Double-headed Nanosystems for Oral Drug Delivery

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Materials:

HPLC grade solvents such as Dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, methanol, and triethylamine were purchased from Fisher Scientific (USA). PLGA 50:50 (Resomer® 503H, viscosity 0.32–0.44 dl/g and MW 24,000–38,000) was purchased from Evonik (Darmstadt, Germany). Trifluoroacetic acid (TFA) were ordered from Acros Organic, Fisher Scientific (USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and Tris(2-aminoethyl) amine (TREN) were obtained from Oakwood Chemicals, Fisher Scientific (USA). N,N-Diisopropylethylamine (DIEA) and Di-tert-butyl dicarbonate were purchased from Alfa Aesar, Fisher Scientific (USA). Gambogic acid was obtained from Broadpharm (San Diego, USA).

Instrumentation:

Nuclear Magnetic Resonance Spectroscopy (NMR): ^{1}H and ^{13}C NMR spectra for materials were recorded on 400/500 MHz Bruker Avance III with HCN Cryoprobe, NMR spectrometers. The spectra were processed and analyzed using Topspin 3.5pl7 software.

High resolution mass spectroscopy (HRMS): The HRMS of materials were obtained on Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass spectrometer.

Fourier Transform Infrared Spectroscopy (FTIR): The FTIR spectra were recorded on NICOLET iS50 FT-IR (Thermo Scientific).

Raman Spectroscopy: The Raman spectra were recorded on NICOLET iS50 FT-Raman (Thermo Scientific).

Gel permeation chromatography (GPC): The GPC analyses were carried on 1220 Infinity LC, Agilent technologies with column (Agilent PLgel 5µm MIXED-D column) at flow rate of 0.5 mL/ min. The samples (1 mg/ 1 mL) were prepared in chloroform. Standard Polystyrene polymer with molecular weight (m. wt. = 1,300/ 2,200/ 4,000/ 5,200/ 13,000/ 25,000/ 50,000/ 65,000/ 1,70,000) were used for calibration purposes.

High-performance liquid chromatography (HPLC): HPLC analysis was done on 1220 Infinity LC, Agilent technologies with column (Thermohyper SILGOLD (150×4.6 mm; 5µm)) at flow rate of 0.8 mL/ min.

Dynamic light scattering (DLS) analysis: A stock solution was prepared by suspending lyophilized polymer sample in 1 mL distilled water. 2 µL aliquots of stock sample were diluted with 2 mL of distilled water for doing a DLS analysis. The samples were analyzed with Malvern Zetasizer Nano-ZS at room temperature.

Scanning electron microscopy (SEM): 10 µL aliquots of polymer samples were deposited onto a silicon wafer (100) and air dried. The samples were dried in-vacuo for 30 min prior to imaging. The samples were gold coated prior to visualization and scanned using a Tescan Vega 3 microscope equipped with a tungsten filament gun, operating at WD 5 mm and 20 kV.

UV-visible (UV-vis) absorption measurements: All samples were prepared in acetonitrile. The UV-vis absorption spectra were obtained on UV-6300 PC double beam spectrophotometer, VWR.

LC-MS/MS method for curcumin detection: The curcumin in samples was detected and quantified on a triple quadrupole mass spectrometer (Quantiva, Thermo Scientific, Waltham, MA) coupled to a binary pump HPLC (UltiMate 3000, Thermo Scientific). MS parameters were optimized for the target compound under direct infusion at 5 µL min-1 to identify the SRM transitions (precursor/product fragment ion pair) with the highest intensity in positive mode as 369.4-177 m/z for the Curcumin, 545.2-369 m/z for Curcumin Glucuronide and 240.2-148.2 m/z for the internal standard, Salbutamol. Samples were maintained at 4 °C on an autosampler before injection. The injection volume was 10 µL. Chromatographic separation was achieved on a Hypersil Gold 5 µm 50 × 3 mm column (Thermo Scientific) maintained at 30 °C using a solvent gradient method. Solvent A was water (0.1% formic acid). Solvent B was acetonitrile (0.1% formic acid). The gradient method used was 0-4 min (20% B to 80% B), 4-4.1 min (80% B to 95% B), 4.1-6 min (95% B), 6-6.5 min (95% B to 20% B) and 6.5-8 min (20% B). The flow rate was 0.5 mL min-1. Sample acquisition and analysis was performed with TraceFinder 3.3 (Thermo Scientific).

Sample preparation for LC-MS/MS:

Standard solutions, calibration, and internal control samples: Stock solutions at a concentration of 1.0 mg/mL were prepared by separately dissolving 1 mg of Curcumin, Curcumin Glucuronide and Salbutamol (Internal Standard) in 1 mL of Acetonitrile. Standard working solutions were then prepared by dilution of Curcumin and Curcumin Glucuronide stock solutions with Acetonitrile to obtain working solutions with concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 250 µg/mL.

Plasma calibration standards were prepared by spiking 30 µL of blank rat plasma with a freshly prepared working solution at concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 250 µg/ml and extracting with 10ng/ml Salbutamol in 100% acetonitrile to achieve standards with concentrations of 0.033, 0.0833, 0.166, 0.33, 0.833, 1.66, 3.33 and 8.33 µg/mL.

