Supporting Information

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

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Supporting Information Contents

1. Synthesis of rGO-C_6H_4-COOH and the Tyndall Effect

rGO-C_6H_4-COOH was synthesized according to the previous method, as shown in Figure S1.[S1]

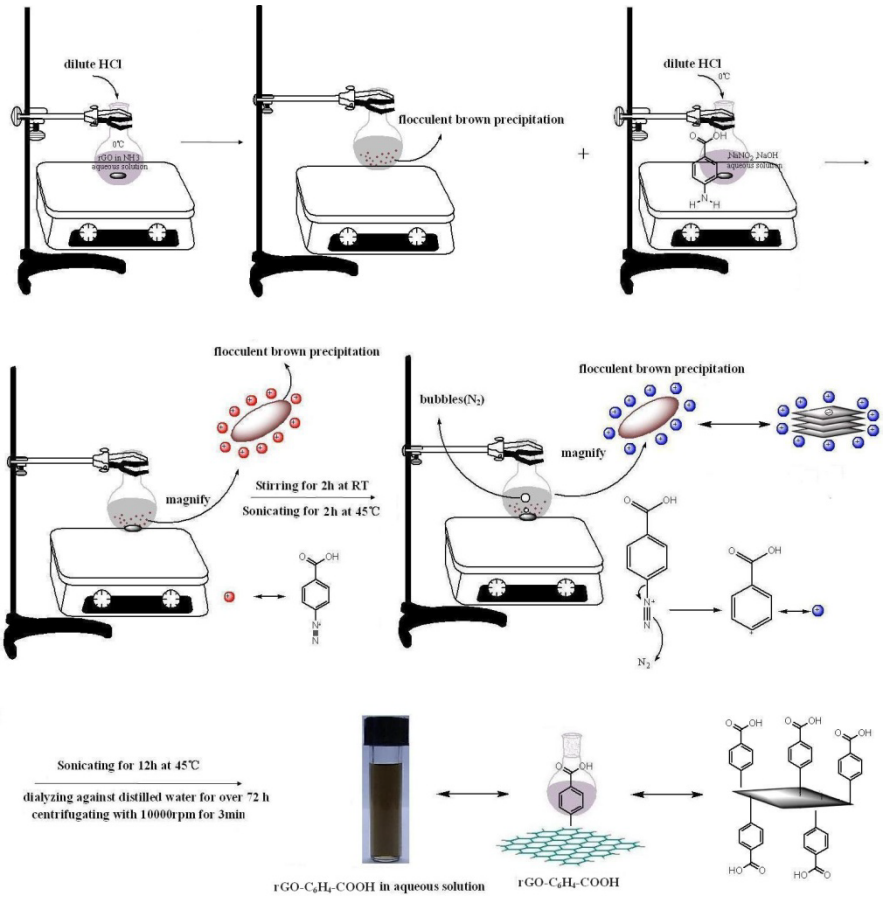
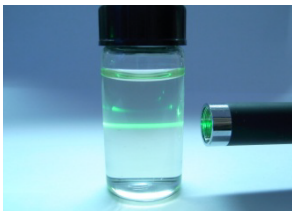
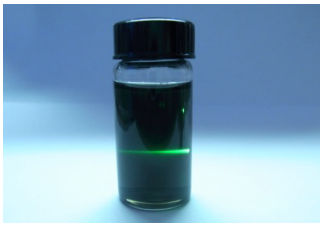


Figure S1. Synthesis route of rGO-C_6H_4-COOH.[S1]

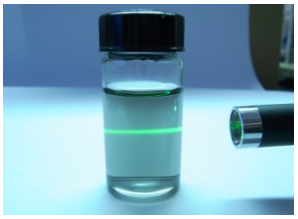
The colloidal nature of GO, rGO and rGO-C_6H_4-COOH was demonstrated by the Tyndall effect, as shown in Figure S2, suggesting excellent water dispersibility.



(a) GO aqueous solution



(b) rGO aqueous solution (0.5% ammonia)



(c) rGO-C_6H_4-COOH aqueous solution

Figure S2. Tyndall effect: GO aqueous solution (a); rGO ammonia aqueous solution (b); rGO-C_6H_4-COOH aqueous solution (c).

