Supporting Information

Functional Materials of Covalent Modification of Reduced Graphene Oxide and β-Cyclodextrin as Drug Delivery Carrier

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1. Synthesis of rGO-C₆H₄-COOH and the Tyndall Effect

rGO-C₆H₄-COOH was synthesized according to the previous method, as shown in Figure S1.¹

![Synthesis route of rGO-C₆H₄-COOH.](image)

**Figure S1.** Synthesis route of rGO-C₆H₄-COOH.¹

The colloidal nature of GO, rGO and rGO-C₆H₄-COOH was demonstrated by the Tyndall effect, as shown in Figure S2, suggesting excellent water dispersibility.

(a) GO aqueous solution

(b) rGO aqueous solution (0.5% ammonia)  (c) rGO-C₆H₄-COOH aqueous solution
**Figure S2.** Tyndall effect: GO aqueous solution (a); rGO ammonia aqueous solution (b); rGO-C₆H₄-COOH aqueous solution (c).

2. The XPS (ESCALAB 250) Survey

2.1. The rGO XPS Survey

![XPS Survey Graph](image)

- **a**
  - Raw line
  - Background
  - O1S: Binding energy = 531.8, area = 28668.86

- **b**
  - Raw line
  - Background line (include the C-OR and O=C-O-R line)
  - C=O, binding energy = 287.8, area = 6025.236
  - C-OR: Binding energy = 285.4 eV, area = 0.1
  - O=C-O-R: Binding energy = 288.9 eV, area = 10.31
  - C1s, binding energy = 285 eV, area = 58085.35

- **c**
  - Raw line
  - Background line
  - N1S: Binding energy = 398.4, area = 2850.5
  - NH4: Binding energy = 402.4, area = 3566.869

![XPS High-resolution Graph](image)

**Figure S3.** High-resolution XPS survey. O1s survey of rGO (a); C1s survey of rGO (b); N1s survey of rGO (c).

Sensitivity factor: C=0.25; N=0.42; O=0.66.

- C total area = 6825.236 + 10.31 + 58085.35 = 64920.896;
- N total area = 2850.5 + 3566.869 = 6417.369;
- O total area = 28668.86;
- Total area = C/0.25 + N/0.42 + O/0.66 = 259683.58 + 15279.45 + 43437.67 = 318400.70;
- Percent of O = C-O- = 10.31/0.25/318400.7 *100% = 0.013%;
- Percent of N = 6417.369/0.42/318400.7 *100% = 4.8%;
- Percent of O = 28668.86/0.66/318400.70 *100% = 13.64%.
2.2. The rGO-C₆H₄-COOH XPS Survey

![Graph showing XPS survey results for rGO-C₆H₄-COOH]

Figure S4. High-resolution XPS survey. O1s survey of rGO-C₆H₄-COOH (a); C1s survey of rGO-C₆H₄-COOH (b); N1s survey of rGO-C₆H₄-COOH (c).

Sensitivity factor: C = 0.25; N = 0.42; O = 0.66.

C total area = 50613.23 + 782.21 + 8300.12 = 59695.56;
N total area = 3005.1 + 6836.73 = 9841.83;
O total area = 48782.53;
Total area = C/0.25 + N/0.42 + O/0.66 = 59695.56/0.25 + 9841.83/0.42 + 48782.53/0.66 = 336128.09;
Percent of O=C-O- = 8300.12/0.25/336128.09 *100% = 9.88%;
Percent of N = 9841.83/0.42/336128.09 *100% = 6.97%;
Percent of O = 48782.53/0.66/336128.09 *100% = 21.99%.

3. The TGA (TGA/SDTA851e) Assay
Figure S5. TGA thermogram of rGO-C₆H₄-COOH.

4. Synthesis of rGO-PEI-CD-Biotin and Characterizations

Figure S6. Synthesis route of rGO-PEI-CD-Biotin.
5. Synthesis of β-CD-NH₂-CO-C₆H₄-COOH[^S2] and Characterizations

Terephthalic acid monomethyl ester (6.6048 g, 36.0 mmol), dicyclohexyl carbodiimide (DCC, 11.1030 g, 54.0 mmol), and N-hydroxysuccinimide (7.2210 g, 54.0 mmol) were added to 180 mL THF with stirring for 24 h at room temperature. Then, the precipitates (dicyclohexylurea) were removed by centrifuging and filtering. The supernatant solution was evaporated to dry in vacuo. The solid sample was dissolved in 180 mL of 2-propanol and recrystallized at 2 °C as a white crystal. 6.0268 (5.30 mmol) 3-Amino-3-deoxy-(2AS,3AS)-β-cyclodextrin hydrate (NH₂-β-CD) was dissolved in 120 mL of DMF, and 1.8046 g (6.06 mmol) terephthalic acid methyl ONSu ester was added. The reaction mixture was stirred at room temperature for 36 h. Then the reaction mixture was poured into 1800 mL of acetone to precipitate methylterephthalamide β-CD. The methylterephthalamide β-CD was collected by centrifuge and washed with acetone. Methylterephthalamide β-CD was dissolved in 60 mL of water and added to 1.2 mL of 1 M NaOH and stirred for an hour. Then, the reaction mixture was neutralized by citric acid and evaporated to dryness. The crude product was purified by column chromatography on DIAION HP-20 (eluted with water/methanol) 100/0 to 50/50). The 70/30 and 60/40 (water/methanol) eluent was concentrated to give terephthalamide β-CD about 0.9002 g.