Tissue calibration standards were prepared by spiking 100 µL of tissue extract with a freshly prepared working solution at concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 µg/mL to achieve standards with concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250 ng/mL.

Sample preparation for plasma: 30 µL of rat plasma samples were placed in a 1.5-mL Eppendorf tube, 120 µL of ice cold 10 ng/mL Salbutamol in 100% Acetonitrile was added, and the mixture was vortexed. Samples were then sonicated in a water bath for 1 minute and then then centrifuged at 15,000 rpm for 5 min. After centrifugation, 100 µL of the supernatant was transferred to a clean 1.5mL Eppendorf tube. 50 µl of the supernatant was then transferred to a 2ml glass vial with a glass insert from where 10 µL was injected into the LC–MS/MS system.

Sample preparation for tissue: 50 mg of rat tissue samples were placed in a pre-chilled and pre-weighed Precellys tissue homogenizing tube, 800 µL of ice cold extraction solvent (10 ng/mL Salbutamol in 100% Acetonitrile) was added, and the mixture was homogenized in a Precellys bead beater at 6000 rpm for 30 seconds. Samples were then kept on ice for 5 minutes and then centrifuged at 15,000 rpm for 5 minutes. After centrifugation, 600 µL of the supernatant was transferred to a 0.2 um filter placed inside a collection tube and then centrifuged at 15,000 rpm for 1 minute to filter particulates. 50 µl of the sample flow through was then transferred to a 2ml glass vial with a glass insert from where 10 µL was injected into the LC–MS/MS system.

Synthesis details:

Polymer synthesis and details:

1. Synthesis of Tris(2-aminoethyl)amine-diBoc, (TREN)-diBoc: TREN-diBoc was synthesized by a general method reported in the literature.[1,2] Briefly, TREN (1.024 mL, 6.83 mmol, 1 eq) was diluted in dry methanol (10 mL) under nitrogen atmosphere in a round bottomed flask. Boc anhydride (3.14 mL, 13.6 mmol, 2 eq) and triethylamine (50 mL) was added to above stirring solution under nitrogen atmosphere. The completion of reaction was monitored by TLC. After completion of reaction, the solvent was dried under vacuum. The crude mixture was dissolved in DCM and washed with 1 N HCl (2×) and deionized water (2×). The organic layer was dried over anhydrous sodium sulphate to get a yellow colored product which was purified through column chromatography (DCM: MeOH) to get final product. (834 mg, 83% yield); ^{1}H NMR (500 MHz, DMSO-d_6, TMS, δ ppm):1.38 (s, 18H), 2.28-2.44 (m, 8H), 2.92-2.95 (t, 4H), 6.64 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 28.7, 38.8, 54.4, 77.96, 156.7; FTIR: 3330, 2976, 2810, 1692, 1503, 1453, 1360, 1282, 1238, 1154, 1072, 1011, 867, 784; HRMS for $C_{16}H_{34}N_4O_4$: ($M+H^+$) 347.2658 (calcd.), 347.2651 (anal.).

2. Synthesis of PLGA-TREN: PLGA (1.0 g, 0.032 mmol) was dissolved in dry DMF in a round bottomed flask under. EDC (0.018 g, 0.096 mmol) was added to the above solution with continued stirring and under nitrogen atmosphere. After 35 min, TREN-diBoc (0.033 g, 0.096 mmol) and DIEA (0.028 mL, 0.161 mmol) was added to the reaction mixture and continued stirring for 24 h. The reaction progress was monitored through TLC. After the completion of reaction, the DMF was washed of through cold water washing (2×). The polymer was purified through precipitation method from DCM into diethyl ether solvent mixture (3×). The purified product was vacuum dried to get the white powder. Further, the white powder (0.900 g) was dissolved in dry DCM (3 mL) under nitrogen atmosphere in a round bottomed flask. Trifluoroacetic acid (TFA, 0.3 mL) was added to the above solution and continued stirring the reaction for 1 h at 4° C under nitrogen environment. The completion of reaction was monitored through TLC. After the completion of the reaction, the solvent was evaporated under vacuum. The polymer was purified through precipitation method from DCM into diethyl ether solvent mixture (3×). The purified polymer was vacuum dried to get the white powder. (0.886 g, 88.6% yield); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.08-1.11 (m, TREN -NH_2), 1.45-1.48

(m, PLGA –CH_3), 2.36 (m, TREN –CH_2–), 2.64 (m, TREN –CH_2–), 2.74 (m, TREN –CH_2–), 3.37-3.41(m, TREN –CH_2–), 4.80-4.95 (m, PLGA –CH_2–), 5.16-5.28 (m, PLGA –CH_2–), 8.23 (s, –NH–);^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 15.6, 16.9, 55.3, 61.1, 65.3, 69.2, 167.1, 169.7; FTIR: 3445, 2999, 2951, 2912, 2853, 1756, 1627, 1535, 1423, 1387, 1262, 1159, 1077, 849.