2. The XPS (ESCALAB 250) Survey

2.1. The rGO XPS Survey

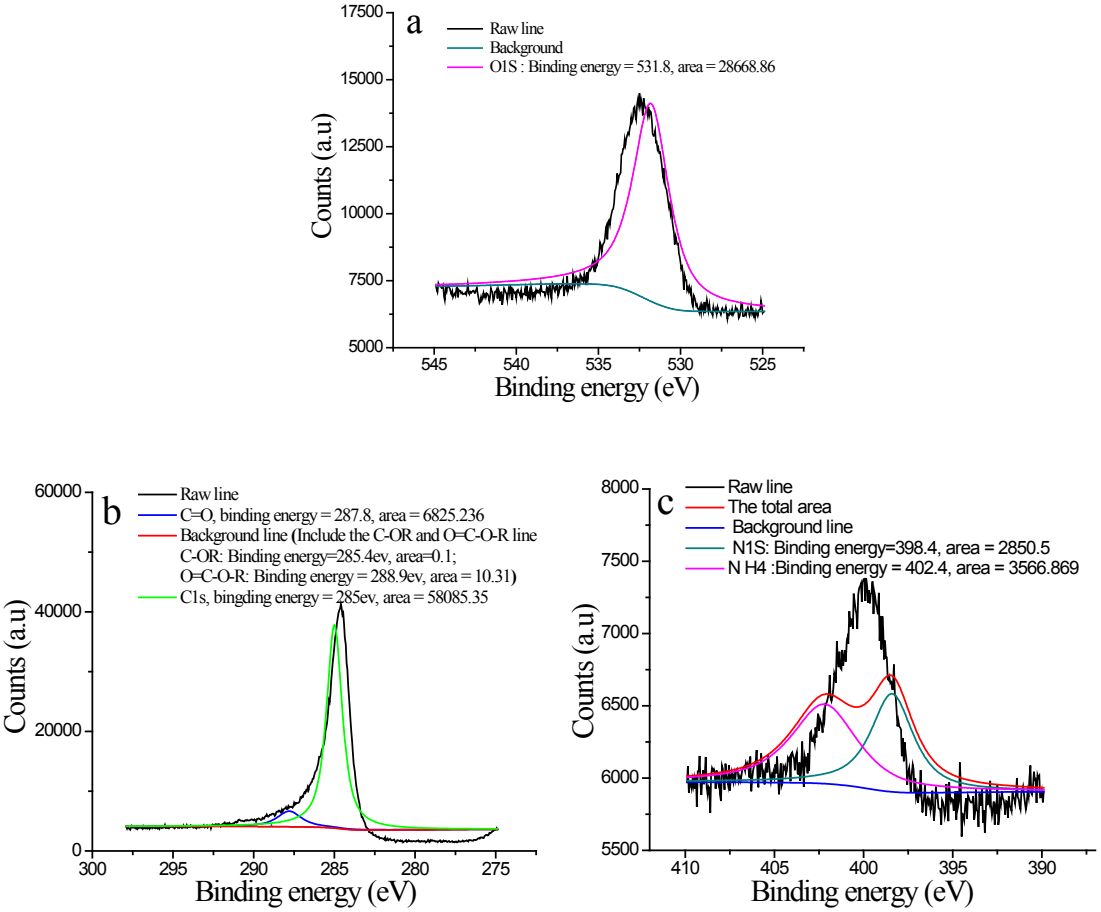


Figure S3. High-resolution XPS survey. O1s survey of rGO (a); C1s survey of rGO (b); N1s survey of rGO (c).

