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Dendritic Nanoconjugate Containing Optimum Folic Acid for Targeted Intracellular Curcumin Delivery

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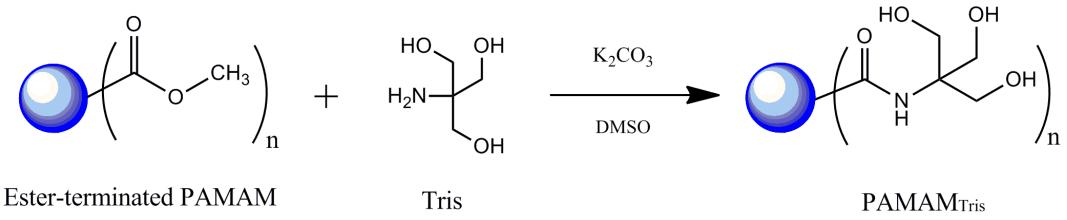
MATERIALS AND METHODS

Materials

Curcumin, poly (ethylene glycol) (OH-PEG-OH) (Mw: 2000), and P-toluenesulfonyl chloride (TsCl) were purchased from Institute of Guangfu Fine Chemical Research (Tianjin, China). Folic acid, glutaric anhydride, 4-dimethylamino pyridine (DMAP), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and potassium phthalimide were obtained from Aladdin (Shanghai, China). TLC silica gel plate (GF254) and silica gel powder (ZCX 2) were sourced from Haiyang Chemicals (Qingdao, China). The human hepatocellular carcinoma cell line (HepG2) was provided by Institute of Biomedical Engineering (Chinese Academy of Medical Sciences & Peking Union Medical College). Dulbecco's modification of eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum were sourced from HyClone Inc. (Logan City, Utah, USA). All other chemicals were of analytical grade from Jiangtian Chemicals (Tianjin, China).

Synthesis of $PAMAM_{Tris}$

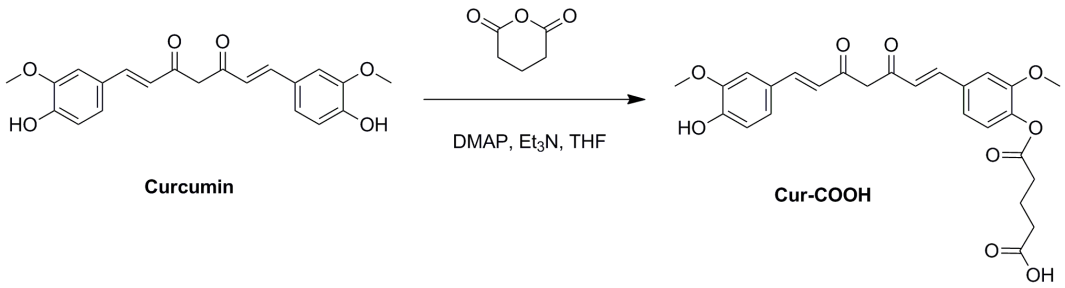
Ester-terminated PAMAM (Generation 2.5) with an ethylenediamine core was synthesized by the divergent growth method.[1] $PAMAM_{Tris}$ was obtained by conjugation between PAMAM and excess Tris in dimethyl sulfoxide (DMSO) in the presence of potassium carbonate (1.5 molar equivalents per terminal ester) at 50^{o}C for 72 h (**Scheme S1**). After filtration and solvent elimination, the crude products were dissolved in water followed by precipitation in acetone. Subsequent dialysis was then carried out against water for 48 h using regenerated cellulose membrane (molecular weight cut-off/MWCO: 1000 Da) followed by lyophilization. The final product was obtained and stored ready for use.



Scheme S1. The synthetic route of $PAMAM_{Tris}$.

