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## Synthesis of pillar[5]arene functionalized graphene as a fluorescent probe for paraquat in living cells and mice

Received 00th November 2015,  
Accepted 00th November 2015

DOI: 10.1039/x0xx00000x

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As the complex organisms vary in vivo, it remains to be a challenge task to get the fluorescence ‘turn on’ imaging for special target. To address this task, we make a new strategy of inducing pillararene based host-guest interaction onto biocompatibility graphene. By means of fluorescence competition displacement, hydrazino-pillar[5]arene modified graphene has been firstly synthesized and provided as a ‘turn on’ probe for paraquat through monitoring the fluorescence signal both in living cells and mice.

Graphene (G), with its peculiar two-dimensional (2D) and single layer structure, has become a hot research topic because of its unique properties,<sup>1</sup> including intriguing electrical performance, high surface areas, inexpensiveness, and low toxicity.<sup>2-4</sup> Recently, graphene has also been increasingly explored as a new excellent quenchers for fluorescent probes in chemical and biological fields due to its remarkable high fluorescence quenching efficiency and biocompatibility.<sup>5,6</sup> For instance, it was reported that the utilization of graphene as a ‘turn on’ fluorescent probe with intracellular analysis receptors for biomolecule detection.<sup>5,6</sup> However, it is still exceptionally challenging to realize ‘turn on’ recognition in mice, especially when taken the living and dynamic nature of the mice into consideration.

Paraquat is one of the widely used herbicides, which is showing an increasing number of scientific and technical applications, such as probes to study DNA and zeolites, components of electrochromic display devices, and pro-oxidants in stress tests.<sup>7</sup> However, its high toxicity processing considerable risks to human health, animals, and

the environment. Absorption of paraquat into the digestive tract, respiratory tract, and skin may result in various diseases or even death.<sup>8</sup> Therefore, design of paraquat probes in biological environments have become increasingly important.<sup>9</sup> Due to the inherently complex organization of cells or mice,<sup>10</sup> the development of a novel and paraquat probe simultaneously possessing highly selectivity and low toxicity in living cells or mice is a challenge task.

To solve this problem, we make a strategy of inducing host-guest system onto the graphene though fluorescent indicator-displacement assay (F-IDA) to obtain a convenient and efficient system for ‘turn on’ fluorescent probe in mice. Pillararenes,<sup>11</sup> composed of hydroquinone units linked by methylene bridges at the para-positions, represent a popular class of macrocyclic hosts. Their intrinsic unique rigid and symmetrical pillar architecture and easy modification have endowed pillararenes with outstanding abilities to selectively bind various types of guests,<sup>12a</sup> thus provided a useful platform for the construction of various interesting molecular receptors, such as the reported<sup>13</sup> host-guest complexation between pillararene and paraquat. To this end, the hydrazino-pillar[5]arene (HP) was designed and synthesized (Scheme 1) for two reasons: 1) pillar[5]arene, with its unique cavity, will offer graphene the potential to ‘turn on’ recognition through competitive inclusion; 2) hydrazino groups, served as immobilized unit, was introduced to rim-differentiation of pillararene.

Hence, in this work, we synthesize hydrazino-pillar[5]arene<sup>14</sup> functionalized graphene (HP-G) as an fluorescent sensor for ‘turn on’ recognition in cells and mice was reported. HP was grafted to graphene through a typical EDC/NHS reaction to obtain HP-G receptor. We show the use of HP-G for fluorescent detection of paraquat via selective host-guest interaction and graphene translates weak interaction into a sensitive fluorescent signal. And the potential application of HP-G sensor for ‘turn on’ fluorescent sensing of both in living cells and in mice was also investigated, thus creating a novel work that combines nanotechnology, host-guest molecular recognition with macrocyclic compounds pillar[5]arene to obtain the efficient recognition in the performance of the new type of graphene biosensor in complex biological samples.

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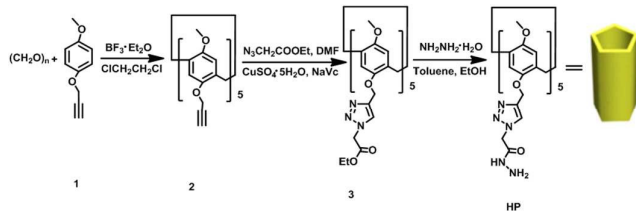
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Electronic Supplementary Information (ESI) available: See  
DOI: 10.1039/x0xx00000x

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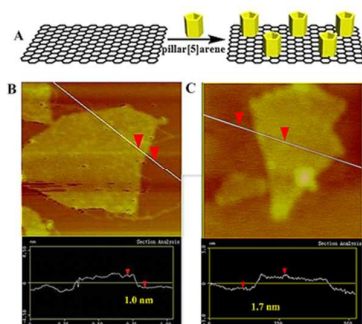
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Scheme 1. Synthesis of HP.