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-C_6H_4-COOH XPS Survey

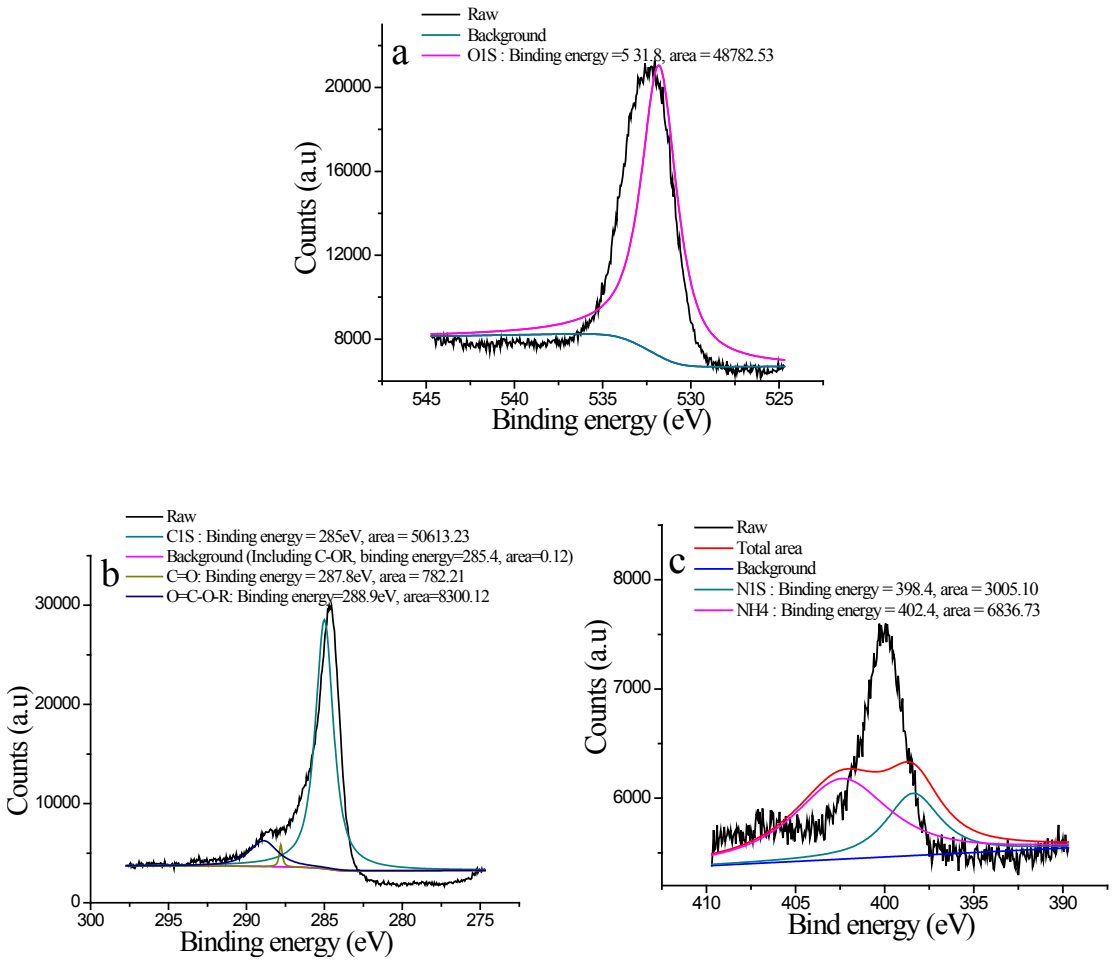


Figure S4. High-resolution XPS survey. O1s survey of rGO-C_6H_4-COOH (a); C1s survey of rGO-C_6H_4-COOH (b); N1s survey of rGO-C_6H_4-COOH (c).

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

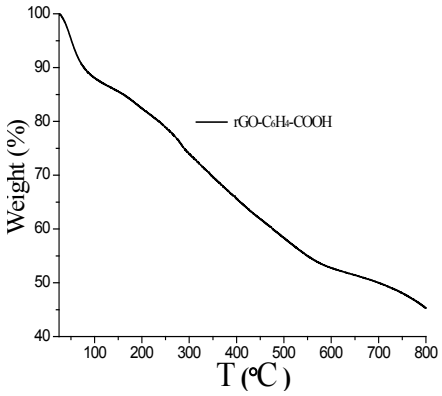


Figure S5. TGA thermogram of rGO-C_6H_4-COOH.

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

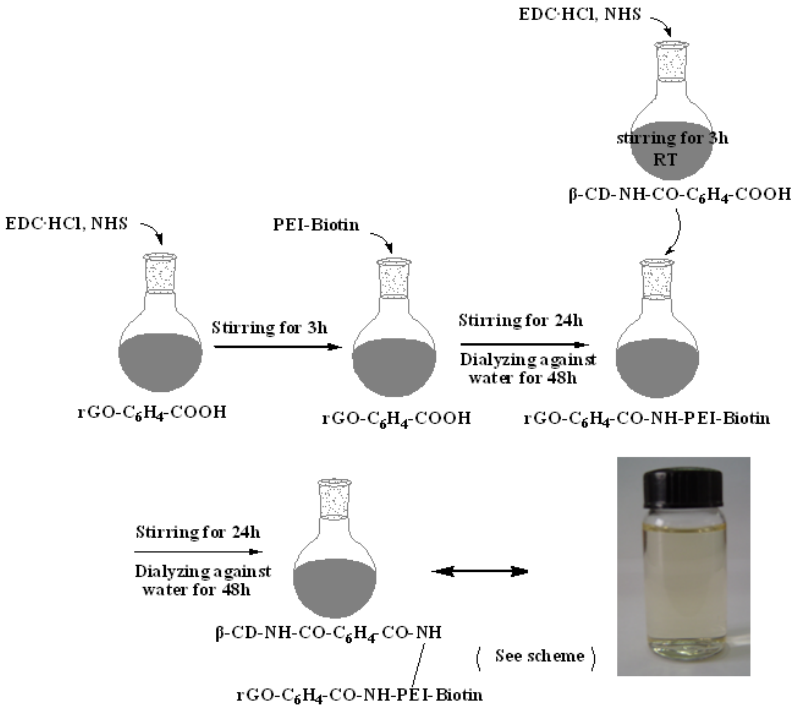
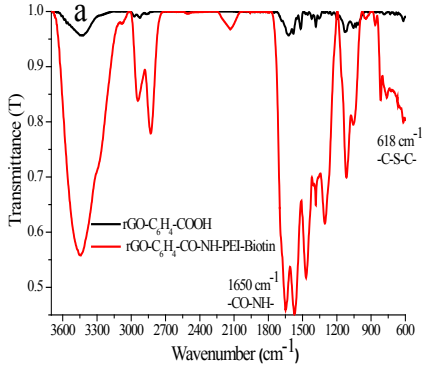


Figure S6. Synthesis route of rGO-PEI-CD-Biotin.



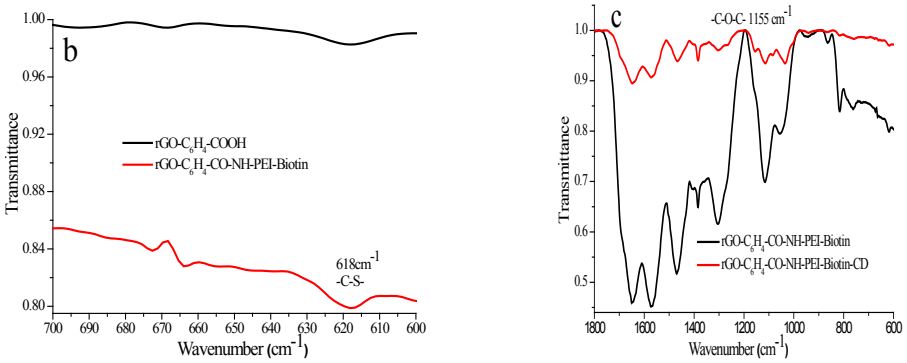


Figure S7. Infrared spectra of rGO-C_6H_4-COOH, rGO-C_6H_4-CO-NH-PEI-Biotin, and rGO-C_6H_4-CO-NH-PEI-Biotin-CD. Infrared spectra of rGO-C_6H_4-COOH and rGO-C_6H_4-CO-NH-PEI-Biotin (a); Magnified infrared spectra of rGO-C_6H_4-COOH and rGO-C_6H_4-CO-NH-PEI-Biotin in the range 600 to 700 wavenumber (b); Infrared spectra of rGO-C_6H_4-CO-NH-PEI-Biotin and rGO-C_6H_4-CO-NH-PEI-CD-Biotin (c).

