Electronic Supplementary Information (ESI)

For

In vivo study of a novel, safe, rapid, and targeted red carbon dot probe for recognition of tumor with high expression of folate enzyme

Zhuoling Zhong,[a] Xingying Li,[b] Shuyao Liu,[c] Chuanwei Zhang,[d] Xiaoping Xu *[a] and Liyun Liao *[b]

a. West China School of Pharmacy, Sichuan University, Chengdu 610041, China. Email: zhongzhuoling@stu.scu.edu.cn

b. West China School of Pharmacy, Sichuan University, Chengdu 610041, China.

c. West China School of Pharmacy, Sichuan University, Chengdu 610041, China.

d. West China School of Pharmacy, Sichuan University, Chengdu 610041, China.

*a West China School of Pharmacy, Sichuan University, Chengdu 610041, China. Email: xuxp319@scu.edu.cn

*b Chengdu Med Coll, Sch Pharm, 783, Xindu Ave, Chengdu 610500, China. Email：llyhx210@tom.com

Calculation of coupling rate

At present, UV method is widely used to calculate the coupling amount in literature. We established a more accurate and scientific method to detect the coupling amount of FA by HPLC. By continuously measuring the content of FA in the external liquid of dialysis bag until the content of FA does not change. The amount of FA outside the dialysis bag was calculated, then, the coupling amount is obtained by subtracting the permeation amount from the feeding amount, and the coupling rate is obtained by dividing the coupling amount by the feeding amount.

Establish a HPLC method for the determination of folic acid outside dialysis bag

Chromatographic method

Referring to the relevant literature conditions and combined with the results of pre-test, the chromatographic conditions were selected as follows: symmetry C18 (4.6X250 mm，5um) The mobile phase was potassium dihydrogen phosphate solution (adjusted to pH 6.3 with 0.1 mol/L potassium hydroxide) - methanol (92:8). The detection wavelength was 280 nm; The flow rate was 1.0ml/min; Column temperature: 30 ℃.
Under these chromatographic conditions, the peak shape of the chromatographic peak is symmetrical, the symmetry factor is 0.96, the number of theoretical plates is 8000, more than 3000, all meet the requirements.

System adaptability test

Under the chromatographic conditions, accurately weigh the right amount of FA reference substance, put it into a volumetric flask, and fix the volume with 0.5% ammonia solution, diluted to per 25 ug/ml FA solution. Shake well to obtain the system adaptability test solution, and use 10 ul microinjector 10 ul. Inject into HPLC, record the chromatogram and inject the sample for 6 times. The result is shown in Figure S1.

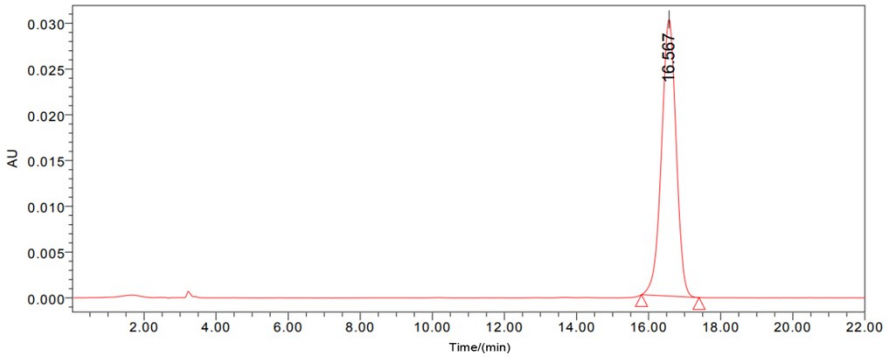

Figure S1 Chromatogram of systemic adaptability test of FA

The number of theoretical plates is more than 8000, the symmetry factor is 0.956, and the RSD of FA peak area is less than 2.0% under the condition of six consecutive injections. In conclusion, the results of system adaptability meet the requirements.