Synthesis of Cur-COOH

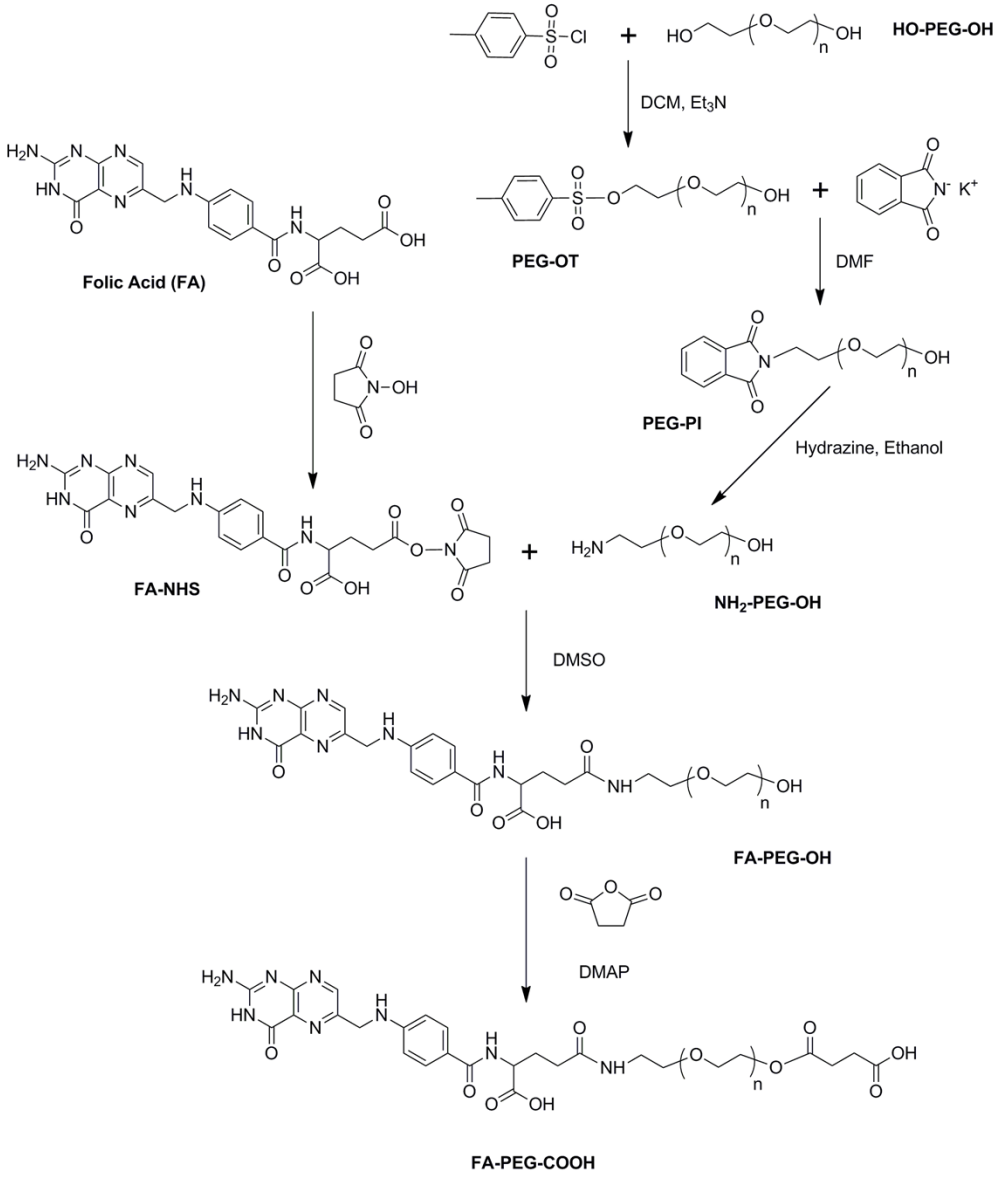
Mono-carboxyl-terminated curcumin (Cur-COOH) was synthesized according to a previously published method (**Scheme S2**).[2] Briefly, curcumin (Cur) was purified by repeated crystallization in methanol and petroleum ether prior to use. After curcumin preparation, Cur (2.00 g), triethylamine (Et_3N) (1.33 mL) and DMAP (0.15 g) were mixed in tetrahydrofuran (THF) (100 mL) followed by addition of glutaric anhydride (0.69 g) which has already been dissolved in THF (5 mL). The reaction was maintained at 90°C for 17 h under nitrogen protection. After solvent evaporation, ethyl acetate (100 mL) was used to re-dissolve the residue reaction product followed by extraction with 1 M hydrochloric acid (15 mL) for three times. Elimination of organic phase was then carried out by a rotary evaporator. The target product (Cur-COOH) was purified by silica gel column chromatography and the mobile phase was a mixture of ethylene dichloride and methanol (97:3, v/v).