5. Synthesis of β-CD-NH_2-CO-C_6H_4-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-hydroxylsuccinimide (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) 3A-Amino-3A-deoxy-(2AS,3AS)-β-cyclodextrin hydrate (NH_2-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 ^{1}H NMR (AVANCE 600) spectrum (Figure S8) was mainly used to verify exist of amide (-CONH-) and pheny group, which suggested the successfull

conjugation of β-CD-NH_2 and HOOC-C_6H_4-$COCH_3$. However the hydrogen atom of -COOH (β-CD-NH-CO-C_6H_4-COOH) could not be demonstrated, and these peaks of CD between 3.0-4.0 ppm were very difficult to disern. So LC-MS(Agilent 6510) was applied to futher verify the product (Figure S9). These datas of ^{1}H NMR and MS suggested that the terephthalamide β-CD had been successfully synthesized.

^{1}H NMR ((DMSO-d_6, 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).

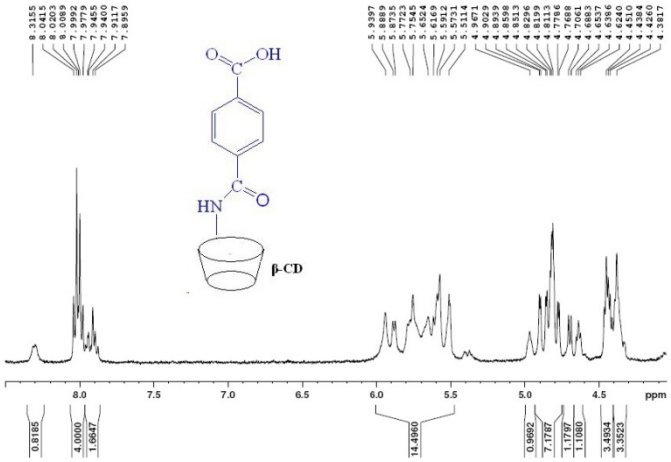


Figure S8. ^{1}H NMR spectrum of terephthalamide β-CD-NH_2-CO-C_6H_4-COOH

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

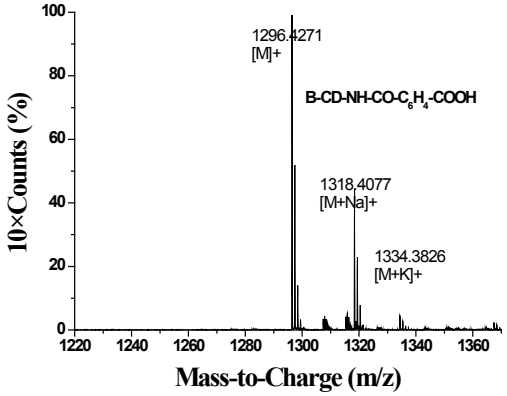
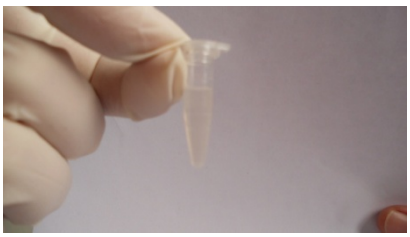
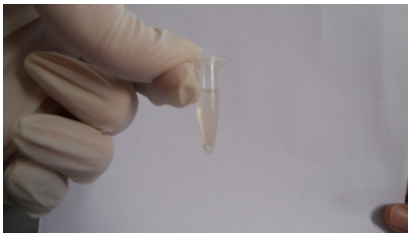


Figure S9. MS spectrum of β-CD-NH_2-CO-C_6H_4-COOH.

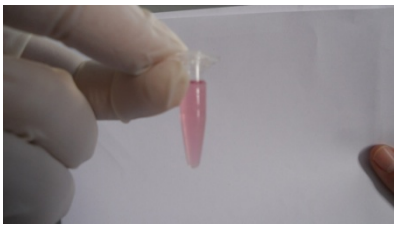
6. The Stability of rGO-PEI-CD-Biotin in Biological Solutions



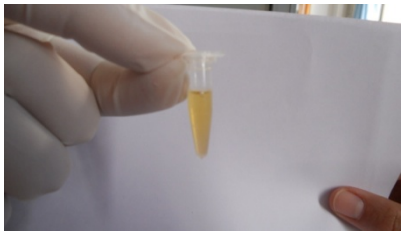
(a) PBS buffer solution



(b) 0.9% NaCl aqueous solution



(c) Cell culture solution



(d) Serum solution

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-NH_2-CO-C_6H_4-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-NH_2-CO-C_6H_4-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-NH_2-CO-C_6H_4-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 3.16×10^4 L mol^{-1} (Figure S11b).