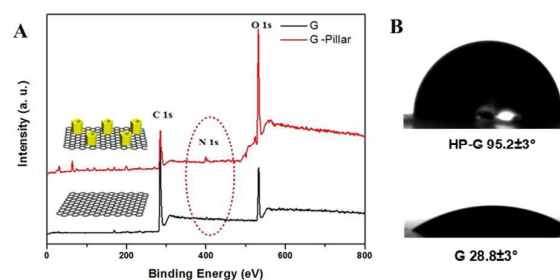
As shown in Scheme 1, a novel HP was designed and synthesized via hydrazinolysis. The G was synthesized referring to Hummer's preparation method<sup>15,16</sup>. The surface of the graphene prepared on this method existed lots of carboxyl groups, which was easy to be functionalized. The synthesized G and HP-G were further characterized by atomic force microscopy (AFM), fourier transform infrared (FT-IR), X-ray photoelectron spectroscopy (XPS), and fluorescence spectroscopy. As shown in Figure 1, the thickness of the G through exfoliation revealed by the AFM image is  $1.0 \pm 0.2$  nm, which clearly demonstrates the single-layer feature of G, according to the previous literature.<sup>17,18</sup> However, a remarkable increase of thickness to  $1.7 \pm 0.2$  nm is observed in the AFM images after the introduction of HP to G. Comparing the thickness of G and HP-G reveals that the thickness increase is a result of the chemical derivatization by the HP on the G. As shown in Figure S6, the alteration of the peak value at  $1729\text{ cm}^{-1}$  of the  $-\text{COOH}$  stretching vibration in G red-shift to  $1645\text{ cm}^{-1}$  in HP-G identified as a result of amide groups.



**Figure 1.** A) Schematic illustration of the preparation of HP-G conjugates. AFM images of G (B), and C) HP-G. The variation in thickness demonstrates the successful assembling of HP on G.

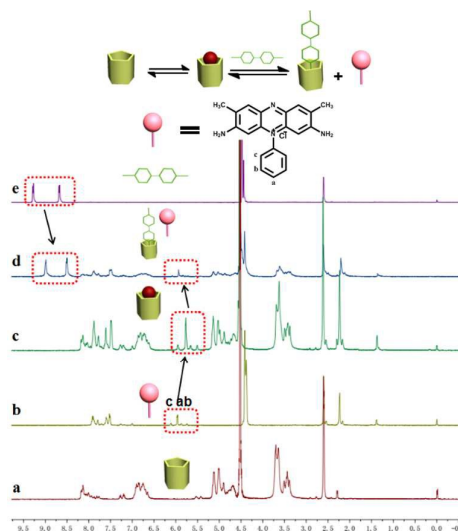
XPS can further provide critical chemical bonding information for G and HP-G surface analysis (Figure 2A). A pronounced N 1s peak was observed for the resultant HP-G, whereas no N signal was detected on the G film, indicating the chemical modification process was taken place. Additionally, the wettability of G and HP-G displays the occurrence of chemical derivation (Figure 2B). A remarkable increase of CA was up to  $95.2^\circ$  after modification, suggesting that HP-G is much more hydrophilic due to integration HP onto G.

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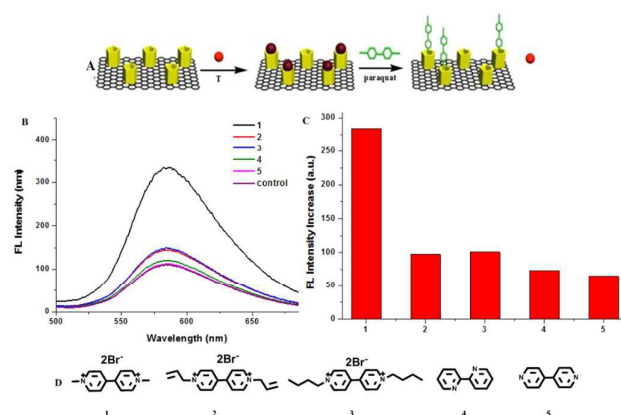
**Figure 2.** (A) XPS spectra of the original G (curve black) and HP-G (curve red); (B) Water drop profile changes of HP-G (top), and G (bottom) measuring on glass slides. It shows the successful assembling of HP on G as well.