3. Synthesis of PLGA-TGA_2: Gambogic acid (GA, 0.015 g, 0.025 mmol) was dissolved in dry DCM in a round bottomed flask under nitrogen atmosphere. To the above stirring solution, EDC (0.004 g, 0.025 mmol) was added in order to activate carboxylic group of gambogic acid. After 30 min, PLGA-TREN (0.250 g, 0.008 mmol) and DIEA (0.007 mL, 0.041 mmol) was added to the reaction mixture and continued stirring for 24 h. The reaction progress was monitored through TLC. After the completion of reaction, the polymer was purified through precipitation method from DCM into cold 50% methanol-diethyl ether solvent mixture (3×). The purified product was vacuum dried to get the light yellow powder. (0.195 g, 78% yield); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.08-1.11 (m, TREN -NH_2), 1.47-1.48 (m, PLGA –CH_3), 2.37 (m, TREN –CH_2–), 2.64 (m, TREN –CH_2–), 2.74 (m, TREN –CH_2–), 3.37-3.41(m, TREN –CH_2–), 4.77-4.95 (m, PLGA –CH_2), 5.18-5.27 (m, PLGA –CH_2–), 5.75 (m, GA), 7.96 (s, –NH–), 8.812 (m, GA); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 15.6, 16.9, 59.7, 61.1, 65.3, 69.1, 110.3, 167.1, 169.7; FTIR: 2997, 2948, 2876, 1747, 1675, 1525, 1431, 1381, 1271, 1171, 1083, 855, 745, 706.

***Ex vivo* and *in vivo* experimental protocols:**

Ex vivo receptor-binding using intestinal sections. In order to understand the PLGA-TGA_2 nanosystems interaction with the intestinal transferrin receptors (TfR) we have used the small intestine (jejunum) cryosections or paraffin sections (4 μm thick). The sections were incubated overnight at 4°C in humidity chamber with primary mouse monoclonal anti-TfR-CD71 antibody diluted 1:200 in PBS containing 3% goat serum, after antigen retrieval process. The sections were then washed with PBS followed by addition of anti-mouse secondary antibody conjugated with Alexa Fluor 555. The sections were finally incubated at room temperature for 2 h and washed three times with PBS. Followed by the above procedure, sections were incubated in humidity chamber at 37°C for 1 h with PLGA, PLGA-EGA or PLGA-TGA_2 fluorescent nanosystems (F-NS 250 μg/ml). Followed by incubation, the sections were washed several times with PBS to remove unbound particles removed by vacuum suction and mounted with VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI for nuclear staining. Images were acquired using confocal laser scanning microscope (Zeiss LSM780). Microscopy images were processed using ZEN2 image processing software. The co-localization analysis was performed with default M1 and M2 coefficients in ImageJ software.

Pharmacokinetics kinetics and bio-distribution studies. Male Sprague Dawley rats (200–250 g) were purchased from ENVIGO. All the experimental protocols were approved by Texas A&M University Institutional Animal Care and Use Committee (protocol number IACUC 2017-0213, Reference 056039). The specific pathogen free animals were kept in standard housing conditions and allowed free access to food and water throughout the experiment.

Rats were randomly divided into 3 groups (n=4). The rats were administered with a single dose of 20 mg/kg (curcumin equivalent) curcumin encapsulated PLGA, PLGA-EGA and PLGA-TGA_2 nanosystems dispersed in water by oral gavage. Blood samples were withdrawn via tail vein and collected in heparinized tubes at different time points up to 48 h post-dosing. Plasma was collected by centrifuging blood samples at 3000 rpm/4 °C/30 min. Supernatant was separated and stored at − 80 °C until analysis. The concentration of curcumin in plasma was measured using LCMS method. In a second study, the rats (n=3) were administered with a single dose of 20 mg/kg (curcumin equivalent) curcumin encapsulated PLGA, PLGA-EGA and PLGA-TGA_2 nanosystems dispersed in water by oral gavage and the study was terminated 30 min post dosing and the tissues were collected for curcumin analysis using LCMS method.