The ¹H NMR (AVANCE 600) spectrum (Figure S8) was mainly used to verify exist of amide (-CONH-) and pheny group, which suggested the successfull

[^S2]: Electronic Supplementary Material (ESI) for New Journal of Chemistry

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conjugation of β-CD-NH₂ and HOOC-C₆H₄-COCH₃. However the hydrogen atom of -COOH (β-CD-NH-CO-C₆H₄-COOH) could not be demonstrated, and these peaks of CD between 3.0-4.0 ppm were very difficult to discern. So LC-MS(Agilent 6510) was applied to further verify the product (Figure S9). These datas of ¹H NMR and MS suggested that the terephthalamide β-CD had been successfully synthesized.

¹H NMR ((DMSO-d₆, 25 °C, 400 MHz, δ): δ8.32 (t, 1H, -CONH-), 8.03 (d, 2H, 2-Ph), 7.98 (d, 2H, 3-Ph), 5.5-6.0 (m, 14H, O(2)H and O(3)H), 4.967 (d, 1H, C(1)’ H), 4.8-4.92 (m, 6H, C(1)H), 4.6-4.8 (m, 3H, O(6)H).

**Figure S8.** ¹H NMR spectrum of terephthalamide β-CD-NH₂-CO-C₆H₄-COOH

MALDI-TOF MS (m/z): 1296.4271 ([M]⁺), 1318.4077 ([M+Na]⁺), 1334.3826 ([M+K]⁺). (Figure S9).

**Figure S9.** MS spectrum of β-CD-NH₂-CO-C₆H₄-COOH.

6. The Stability of rGO-PEI-CD-Biotin in Biological Solutions
Figure S10. Micrographs of rGO-PEI-CD-Biotin in physiological solutions. rGO-PEI-CD-Biotin in PBS (phosphate buffered saline, pH = 7.4) buffer solution (a); rGO-PEI-CD-Biotin in 0.9% (wt%) NaCl solution (b); rGO-PEI-CD-Biotin in cell culture solution (c); rGO-PEI-CD-Biotin in serum solution (d). These micrographs demonstrate that no sediments produce, suggesting the excellent stability of rGO-PEI-CD-Biotin in physiological solutions.

7. The Inclusion Ratio and Inclusion Constant of DOX and β-CD-NH₂-CO-C₆H₄-COOH

Taking five colorimetrical cylinders, each colorimetrical cylinder was added to 1.0 mL DOX solution (0.10 mmol L⁻¹ ethanol solution), and 0, 1.0, 2.0, 3.0, 4.0, 5.0 mL β-CD-NH₂-CO-C₆H₄-COOH solution (1.0 mmol L⁻¹) was added to each colorimetrical cylinder, and finally was diluted with water to 10 mL. The solution was sonicated for 6 hrs with 500 W sonicator (HS-3120). After the prescribed time, these solutions were dialyzed against water for 6 hrs to remove ethanol separately, and precipitates were decanted through filtration. Then these solutions were dried through freeze-drying to obtain these solid samples. Finally, these solid samples were dissolved in water. Fluorescence spectra and intensity was measured with Fluorescence spectrometer (LS-55 Perkin-Elmer).

According to Benesi-Hildebrand method,[S3] the inclusion ratio and inclusion constant of DOX and β-CD-NH₂-CO-C₆H₄-COOH (β-CD) were obtained. Seen from
the Figure S11, under the constant concentration of DOX, the intensity of fluorescence increases with the increase of β-CD concentration. The intensity of fluorescence in 555 nm was obtained (Table S1). According to the formula of Benesi-Hildebrand, the Benesi-Hildebrand plot was obtained and the plot is linear, which suggested the inclusion ratio of β-CD with DOX is 1:1, the inclusion constants is $3.16 \times 10^4$ L mol$^{-1}$ (Figure S11b).

Figure S11. Fluorescence spectra of DOX was included by the β-CD-NH$_2$-CO-C$_6$H$_4$-COOH (a); Benesi-Hildebrand plot of inclusion complexes from DOX with β-CD-NH$_2$-CO-C$_6$H$_4$-COOH (b).