We further demonstrated the potential application of HP-G as fluorescent sensor. Considering the electron-rich cavity of HP, safranin T acted as an electron-deficient dye indicator, was used in vitro to label G. The HP based host-guest interaction was strongly supported by  $^1\text{H}$  NMR spectra in Figure 3. The aromatic protons of safranin T and underwent upfield  $0.07$  ppm shifting that exhibited HP can bind safranin T, significantly; After addition of paraquat, Figure 3d shows the aromatic protons of safranin T underwent downfield shifting ( $0.07$  ppm, recovering), which indicates the release of safranin T. Moreover, compared Figure 3d with c, the protons of paraquat underwent upfield shifting ( $0.05$  ppm) shows HP can bind paraquat more affinity to release safranin T. Therefore, safranin T was incubated with HP-G to form safranin T /HP-G. After 5 min incubation, nearly 95% fluorescence quenching with very fast kinetics was observed on safranin T /HP-G (Figure S7, A). It is clear that the electrostatic interaction between safranin T and HP caused safranin T to attach graphene, accompanied with indicator fluorescence 'turn off' because of fluorescence resonance energy transfer (FRET) between safranin T and graphene.



**Figure 3.**  $^1\text{H}$  NMR spectra (DMSO, 600MHz, 298K) of (a)HP (15 mM each), (b)safranin T (15 mM), (c)HP and Safranin T (15 mM) (d)HP, Safranin T and paraquat, (e)paraquat (15 mM each). It shows that paraquat can insert into HP to release safranin T.

To confirm the selectivity of paraquat towards competitive binding analytes, five paraquat analogs (Figure 4) of the same concentration ( $5 \times 10^{-4}$  M) were added to the solution of the safranin T/HP-G complex (Figure 4B). Figure 4B displays the changes in the fluorescence ratio  $I/I_0$  of the safranin T/HP-G complex upon addition of a particular competitive binding analyte. Upon interaction with various competitive binding analytes, the fluorescence of the safranin T/HP-G complex increased selectively by adding paraquat, while adding other competitive binding analytes caused no significant fluorescence changes. Under the employed conditions, other four paraquat analogs induced little interference with the paraquat selective responses. The fluorescence recovery by paraquat is possibly due to the stronger intermolecular electrostatic binding between paraquat and HP, which could cause a displacement of safranin T to inhibit further the energy transfer between graphene and safranin T. So we can deduce that safranin T first formed inclusion complexes with HP-G and then they were released from HP-G upon the addition of paraquat accompanied with a phenomenon of fluorescence 'off' to 'on'.

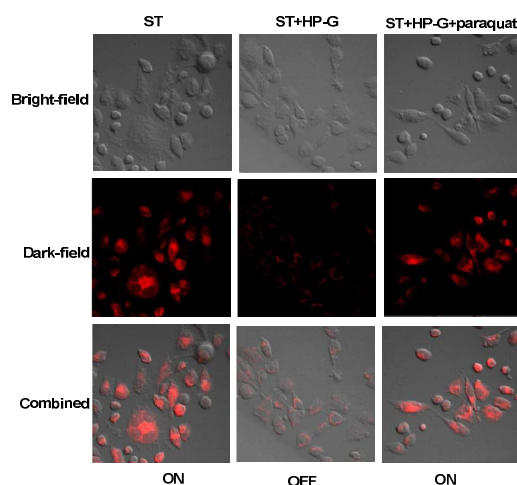


**Figure 4.** A) Schematic demonstration of the fluorescence 'off-on' mechanism for detecting paraquat. B) Fluorescence spectra of HP-G/safranin T in presence of paraquat molecules, respectively. C) Relative fluorescence intensity ( $I/I_0$ ) is calculated by  $I/I_0$ , where  $I_0$  and  $I$  are the fluorescence intensity without and with the presence of paraquat, respectively. D) Chemical structures of five paraquats. It can be seen that HP modified G can be used as fluorescent probe for paraquat.

A series of different concentrations of paraquat were incubated with HP-G/safranin T solution, as displayed in Figure S8, to gain insight into the release of dye indicators. It is obvious that as the concentration of paraquat increased, the fluorescence intensity progressively enhanced. The growth of fluorescence with increasing concentration of the additive was fitted by a competitive binding scheme. A detection limit as low as 1.04 mM is obtained based on the HP-G/safranin T fluorescent probe. Results found here could also suggest some possible competitive binding and fluorescence recovery mechanisms.

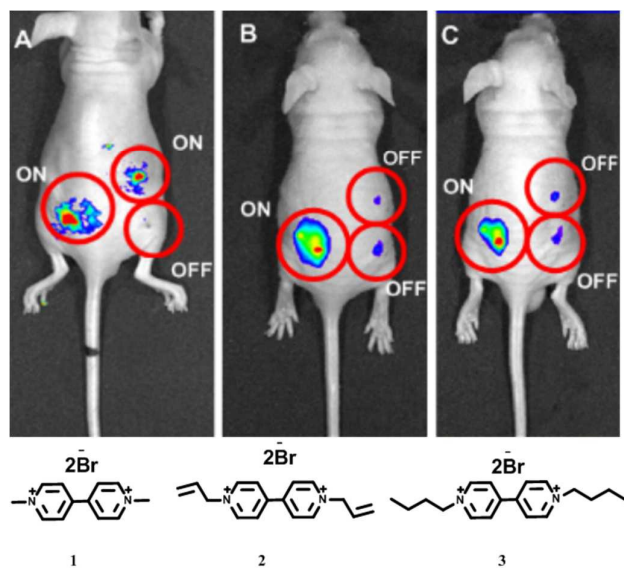
For the sake of determining the cell permeability and biocompatibility, the as-prepared HP-G probe was incubated with living cells. For biological applications, the cytotoxicity of HP-G