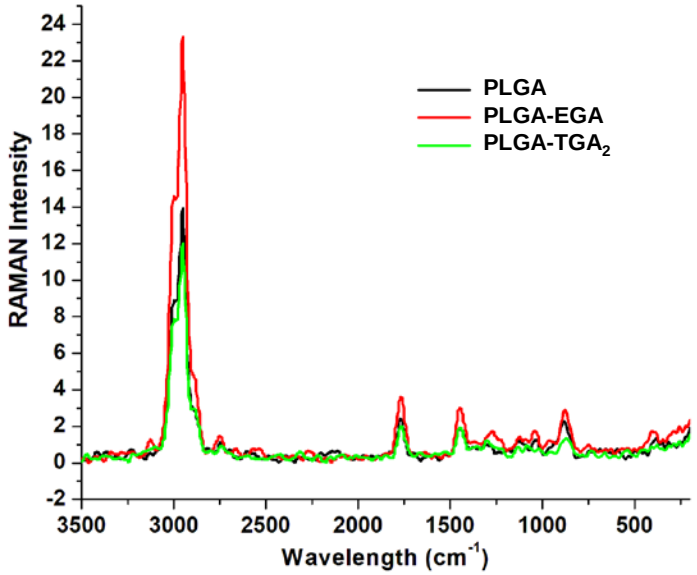
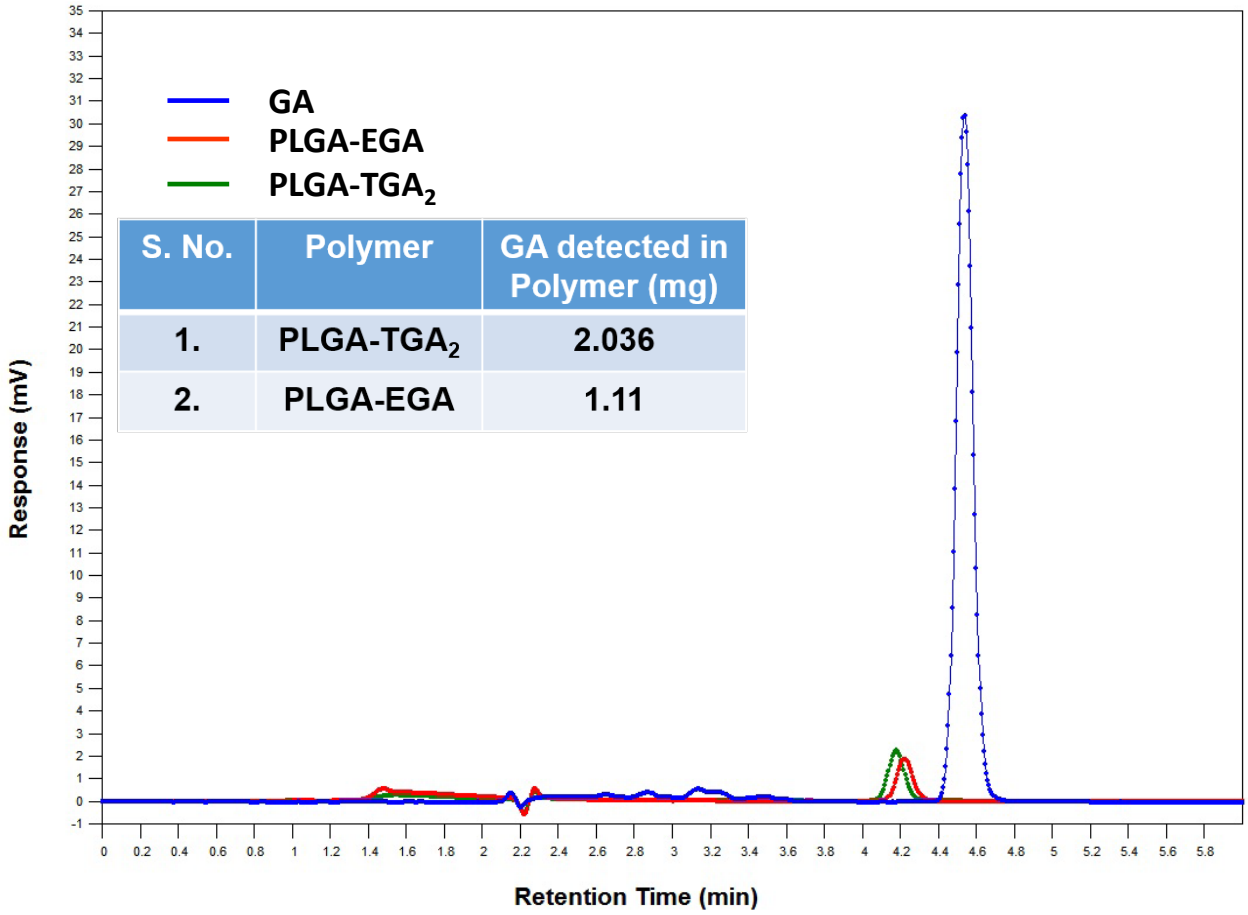


Fig S1. Raman Spectra of PLGA, PLGA-EGA and PLGA-TGA_2.



S. No.	Polymer	GA detected in Polymer (mg)
1.	PLGA-TGA_2	2.036
2.	PLGA-EGA	1.11

Fig S2. Quantification of GA in the polymers using HPLC method. HPLC traces are used to confirm GA in the polymers where TGA_2 shows an increase in area under the curve compared to EGA. The HPLC traces further confirm the purity of the polymers, also seen is pure GA peak.

S. No.	Polymer (Batch I)	Diameter (nm) / PDI
A1	PLGA-EGA (Fresh particles)	218.4 / 0.137
		220.3 / 0.081
A2	PLGA-EGA (Re-suspended particles)	213.5 / 0.071
		211.0 / 0.082

S. No.	Polymer (Batch I)	Diameter (nm) / PDI
B1	PLGA-TGA_2 (Fresh particles)	213.2 / 0.097
		212.7 / 0.094
B2	PLGA-TGA_2 (Re-suspended particles)	244.1 / 0.211
		250.2 / 0.189

