                                                        | References                        |
|--------|-------------------------------------------------------------------|-----------------------------------|
| PAGE   | Gel electrophoresis and radioimaging                              | Tai et al. (1996)                 |
| SPA    | SPA beads, microplate scintillation counter                       | Kyono et al. (1998)               |
| FlashPlate | FlashPlates, microplate scintillation counter                   | Hichem Alaoui-Ismaili et al. (2000) |
| ECL    | Magnetic flow cell, ECL instrument                               | Zhang et al. (2001)               |
| ELISA  | UV-vis spectrophotometer (A450)                                   | Hsu et al. (1998)                 |
| FRET   | Fluorescence spectrophotometer                                    | Porter et al. (1998)              |
| TR-RET | Fluorescence spectrophotometer                                    | Earnshaw et al. (1999)            |
| SSB    | Gel electrophoresis and fluorescence spectrophotometer           | Rajagopal et al. (2008)           |
| 3-AP   | Fluorescence spectrophotometer                                    | Raney et al. (1994)               |
| MBHA   | Fluorescence spectrophotometer                                    | Belon and Frick (2008)            |

See text for explanation on abbreviations

Fig. 2. mGOHA. Two different helicases, SCV helicase and HCV NS3 helicase, selectively recognize cognate dsDNA substrates labeled with two distinct fluorescent dyes. In a mixed solution, activities of the individual helicases are quantitatively measured with real-time monitoring.

Fig. 3. Procedure for small-molecule screening against two helicases, SCV helicase and HCV NS3 helicase, based on mGOHA.
Helicase Assay Based on Graphene for Viral Drug Discovery

Hongje Jang et al.

Helicase to ensure orthogonal reactivity. Approximately 10,000 small molecules underwent a high-throughput screen using the mGOHA platform to identify potent inhibitors of target helicases (Fig. 3). After the primary and secondary screens, five inhibitors specific to each helicase and five inhibitors active towards both helicases were identified. The inhibitors exhibited 50-500 μM of IC50 values on target helicases. Furthermore, most directly inhibited the ATPase activity of the target helicase. Among the five compounds identified as HCV NS3 helicase inhibitors, two compounds showed a considerable inhibitory effect on HCV gene replication following infection of the human liver cell line HuH-7. Collectively, mGOHA was demonstrated to be a successful high-throughput screening method that yields highly potent antiviral candidates without interfering with the assay itself and producing false positives. This novel assay also overcame the limitations of existing assay methods by providing reliable, reproducible, real-time quantitative monitoring of helicase activity.

CONCLUSION

A helicase assay based on GO, called GOHA, was developed and successfully implemented in multiplexed high-throughput chemical screening to identify potent helicase inhibitors, which are potentially of clinical interest to treat hepatitis C and SARS. The new assay enables quantitative, reliable, and real-time monitoring of helicase activity by simply tracking changes in fluorescence intensity. The ability to perform multiplexed screening is also advantageous because it reduces costs and labor while providing near instantaneous information on the relative selectivity of the identified inhibitors.

To effectively treat HCV infection, direct-acting antiviral agents should be developed to complement the standard interferon- and ribavirin treatments. In this regard, discovery of new inhibitors of HCV NS3 helicase will be a great addition to existing options for direct-acting antiviral agents. The GOHA will be further harnessed to identify other helicase inhibitors to treat additional viral infections in the future.

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272 Mol. Cells http://molcells.org
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