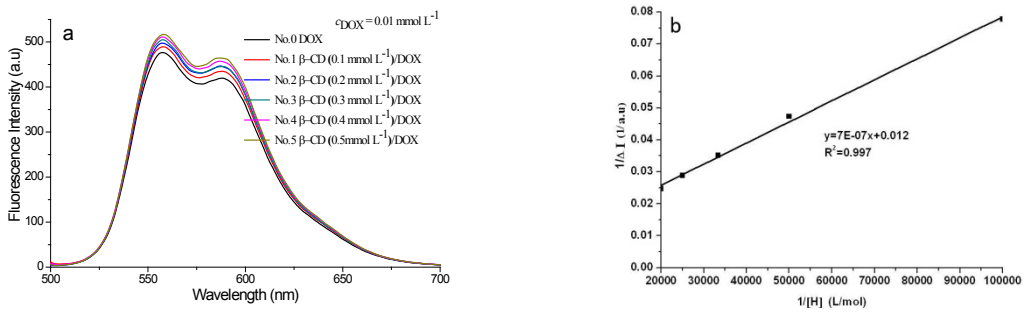


Figure S11. Fluorescence spectra of DOX was included by the β-CD-NH_2-CO-C_6H_4-COOH (a); Benesi-Hildebrand plot of inclusion complexes from DOX with β-CD-NH_2-CO-C_6H_4-COOH (b).

Test sequence of DOX-CD complex	Fluorescence intensity (a.u)
0	476.44
1	489.29
2	497.57
3	504.89
4	511.14
5	516.91

Table S1. The fluorescence intensity of DOX included by β-CD-NH_2-CO-C_6H_4-COOH at 555 nm

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

The weight extinction co-efficiency of rGO-PEI-CD-Biotin and DOX was calculated by the method proposed.[S4] The DOX weight extinction co-efficiency is ~8.36 L g^{-1} cm^{-1} at 485 nm, and the rGO-PEI-CD-Biotin is ~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 coefficiency of ~1.16 L g^{-1} cm^{-1}.

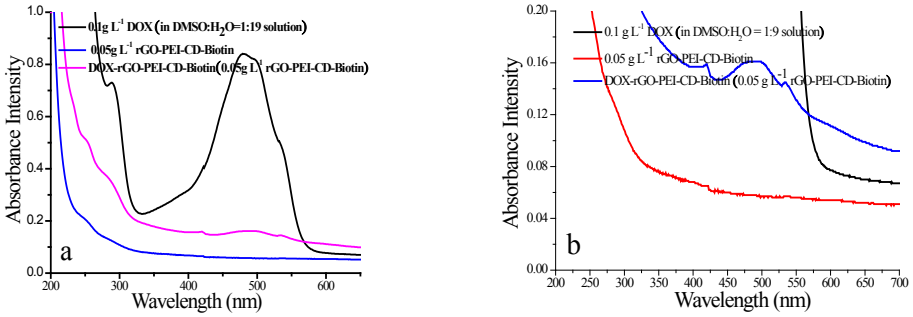


Figure S12. UV-vis spectra of DOX, rGO-PEI-CD-Biotin, and DOX-rGO-PEI-CD-Biotin (a); The magnified UV-vis spctra of DOX, rGO-PEI-CD-Biotin, and DOX-rGO-PEI-CD-Biotin (b).

9. Drug Releasing Behavior

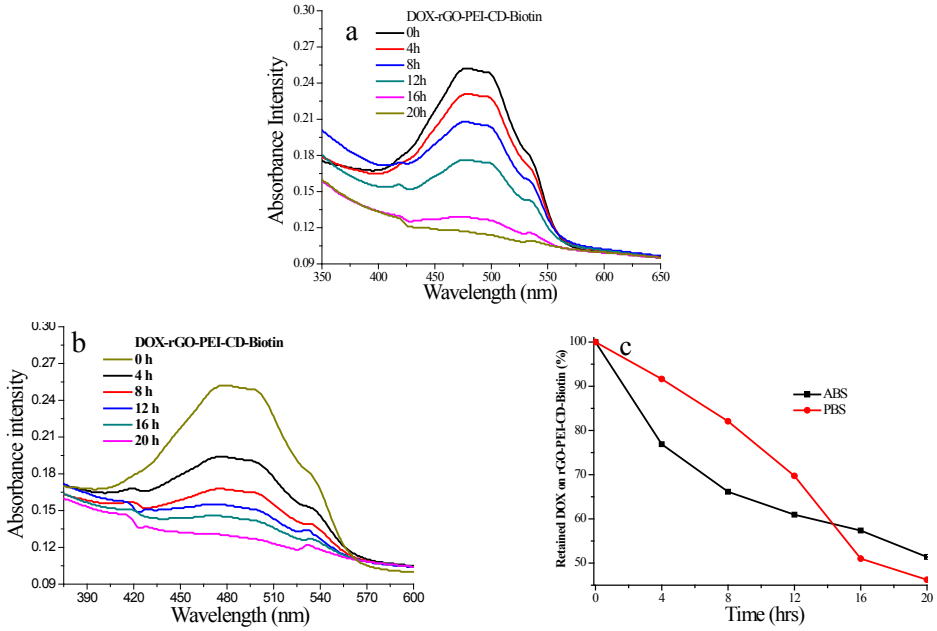
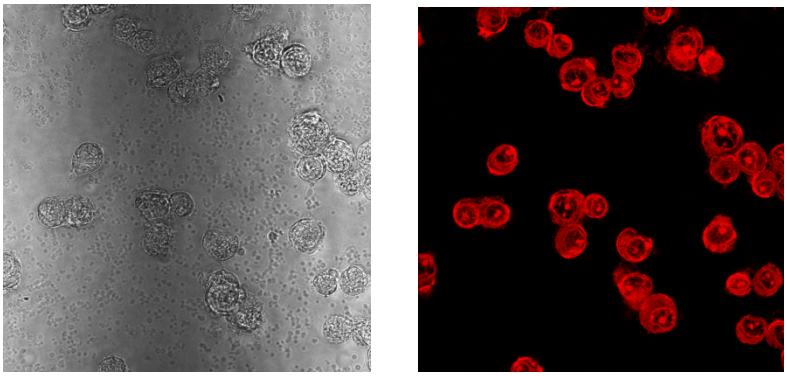