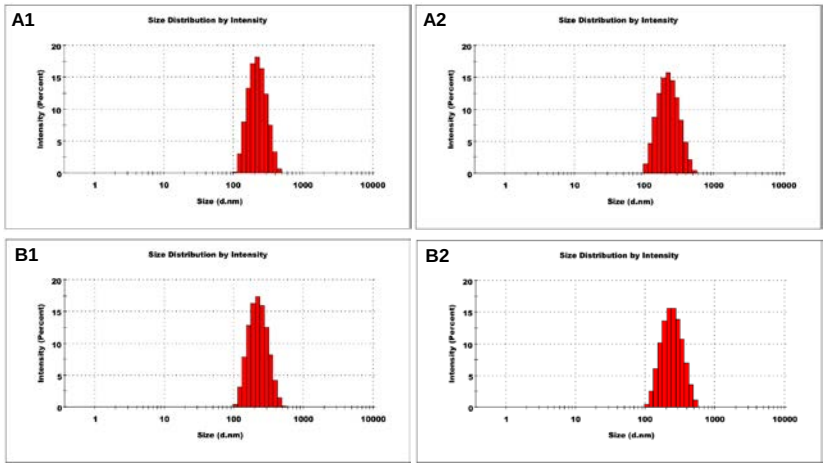
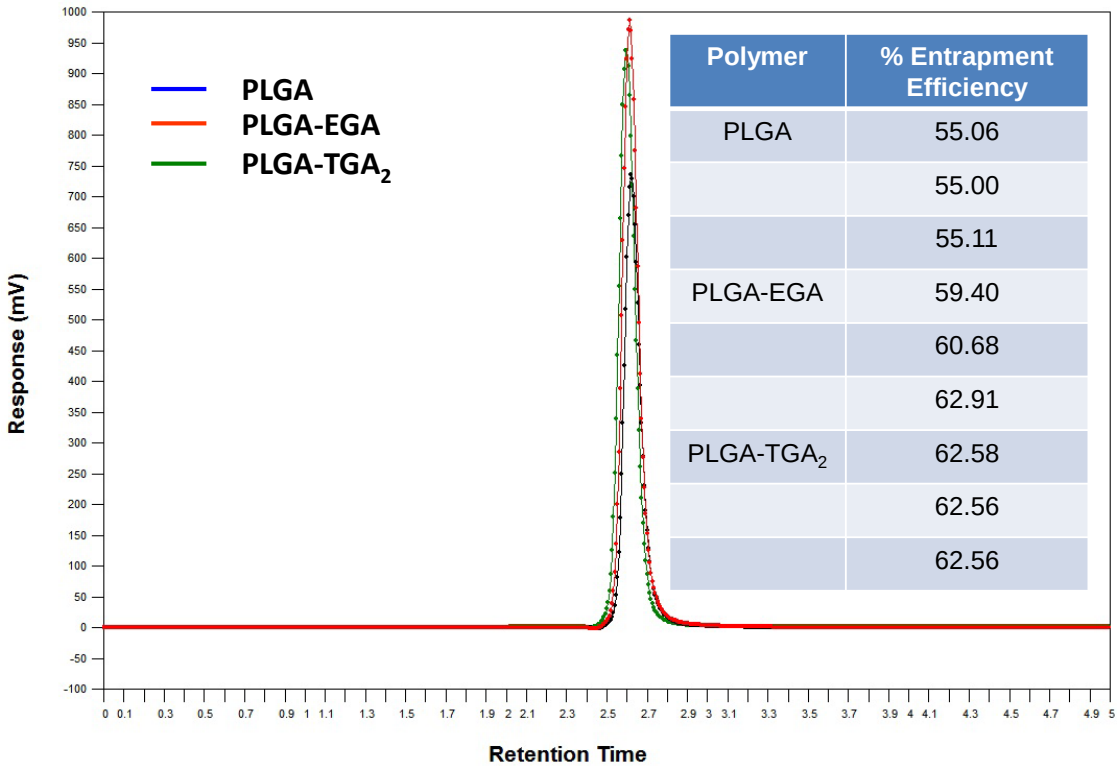


Fig S3. DLS analysis of fresh and re-suspended polymer nanosystems.



Polymer	% Entrapment Efficiency
PLGA	55.06
	55.00
	55.11
PLGA-EGA	59.40
	60.68
	62.91
PLGA-TGA_2	62.58
	62.56
	62.56

Fig. S4. Quantification of curcumin in the nanoparticles using HPLC method. Also seen are curcumin traces in all the three formulations used in the study.