Scheme S2. The synthetic route of mono-carboxyl-terminated curcumin (Cur-COOH).

Synthesis of NH_2-PEG-OH

Heterofunctional PEG (NH_2-PEG-OH) was generated in three main steps (**Scheme S3**).[3] First, HO-PEG-OH (15.0 g), TsCl (2.14 g), Et_3N (4 mL) were mixed in 100 mL dichloromethane (DCM) for 24 h at ambient temperature. The remaining Et_3N was cleared by repeated washing with hydrochloric acid (1 M) before addition of excess sodium sulphate to the organic phase. The mixture was stirred overnight. The intermediate product (PEG-OT) was obtained by precipitation in ice-cold diethyl ether followed by vacuum-drying. Subsequently, the PEG-OT (14.4 g) was reacted with potassium phthalimide (7.15 g) in 100 mL dimethylformamide (DMF) at 120^oC for 6 h followed by filtration and the removal of DMF. The remaining reaction product was re-dissolved in water (40 mL) and the filtrate was repeatedly extracted with DCM. The organic phase was de-hydrated by sodium hydrate and precipitated in diethyl ether for the generation of an intermediate product, PEG-PI. The obtained PEG-PI was then dissolved in 100 mL ethanol followed by subsequent addition of 8 mL hydrazine hydrate under nitrogen atmosphere. After 12 h, the crude products were purified by the method of PEG-PI purification described above. The target product (NH_2-PEG-OH) was obtained by silica gel column chromatography followed by elution with a mixture of DCM and methanol (8:1, v/v) which also contains 0.3% (v/v) Et_3N.



Scheme S3. The synthetic route of heterofunctional PEG (NH_2-PEG-OH) and FA-PEG-COOH.

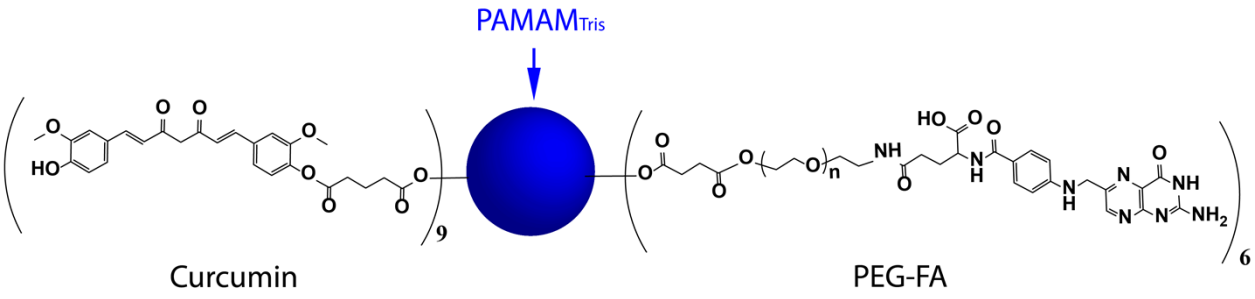
Synthesis of FA-PEG-COOH

FA-PEG-COOH was synthesized via typical DCC/NHS chemistry (**Scheme S3**).[4] Briefly, FA (126 mg) was activated by DCC (61 mg) and NHS (35 mg) in the presence of Et_3N at ambient temperature for 24 h. The product (FA-NHS) was then coupled with NH_2-PEG-OH (622 mg) in DMSO under alkinic condition via Et_3N addition. The reaction was allowed to be carried out for 48 h at ambient temperature and the obtained FA-PEG-OH was then mixed with succinic

anhydride (412 mg) in the presence of DMAP catalyst (38 mg) and Et_3N (45 μL) for 24 h. The products were then dialyzed against water through cellulose membrane (MWCO: 1000 Da) for 48 h followed by lyophilization for FA-PEG-COOH.