Figure S13. The releasing behavior of DOX on rGO-PEI-CD-Biotin in pH=7.4 PBS (a); The releasing behavior of DOX on rGO-PEI-CD-Biotin in pH=5.5 ABS (b); The releasing plot of DOX on rGO-PEI-CD-Biotin in PBS and ABS buffer solution (c).

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.

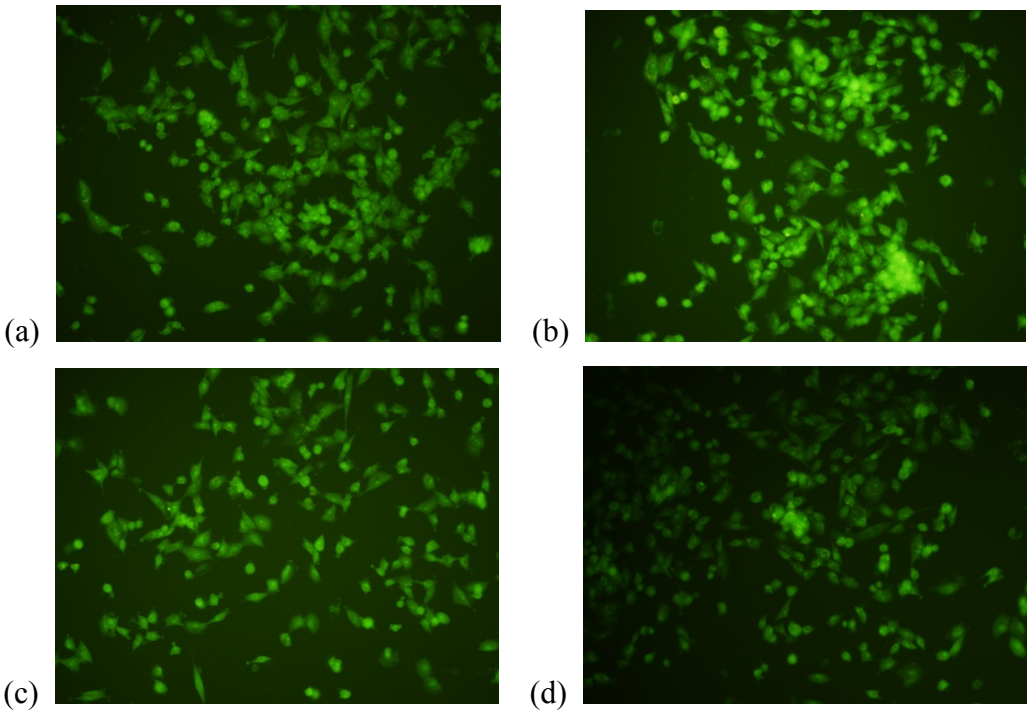


(a) Bright field (b) Darkness field

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 (1×10^5 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).



(a) (b)

(c) (d)