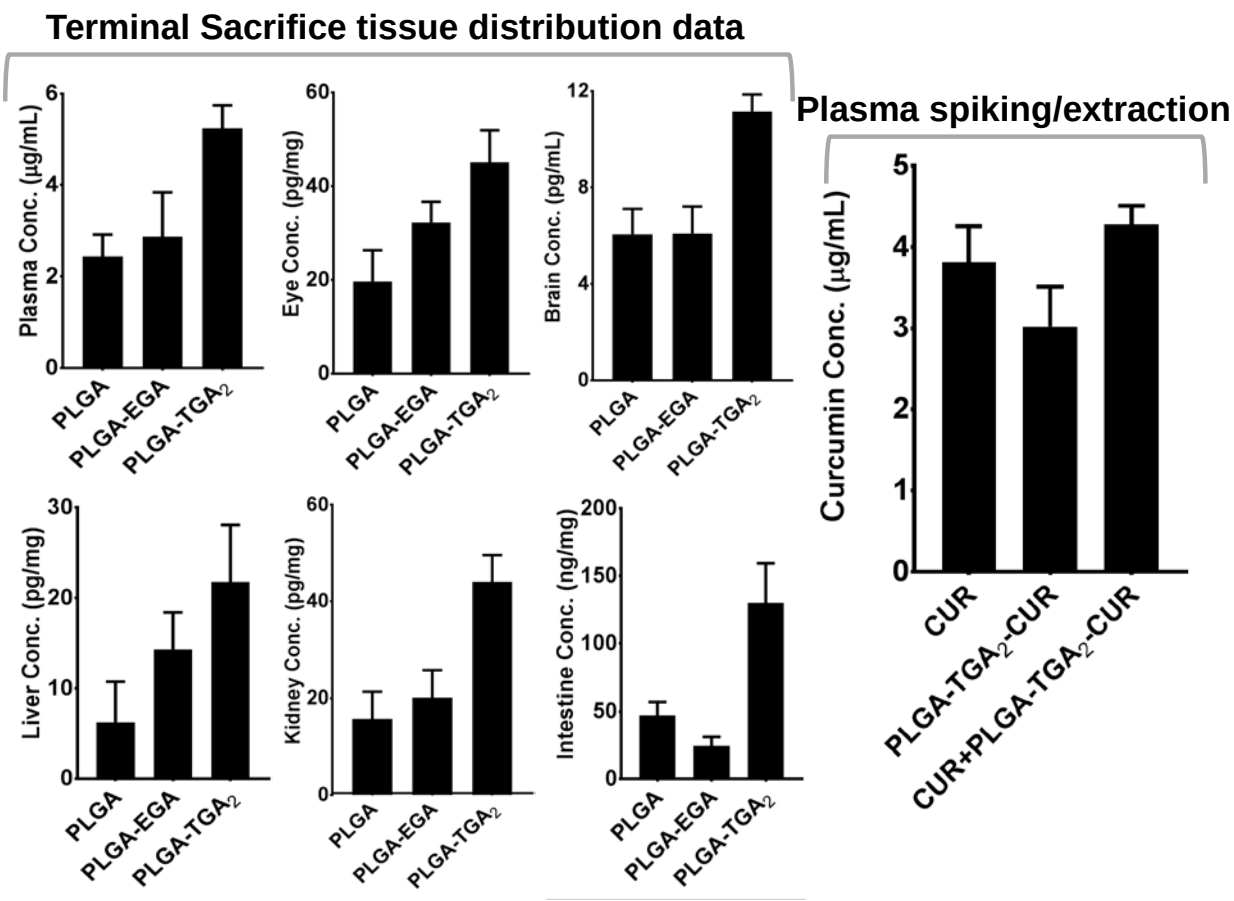


Fig. S5. Curcumin (CUR) distribution at 30 min (n=3) in intestine, plasma, liver, brain, eye, and kidney. The extraction process involved in the *in vivo* assay of curcumin takes into account free and encapsulated curcumin. Particularly this reflects in the curcumin levels in the terminal sacrifice study. This was demonstrated by spiking plasma with equal portions of CUR or PLGA-TGA_2-CUR or mixing 50-50 CUR+ PLGA-TGA_2-CUR followed by subjecting to CUR extraction process used for unknown samples. The data presented are in triplicates and are not significant. Plasma **P < 0.001, PLGA vs PLGA-TGA_2; **P < 0.002, PLGA-EDA vs PLGA-TGA_2; Eye: **P < 0.003, PLGA vs PLGA-EDA; ***P < 0.0002, PLGA vs PLGA-TGA_2; **P < 0.003, PLGA-EDA vs PLGA-TGA_2; Brain: **P < 0.003, PLGA vs PLGA-TGA_2; **P < 0.003, PLGA-EDA vs PLGA-TGA_2 ; Liver: *P<0.05, PLGA vs PLGA-TGA_2; Kidney: **P < 0.009, PLGA vs PLGA-TGA_2; *P < 0.02, PLGA-EDA vs PLGA-TGA_2; Intestine: ***P < 0.0001, PLGA/PLGA-EDA vs PLGA-TGA_2. At 30 min, curcumin was not detected in spleen.

Characterization:

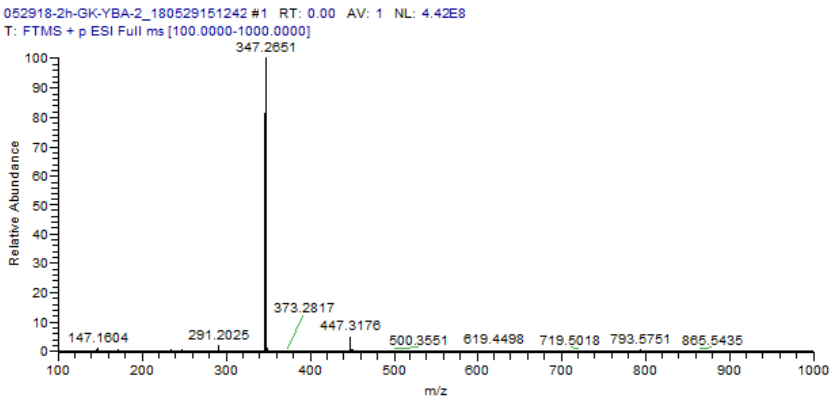


Fig. S6. HRMS of TRENdiboc

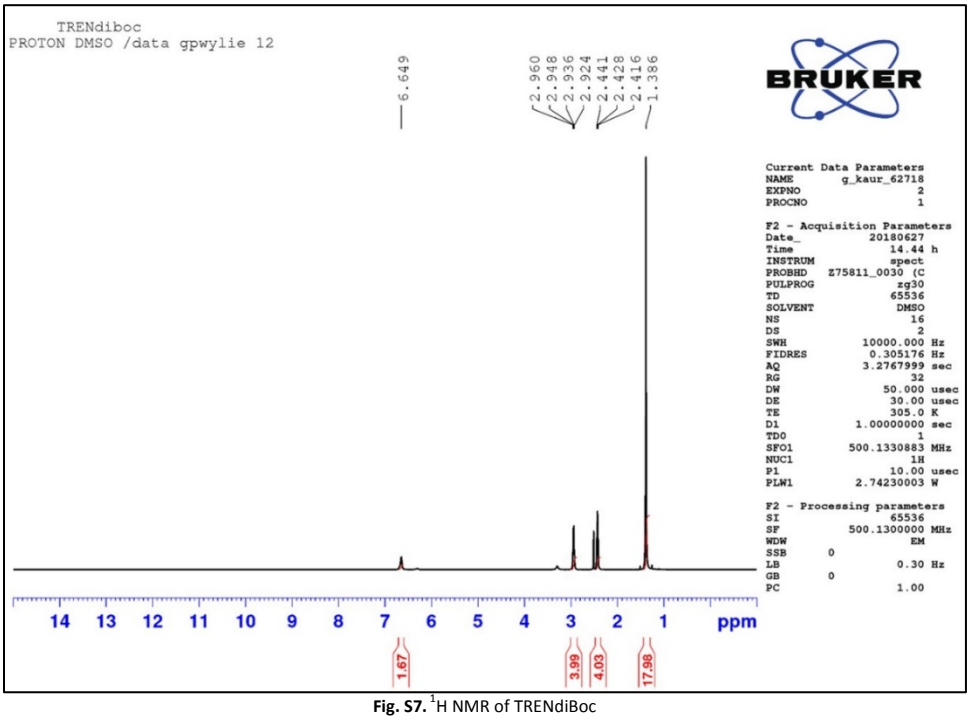


Fig. S7. ^{1}H NMR of TRENdiBoc

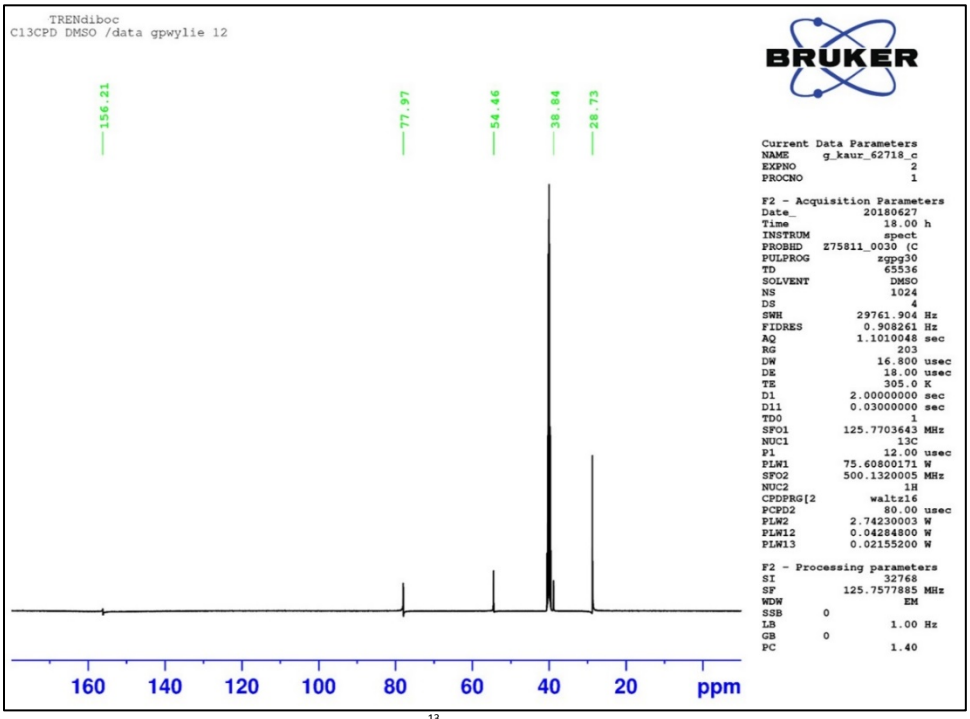


Fig. S8. ^{13}C NMR of TRENdiBoc

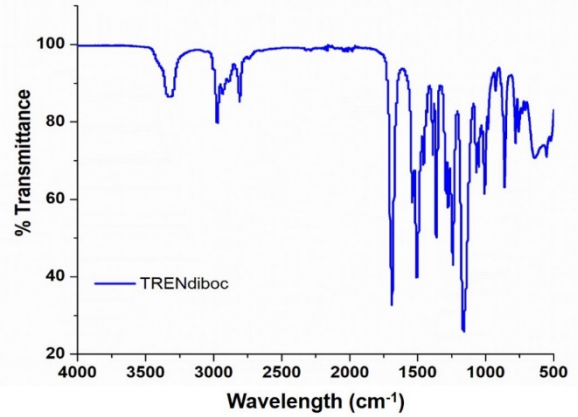


Fig. S9. FT-IR of TRENdiBoc

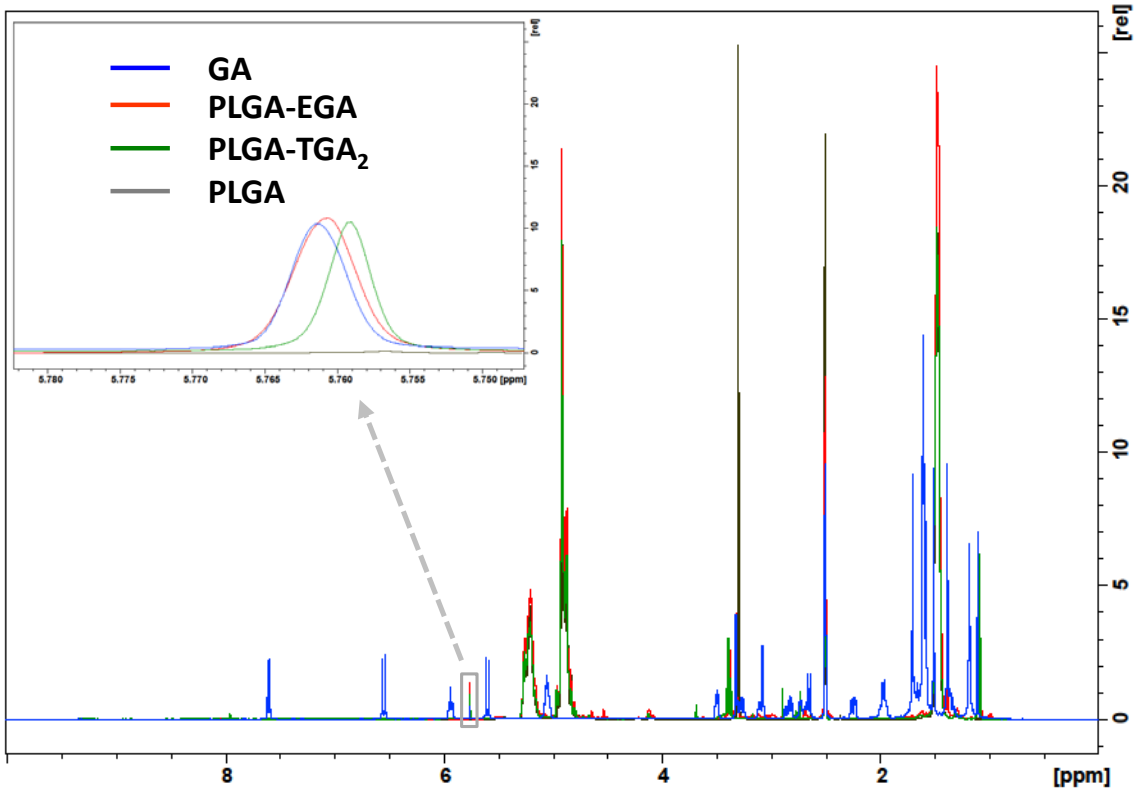


Fig. S10. ^{1}H NMR of GA, PLGA-EGA, PLGA-TGA_2 and PLGA with the inset highlighting GA resentative peaks.