Synthesis of $PAMAM_{Tris}$-Cur and $PAMAM_{Tris}$-Cur-*co*-(PEG-FA)

The synthesis of $PAMAM_{Tris}$-Cur was carried out using a previously published method.[5] In detail, $PAMAM_{Tris}$ (191 mg), Cur-COOH (554 mg), DMAP (148 mg), and DCC (374 mg) were mixed in DMSO (5 mL) at ambient temperature under nitrogen atmosphere. After 120 h, the reactants were transferred to 20 mL of blended solvent composed of methanol and water (1:1, v/v) followed by centrifugation. The remaining supernatant was dialyzed against water using cellulose membrane (MWCO: 3500 Da) for 48 h followed by lyophilization. $PAMAM_{Tris}$-Cur-*co*-(PEG-FA) was produced and purified using the same method described above (**Scheme S4**). The molar ratio between $PAMAM_{Tris}$ (50 mg) and FA-PEG-COOH (500 mg) was ca. 1:24. The mass of DMAP and DCC was 21 mg and 52 mg, respectively.



Scheme S4. The schematic illustration of the $PAMAM_{Tris}$-Cur-*co*-(PEG-FA).

Characterization of Nanoconjugates

The structures of nanoconjugates and relevant intermediate products were verified by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). The spectra were recorded via a Varian

INOVA 600 MHz NMR spectrometer using deuterated DMSO-d_6 or chloroform-d ($CDCl_3$) as solvent (**Fig. S1-S2**). An Agilent gel permeation chromatography (GPC) system was used to determine the molecular weight of synthesized products using polystyrene standards at ambient temperature. The flow rate of mobile phase (THF) was 1 mL/min. The hydrodynamic diameter and zeta potential of nanoconjugates were analyzed by a Malvern Zetasizer Nano ZS instrument (**Fig. S3**). The morphologies of all nanoconjugates were assessed by a FEI Tecnai G2 F20 transmission electron microscope (**Fig. S4**). Drug loading was determined by an Agilent Cary 60 UV-vis spectrometer at a wavelength of 426 nm in methanol. Drug release was carried out at 37^{o}C in a Franz type diffusion cell with a receiver fluid volume of ca. 17 mL. The regenerated cellulose membrane (MWCO: 3500 Da) was used to separate the nanoconjugates which had been dissolved in 2 mL phosphate buffered saline (PBS, pH 7.4), and the receiver fluid composed of PBS (pH 7.4) and 5% (v/v) sodium dodecyl sulfate. A mass balance study was also performed to measure curcumin recovery at the end of the drug release experiments.