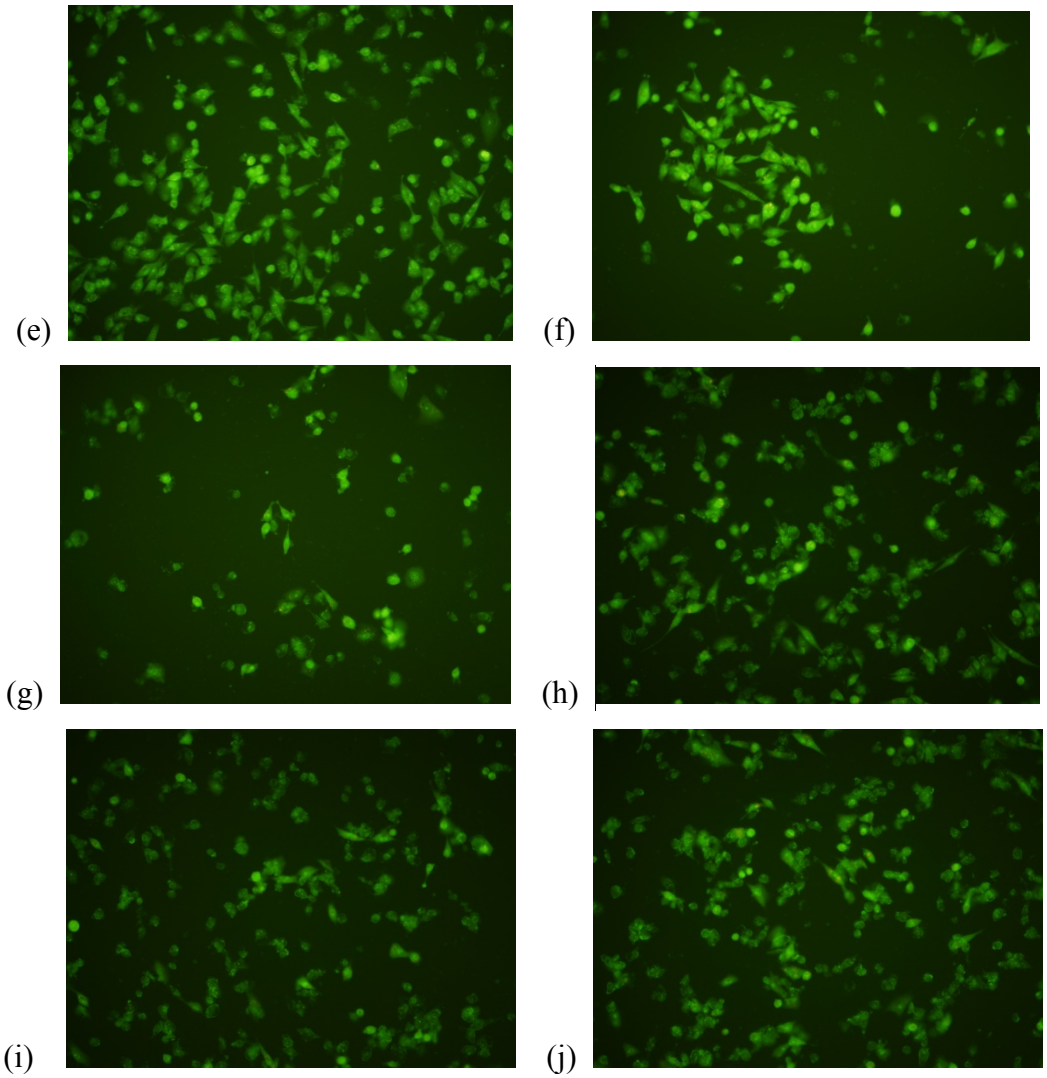
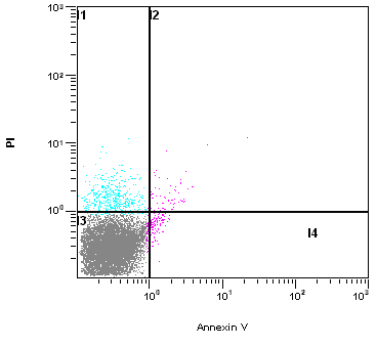


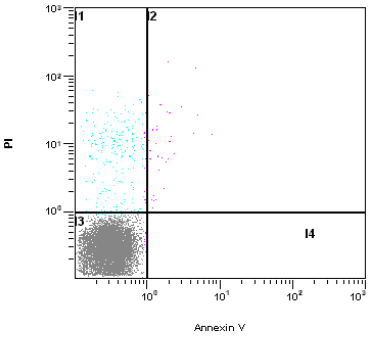
Figure S15. Morphology pictures of neurogliocyte cells cocultured with the rGO-PEI-CD-Biotin. The concentration of rGO-PEI-CD-Biotin from (a) to (j): 0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 mg L^{-1}, respectively. The magnified multiple is 400 (400×).

12. Cell Viability Assay (FCM assay)



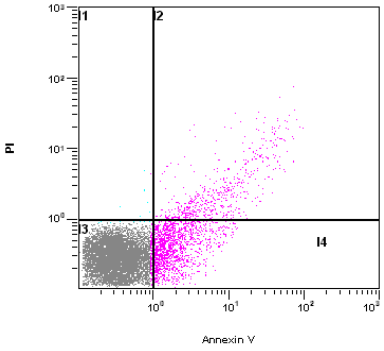
I1	446	4.46%
I2	59	0.59%
I3	9397	93.97%
I4	98	0.98%

(a) Control group



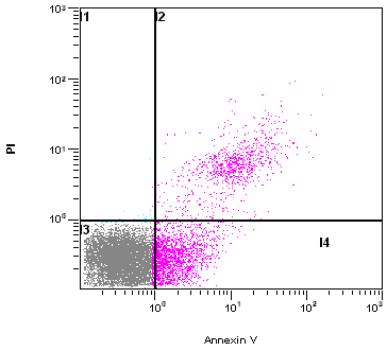
I1	246	2.46%
I2	31	0.31%
I3	9722	97.22%
I4	1	0.01%

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



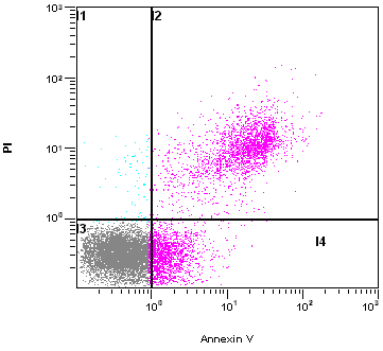
I1	16	0.16%
I2	543	5.43%
I3	8083	80.83%
I4	1358	13.58%