Methodology investigation

Linearity and range

Accurately weigh 25 mg FA reference substance, put it into a 250 ml volumetric flask, add 0.5% ammonia solution to dissolve, and then fix the volume, made into 100 ug/ml FA stock solution. Then dilute with 0.5% ammonia solution to 0.78, 1.56, 3.13, 6.25, 12.50, 25 and 50 ug/ml of test solution.

Table S1 The linear test results of FA

Sample Concentration(μg/mL)	A (mAU)	Linear Equation	Linear Range
0.78	28506		
1.56	56312		
3.13	110804		
6.25	228055	y = 35747x + 4791.6 R^2 = 0.9993	0.78 μg/mL-50.00 μg/mL
12.50	485448		
25.00	875372		
50.00	1795799		

According to the chromatographic conditions, 10 ul micro injection needle took 10 ul samples of test solution. The peak area (a) of folic acid was determined by HPLC, and in the concentration (ug/l) is the abscissa, and the peak area (A) is the ordinate, the linear equation y=35747x+4791.6, and the correlation coefficient R^2= 0.9993, the linear range is 0.78 ug/ml-50.00 ug/ml. The linear relationship is good and meets the requirements. The results are shown in Table S1 and Figure S2.

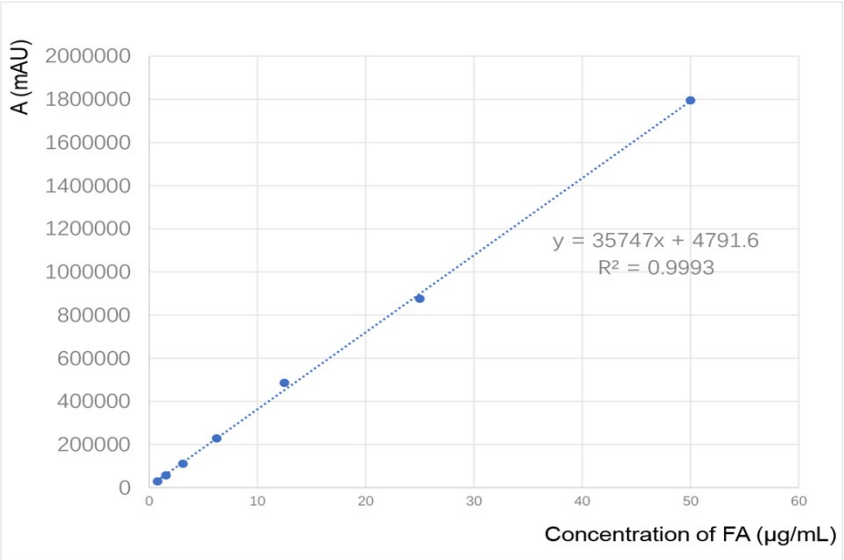

Figure S2 Linear relationship of FA

Table S2 LOD and LOQ of FA

	FA
LOD (S/N≥10)	78ng/ml
LOQ (S/N≥3)	7.8ng/ml

Limit of detection (LOD) and limit of quantitation (LOQ)

The lowest concentration point used in the above linear study was 0.78 ug/ml FA reference solution was diluted step by step, in turn, use a micro injection needle to

precisely measure the above diluted solution for 10 ul was injected into HPLC, and the chromatogram was recorded for calculation. According to the signal-to-noise ratio method, when S/N≥10, determine the concentration as the LOQ, and determine the concentration as LOD in S/N≥3. The results are shown in Table S2. The LOD of folate is 78 ng/ml, and the LOQ of folic acid is 7.8 ng/ml

Injection precision test

Precisely weigh the right amount of FA reference substance, add 0.5% ammonia solution to dissolve and dilute it to make 25.00 ug/ml FA solution, as the reference solution, according to the chromatographic conditions, accurately measure 10 ul was injected into the HPLC for 6 consecutive times. The chromatogram was recorded and the change of peak area was investigated. The test results are shown in Table S3. The results showed that the RSD was 1.37%. The precision of this method is good.