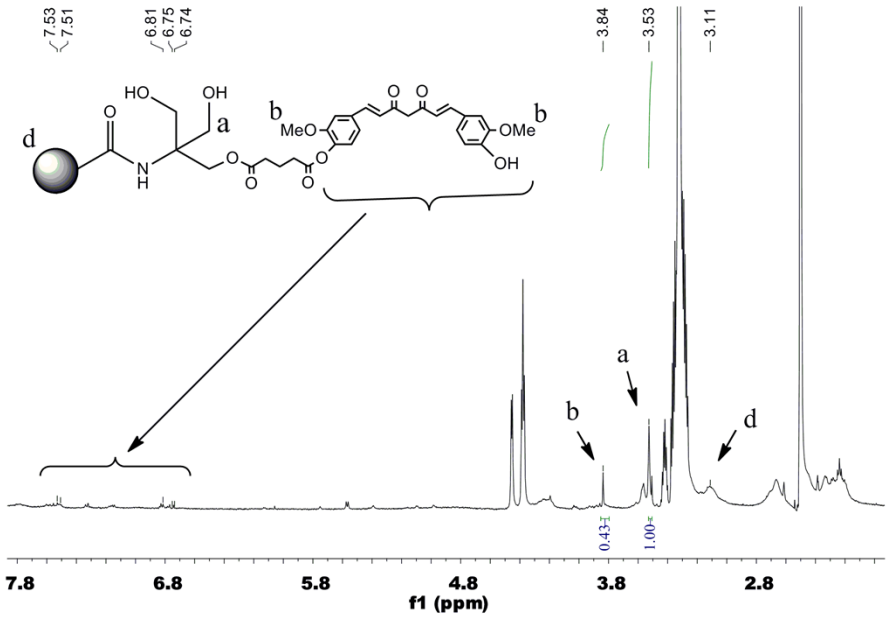


Figure S1. The ^{1}H-NMR of $PAMAM_{Tris}$-Cur.

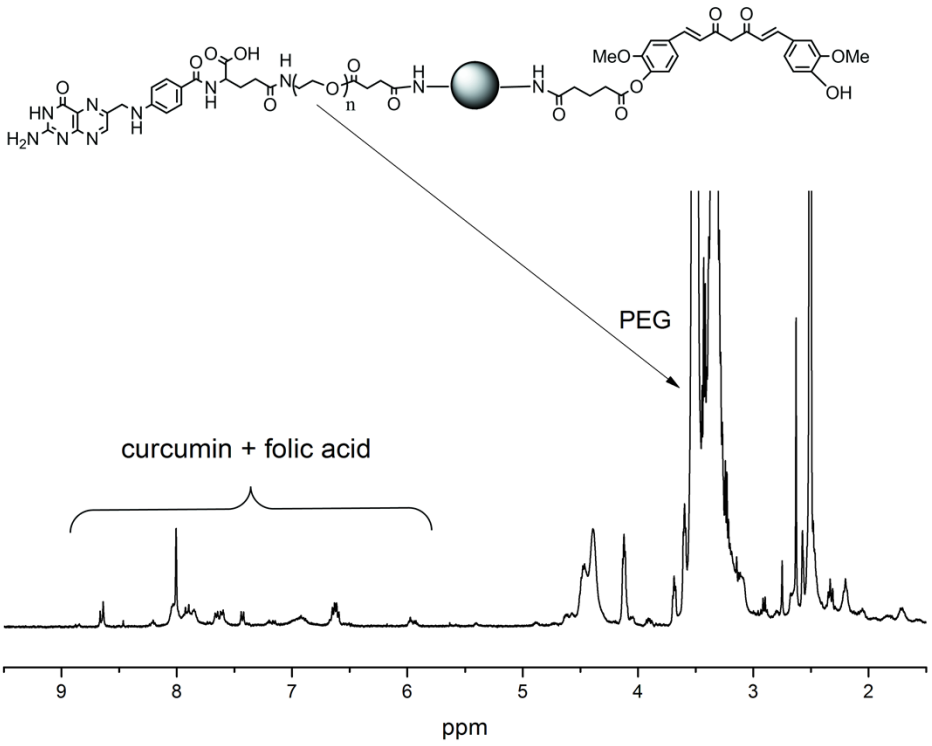


Figure S2. The ^{1}H-NMR of PAMAM$_{Tris}$-Cur-*co*-(PEG-FA).