was evaluated via classic thiazolyl blue tetrazolium bromide (MTT) assays with HeLa cells line (Figure S9). After 24h incubation with HeLa cells, we found that this graphene derivative did not lead to serious acute cytotoxicity. In this case, HeLa cells were incubated with safranin T in growth media up to 1h at 37 °C. As can be seen from Fig 5, HeLa cells upon incubation with safranin T exhibited a bright-red colour. Significantly weaker fluorescence of HeLa cells could be observed after the addition of HP-G into the medium followed by incubation for 2h, and we serially treated the cell samples by washing with PBS three times.<sup>19a</sup>



**Figure 5.** (A) Cellular uptake of HP-G hybrid shown by Bright-field (top) and dark-field fluorescence images (bottom). HeLa cells were incubated with safranin T without HP-G (ST) or with HP-G (ST+HP-G); and safranin T, HP-G, paraquat (ST+HP-G+paraquat) for 1–2h at 37°C. Images were obtained after extensive washing of cells with PBS. The results show that the fluorescence detection method has better anti-interference and specificity, can be used for sensitive and rapid detection of paraquat in the cell.

Previous studies<sup>20a</sup> demonstrated that graphene-based materials could efficiently be transported into living cells, and the cellular uptake of graphene materials possibly relies on direct penetration of cell membranes. So we can reasonably conclude that the internalization of HP-G and energy transfer from dye to graphene was taken place in cells from observing fluorescence quenching of HeLa cells. Then HeLa cells were incubated with paraquat for 1h, which had been incubated with HP-G/safranin T for 3h, and were observed by fluorescence microscopy. The fluorescence of HeLa cells evidently recovered, indicating the release of the dye indicator in cells when paraquat was added. However, no obvious change of fluorescence intensity was observed for the cells cultured with other four paraquat analogs (Figure S10), suggesting that a good selective fluorescence recovery was obtained in cells. On the basis of the experimental results described above, it is reasonable to draw a conclusion that the designed graphene-based fluorescent probe can be used to image intracellular paraquat in living cells by using a general fluorescence technique, which should be potentially useful for the study of intracellular imaging and drug delivery in living cells.



**Figure 6.** In vivo release of safranin T ( $5 \times 10^{-4}$ M,  $25 \mu\text{L}$ ) from HP-G ( $0.05 \text{ mg/mL}$ ,  $25 \mu\text{L}$ ) with or without treatment of (A) paraquat **1**, ( $10^{-3}$ M,  $25 \mu\text{L}$ ) respectively. Safranin T fluorescence, ON (left, fluorescence normal) after treating HP-G, OFF (lower right, fluorescence quenching) and safranin T/HP-G with paraquat **1**, ON (upper right, fluorescence recovery). The total fluorescence intensities increased after the treatment with paraquat, showing that the release of dye molecule in mice. While the other that in vivo release of safranin T ( $5 \times 10^{-4}$ M,  $25 \mu\text{L}$ ) from HP-G ( $0.05 \text{ mg/mL}$ ,  $25 \mu\text{L}$ ) with or without treatment of paraquat analogs **2**(B), **3**(C), ( $10^{-3}$ M,  $25 \mu\text{L}$ ), respectively. B and C shows the analogous phenomenon that safranin T fluorescence, ON (left, fluorescence normal) and after treating HP-G, OFF (lower right, fluorescence quenching), differently, when treating the safranin T/HP-G with paraquat **2**, **3** respectively, still OFF (upper right, fluorescence quenching, no recovery).

Owing to the good performance of HP-G for fluorescence recognition in vitro, we reason that this probe would function in mice. To this end, this recognition system was administered via subcutaneous injection in mice. As shown in Figure 6, the total fluorescence intensities increased after the treatment with paraquat, showing that the release of dye molecule in mice. Our results indicate that HP-G conjugate systems can be utilized as an effective fluorescence recognition platform for paraquat both in vitro and in vivo with low cellular toxicity. This method will be useful in the development of a new method for quantifying intracellular imaging in live cells.

This work was financially supported by the National Natural Science Foundation of China (21572076, 21372092), Wuhan scientific and technological projects (2015020101010079).

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