Table S3 The results in injection precision test of FA

	t_R(min)	A(mAU)	RSD(%)
1	17.371	840017	1.37
2	17.815	830624	
3	16.766	861837	
4	16.651	856861	
5	16.701	846360	
6	16.567	853926	

Intra assay precision

Take 6 parts outside the same dialysis bag, as the test solution, according to the above chromatographic conditions, take 10 ul was injected into HPLC for 6 consecutive times. The chromatogram was recorded and the change of peak area was investigated. The test results are shown in Table S4. The results showed that the average FA concentration in the external fluid of the dialysis bag was 8.88 ug/ml The RSD was 1.33% and the intra assay precision of this method is good.

Table S4 The results in intra assay precision test of FA

	t_R(min)	A(mAU)	RSD(%)
1	18.718	320491	1.33
2	17.269	320193	
3	17.24	327401	
4	17.264	321919	
5	17.355	327160	
6	17.397	316490	

Inter-day precision

Take 6 parts outside the same dialysis bag, as the test solution, according to the above chromatographic conditions, take 10 μl was injected into the HPLC for 6 consecutive injections, and the same dialysate was used the next day. The above steps were repeated, and the inter day precision was calculated with the data of two days. Record the chromatogram and investigate the peak area change. The test results are shown in Table S5, the results showed that the RSD of inter day precision was 2.22%, so the inter day precision of this method is good.

Table S5 The results in inter-day precision test of FA

		tR(min)	A(mAU)	RSD(%)
The first day	1	18.718	320491	2.22
	2	17.269	320193	
	3	17.24	327401	
	4	17.264	321919	
	5	17.355	327160	
	6	17.397	316490	
The second day	1	18.161	311228	
	2	17.963	308276	
	3	17.452	309226	
	4	17.342	313859	
	5	17.394	309928	
	6	17.346	308995	

Recovery (%)

A certain amount of FA was accurately weighed and 0.5% ammonia solution was used to prepare15.68 ug/ml,8.7 ug/ml,1.74 ug/ml of the three FA control solutions, 5 ml of the above reference solution was accurately measured and put into a 10 ml volumetric flask, and then the external liquid of the above dialysis bag was used to fix the volume. Three copies of the test solution were prepared in parallel with high, medium and low concentrations to obtain the test solution. According to the above chromatographic conditions, 10 ul was injected into the HPLC and the chromatogram was recorded. Calculate the recovery rate of sample addition, and the results are shown in Table S6. The results showed that the high, middle and low group average recoveries were

Table S6 Results of the recovery rate of FA

Concentration of FA	Added quantity(ug/ml)	Detection quantity(ug/ml)	Recovery (%)	Average Recovery (%)	RSD (%)
20%	0.87	0.90	102.94	99.71	1.88
	0.87	0.87	99.43		
	0.87	0.84	96.76		
100%	4.36	4.28	98.17	100.15	
	4.36	4.42	101.44		
	4.36	4.39	100.82		
180%	7.84	7.76	98.96	99.67	
	7.84	7.77	99.07		
	7.84	7.92	100.99		

99.67%, 100.15% and 99.71% with RSD of 1.88%. The recovery rate meets the requirements, and the method has high accuracy.

Determination of coupling rate

In order to determine the best dialysis time, the dialysate outside the dialysis bag was collected at several time points, and according to the above chromatographic conditions 10μl of the dialysate accurately measured was injected into the HPLC, the chromatogram was recorded, and the peak area was calculated. The results are shown in Figure S3 below. The results show that in the first 1-6 h, the dialysis rate is the fastest, and a large amount of FA permeates out, and then the rate gradually slows down. After 24 h, the FA permeates out basically reaches the platform. Therefore, 24 h is the best dialysis time based on the time factor and dialysis volume.