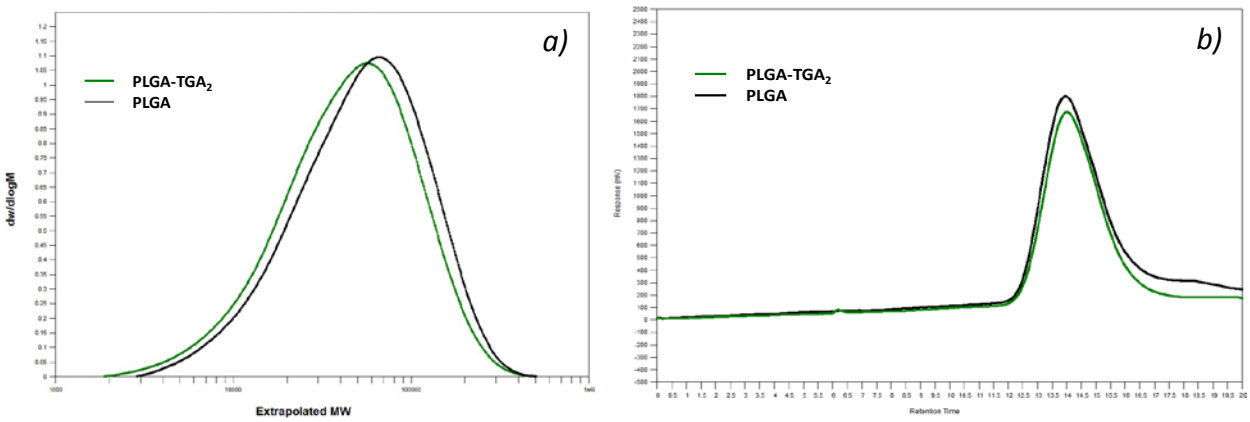


Fig. S11. a) Molecular weight and b) distribution profiles of PLGA and PLGA-TGA_2 obtained from GPC

References:

1 S. M. Cohen, B. O'Sulliva and K. N. Raymond, *Inorg. Chem.*, 2000, **39**, 4339–4346.

2 E. J. Werner, M. Botta, S. Aime and K. N. Raymond, *Contrast Media Mol. Imaging*, 2009, **4**, 220–229.