<table>
<thead>
<tr>
<th>Test sequence of DOX-CD complex</th>
<th>Fluorescence intensity (a.u)</th>
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<tr>
<td>0</td>
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<tr>
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Table S1. The fluorescence intensity of DOX included by β-CD-NH$_2$-CO-C$_6$H$_4$-COOH at 555 nm

8. The Ratio of DOX Loaded on rGO-PEI-CD-Biotin

The weight extinction co-efficient of rGO-PEI-CD-Biotin and DOX was calculated by the method proposed.$^{[54]}$ The DOX weight extinction co-efficient is $\sim 8.36$ L g$^{-1}$ cm$^{-1}$ at 485 nm, and the rGO-PEI-CD-Biotin is $\sim 1.16$ L g$^{-1}$ cm$^{-1}$ at 485 nm. The DOX concentration was determined at 485 nm (after subtracting the absorption contribution from rGO-PEI-CD-Biotin) with weight extinction coefficient of $\sim 1.16$ L g$^{-1}$ cm$^{-1}$. 
Figure S12. UV-vis spectra of DOX, rGO-PEI-CD-Biotin, and DOX-rGO-PEI-CD-Biotin (a); The magnified UV-vis spectra of DOX, rGO-PEI-CD-Biotin, and DOX-rGO-PEI-CD-Biotin (b).

9. Drug Releasing Behavior

Figure S13. The releasing behavior of DOX on rGO-PEI-CD-Biotin in pH=7.4 PBS (a); The releasing behavior of DOX on rGO-PEI-CD-Biotin in pH=5.5 ABS (b); The releasing plot of DOX on rGO-PEI-CD-Biotin in PBS and ABS buffer solution (c).

10. Uptake of rGO-PEI-CD-Biotin-Cy7

The HepG2 cell solution (200 μL, ~1 million mL⁻¹) was incubated with rGO-PEI-CD-Biotin-Cy7 (50 μL) solution in PBS buffer solution (pH=7.4) for 1 hr at 37 °C. The concentration of rGO-PEI-CD-Biotin-Cy7 solution during incubation was about 1.0 mg mL⁻¹. Before confocal fluorescence imaging (TCS CONFOCAL) of HepG2 cell treated, the cells should be washed three times with PBS buffer solution to remove excess rGO-PEI-CD-Biotin-Cy7.
Figure S14. Confocal laser fluorescence images of HepG2 cells incubated with rGO-PEI-CD-Biotin (1.0 mg mL⁻¹) for 1 hr. Bright field (a) and darkness field (b) at Ex = 532 nm.

11. Cell Morphology Observations

Exponentially growing neurogliocyte cells were seeded (1×10⁵ cell per well) in 12-well culture plates and preincubated for 24 hr and then co-incubated with different rGO-PEI-CD-Biotin concentrations for 24 hr again. After the prescribed time, the cells were stained with acridine orange, a drop of cell suspension was dripped to the slide and then the cell solution was covered with the cover glass. The morphology of neurogliocyte cells treated with different concentration carriers were observed with the fluorescence microscope (IX-71, Olympus, Japan).
Figure S15. Morphology pictures of neurogliocyte cells cocultured with the rGO-PEI-CD-Biotin. The concentration of rGO-PEI-CD-Biotin from (a) to (j): 0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 mg L⁻¹, respectively. The magnified multiple is 400 (400×).
12. Cell Viability Assay (FCM assay)

(a) Control group

(b) 20 mg L$^{-1}$ rGO-PEI-CD-Biotin

(c) 40 mg L$^{-1}$ rGO-PEI-CD-Biotin

(d) 60 mg L$^{-1}$ rGO-PEI-CD-Biotin

(e) 80 mg L$^{-1}$ rGO-PEI-CD-Biotin

(f) 100 mg L$^{-1}$ rGO-PEI-CD-Biotin

Figure S16. FCM Figures, I1, I2, I3, and I4 zones denote the cell necrosis period, the late period of the apoptotic cells, the early period of the apoptotic cells, and the
normal cell death period, respectively. From (a) to (f) is the FCM data of neurogliocyte cells, the concentration is 0, 20, 40, 60, 80, 100 mg L$^{-1}$, respectively.

13. rGO-PEI-CD-Biotin/DOX Induces HepG2 Cells to Be Apoptosis

Figure S17. FCM data of HepG2 cells apoptosis assay. The control group (a); The group treated with the 10 mg L$^{-1}$ rGO-PEI-CD-Biotin (b), 1.0 mg L$^{-1}$ DOX (c), and 1.0 mg L$^{-1}$ DOX and 10 mg L$^{-1}$ rGO-PEI-CD-Biotin (d).
14. The Cell Cycle

The cell cycle is a series of events that lead to cell division and replication. It consists of four phases: G1, S, G2, and M (Figure S18). The activation of each phase depends on the proper completion of the previous one. The cell cycle commences with the G1 phase, during which the cell increases its size. During the S phase the cell synthesizes DNA, and in the G2 phase it synthesizes proteins to prepare for cell division. Finally, during the M phase, the cell divides and the two daughter cells enter the G1 phase. Cells that have temporarily stopped dividing can enter a resting phase called G0.

![Figure S18](image-url) The schematic illustration of cell cycle.

Statistical analysis: Experimental data were processed using OriginPro8.SR3 software. Statistical analysis of the data was carried out using Student’s t-test, and the analysis was performed using SPSS Statistics V17. Differences were considered statistically significant when the P value was less than 0.05. The data are presented as the mean-standard deviation.

15. References