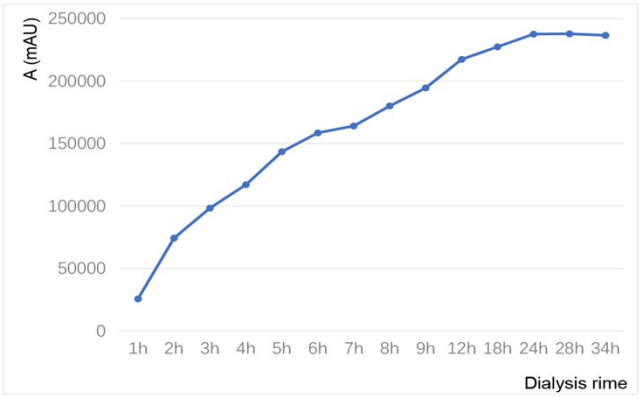

Figure S3 Peak area of FA outside the bag in different dialysis time

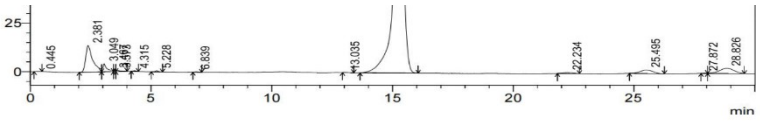

Figure S4 Chromatogram of conjugates in dialysis bag

In order to further determine the scientific nature of the purification method, the conjugates with a certain concentration after dialysis for 24 h were injected into the HPLC with DAD detector. The chromatographic conditions were exactly the same. The results were shown in Figure S4 and S5. It was found that there was no obvious FA Peak in the HPLC peak, and the UV spectrum of the main peak was consistent with that of the previous conjugates, so it could be further determined, FA was successfully modified on the carbon dots, and dialysis was also a feasible and effective purification method. Therefore, dialysis bag (MWCO=500 Da) for 24 h was selected as the purification method.

Finally, the coupling rate was calculated by the above method, the content was calculated by the standard curve method, and the coupling rate was calculated by equation S1.

$$Coupling\ Rate\ (\%) = \frac{(m_{FAtotal} - m_{FA\ outside\ the\ bag})}{m_{FA}} \times 100\%$$

equation S1

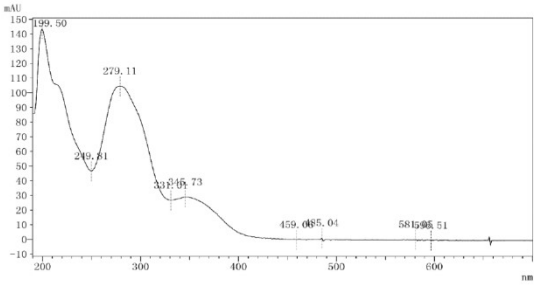

Figure S5 UV spectrum of main peak of conjugates in dialysis bag

$m_{FAtotal}$- Total FA dosage(mg)

$m_{FA\ outside\ the\ bag}$- Total FA dosage outside the dialysis bag(mg)

The coupling rate of FA-DCCDs was 75.32%.

Quality control of conjugates

Effect of salt concentration on FA-DCCDs

Take 1ml FA-DCCDs solution into 10 ml volumetric flask, add different concentrations of NaCl solution, fix the volume, and measure the fluorescence intensity. Results as shown in Figure S6, in 0~1.3 mg/l NaCl solution, with the increase of NaCl solution concentration, the fluorescence intensity of FA-DCCDs was stable, indicating that the fluorescence intensity of FA-DCCDs as less affected by salt solution and had good stability.

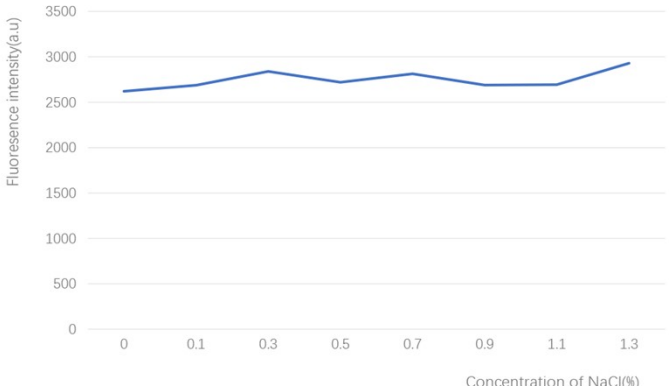

Figure S6 The influence of salt ion concentration on fluorescence intensity of FA-DCCDS