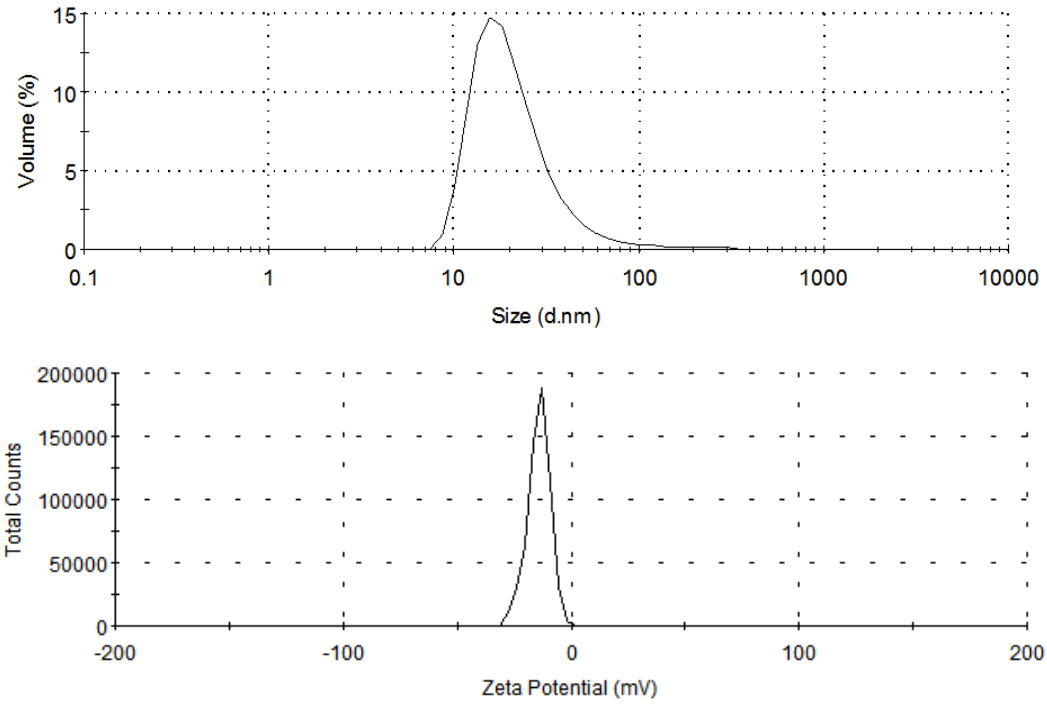


Figure S3. The hydrodynamic size and zeta potential analysis of PAMAM$_{Tris}$-Cur-*co*-(PEG-FA).

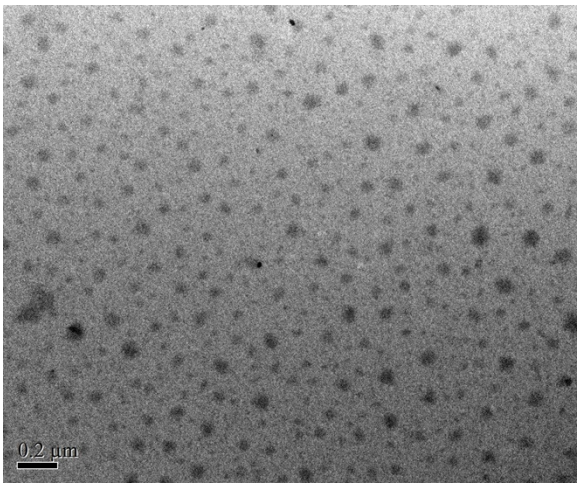


Figure S4. The size and morphology analysis of $PAMAM_{Tris}$-Cur-*co*-(PEG-FA) nanoconjugate.

Intracellular uptake of nanoconjugates

Human liver hepatocellular cells (HepG2) were used as an *in vitro* model to monitor the intracellular uptake of curcumin nanoconjugates. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin and maintained under standard culturing conditions. 24 h prior to the experiments, the cells were seeded in 24-well plate with a density of 2.5×10^4 cells per well before media replacement and the addition of nanoconjugates. The concentration of curcumin used in the experiment was standardised as 50 µg/mL for all samples. After an incubation period of 6 h, the cells were washed by PBS by three times. Curcumin uptake and the intracellular distribution of nanoconjugates were examined and quantified by a Zeiss LSM 710 confocal laser scanning microscope (CLSM) at an excitation wavelength of 425 nm. Quantitative analysis of the fluorescence intensity of curcumin was carried out using a BD Accuri® C6 flow cytometer.

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