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



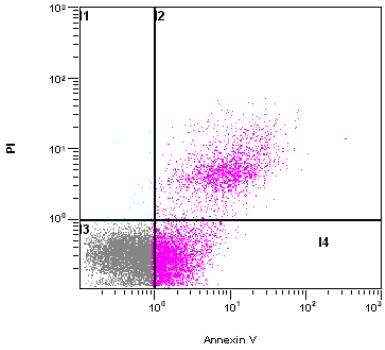
I1	23	0.23%
I2	896	8.96%
I3	7363	73.63%
I4	1718	17.18%

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



I1	74	0.74
I2	2328	23.28
I3	6110	61.10
I4	1488	14.88

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



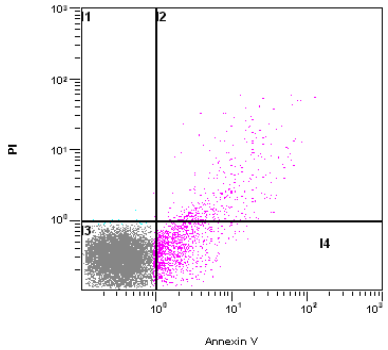
I1	25	0.25
I2	1758	17.58
I3	5399	53.99
I4	2818	28.18

(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

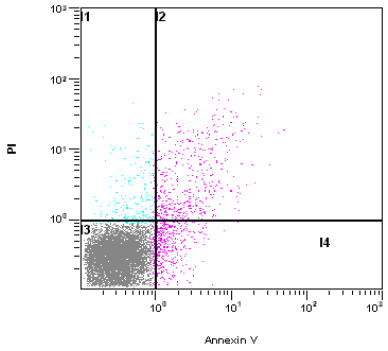
normal cell death period, respectively. From (a) to (f) is the FCM data of neurogliocyte cells, the concentration is 0, 20, 40, 60, 80, 100 mg L^{-1}, respectively.

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



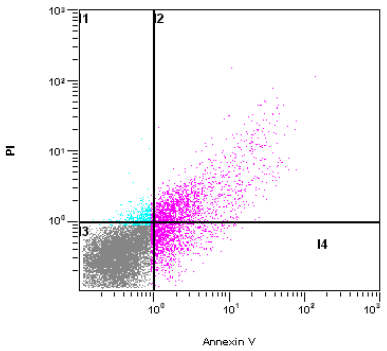
I1	13	0.13%
I2	360	3.60%
I3	8702	87.02%
I4	925	9.25%

(a) Control group



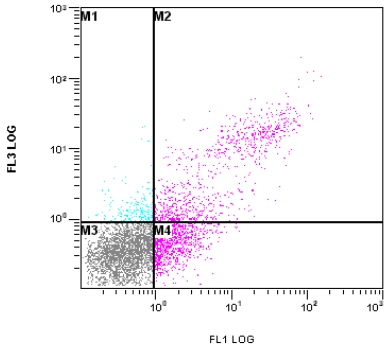
I1	217	2.17%
I2	465	4.65%
I3	8941	89.41%
I4	377	3.77%

(b)10 mg L^{-1} rGO-PEI-CD-Biotin



I1	401	4.01%
I2	1439	14.39%
I3	7148	71.48%
I4	1012	10.12%

(c) 1.0 mg L^{-1} DOX



M1	409	4.09
M2	1927	19.27
M3	5687	56.87
M4	1977	19.77

(d) 1.0 mg L^{-1} DOX + 10 mg L^{-1} rGO-PEI-CD-Biotin

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.[S5] 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.[S6]

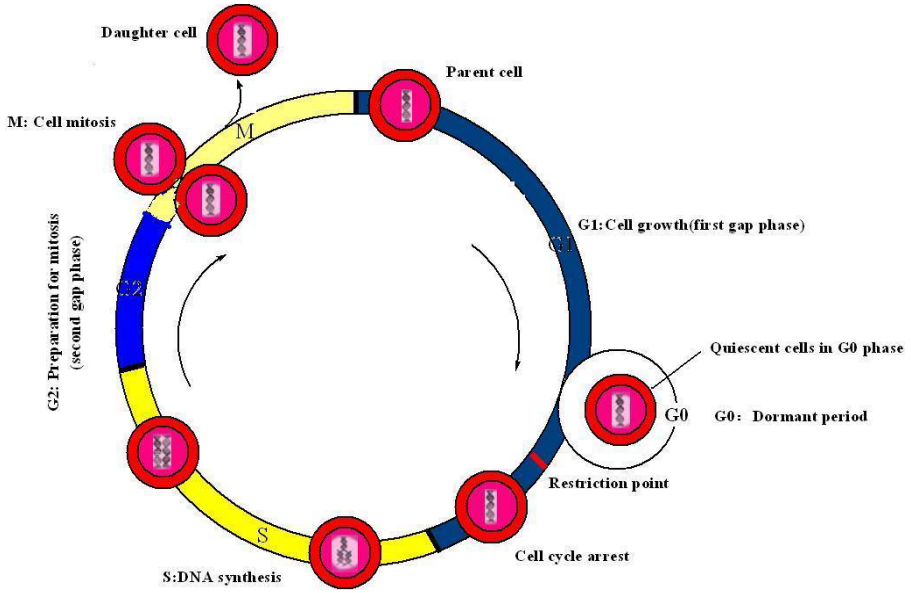


Figure S18. The schematic illustration of cell cyle.[S1]

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

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