Effect of pH on FA-DCCDs

Take 1ml FA-DCCDs into a 10ml volumetric flask, add buffer salt solutions with different pH values, fix the volume, and measure the fluorescence intensity. As shown in Figure S7, in BR buffer solution with pH2-11, the fluorescence intensity of FA-DCCDs increases slowly with the increase of pH in acidic to neutral environment, and increases sharply in neutral to alkaline environment (pH 9), which is stable in strong alkaline environment, and still has strong fluorescence intensity near cell environment (pH7.3). This carbon dot is greatly affected by strong acid and strong alkaline environment, the acid-base stability is average.

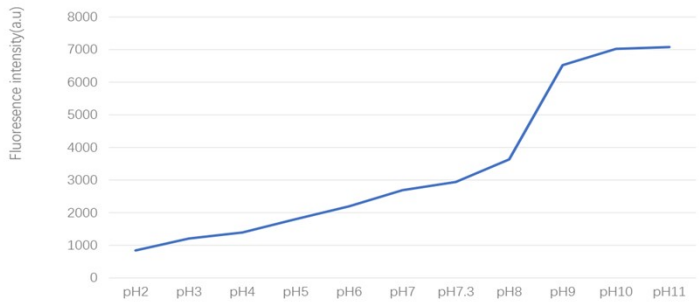

Figure S7 The influence of pH on fluorescence intensity of FA-DCCDS

Effect of UV irradiation time on FA-DCCDs

The FA-DCCDs solutions were irradiated by UV lamp, and the fluorescence intensity was measured at different irradiation time to investigate the effect of UV irradiation

time on the fluorescence intensity. As shown in Figure S8, the fluorescence intensity of FA-DCCDs decreased slightly after UV irradiation for 2 h, and decreased by 30% after 8 h. The results showed that the fluorescence intensity of FA-DCCDs was greatly affected by UV irradiation.

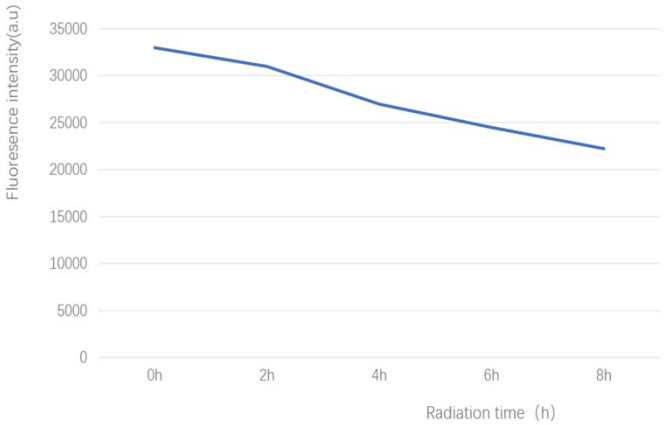

Figure S8 the influence of UV irradiation time on fluorescence intensity of FA-DCCDS

Effect of storage time on FA-DCCDs

The FA-DCCDs solutions were stored at room temperature for a period of time, and then the fluorescence intensity was measured to investigate the effect of storage time on the fluorescence intensity. As shown in Figure S9, the fluorescence intensity of FA-DCCDs only slightly decreased after 8h at room temperature, indicating that the carbon dots can be stored stably at room temperature.

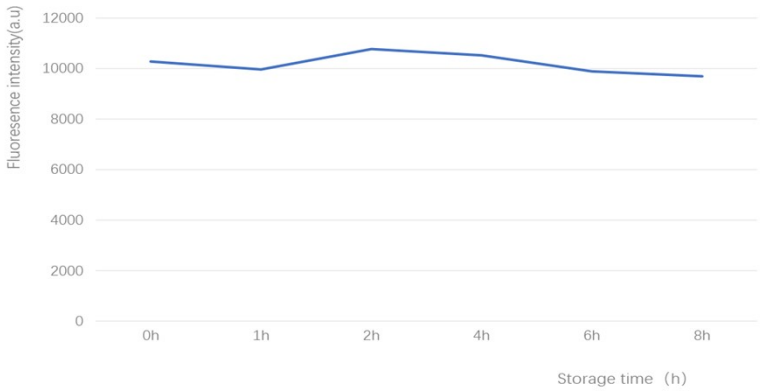

Figure S9 the influence of storage time on fluorescence intensity of FA-DCCDS

Effect of common potential coexisting substances on fluorescence intensity of FA-DCCDs

In order to investigate the effect of common cell cations, trace metal ions, sugars, amino acids and other potential coexisting substances on the fluorescence intensity of FA-DCCDs. The potential coexisting substances are 1000 times of K^+, Mg^{2+}, Na^+, Ca^{2+}, sucrose, glucose and glycine; 100 times of leucine, histidine and alanine; 50 times of Co^{2+}; 10 times of Cr^{2+}; 5 times of Ni^+, Zn^{2+}, Fe^{2+}; 2 times Cu^{2+}. The concentration of FA-DCCDs was 5 ug/ml.

Table S7 the influence of coexisting substances on fluorescence intensity of FA-DCCDs

Co-existing substances	Relative Error (%) FA-DCCDs	Co-existing substances	Relative Error (%) FA-DCCDs
×1000 K^+	-3.23	×1000 sucrose	0.54
×1000 Mg^{2+}	-4.12	×1000 glucose	-0.90
×1000 Na^+	-2.56	×100 leucine	-3.12
×1000 Ca^{2+}	-3.12	×1000 glycine	-2.78
×10 Cr^{2+}	-2.85	×100 histidine	0.31
×50 Co^{2+}	-0.90	×100 alanine	0.60
×5 Ni^+	-4.20		
×5 Fe^{3+}	-3.71		
×2 Cu^{2+}	-2.77		
×5 Zn^{2+}	-3.39		

The results are shown in Table S7, the fluorescence intensity of FA-DCCDs is not affected by the potential coexisting substances. The relative error was less than ±5%.

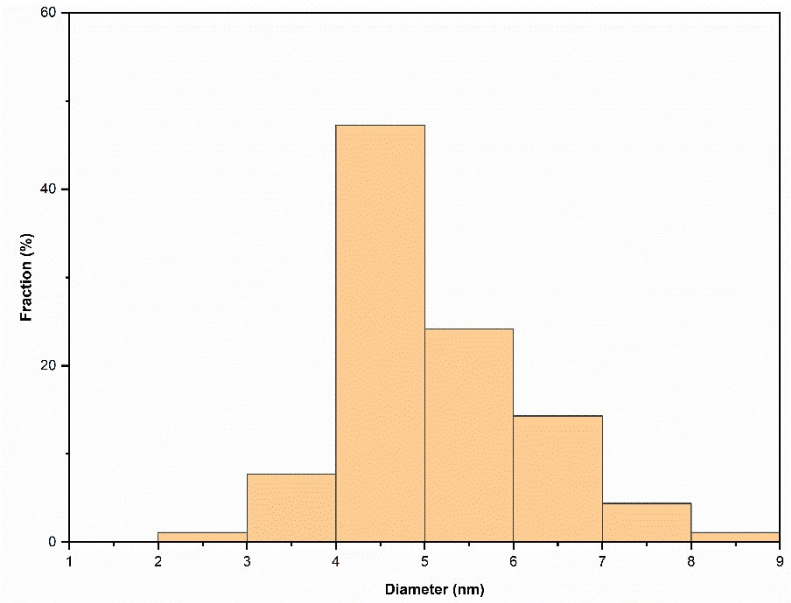

Figure S10 Size distribution of DCCDS