Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information

for

Peptide decorated glycolipid nanomicelles for the drug delivery across blood-brain barrier (BBB)

S. Meenu Vasudevan[a,b], N. Ashwanikumar [c], G.S.Vinod Kumar[a]*

[a.] *Nano Drug Delivery Systems Lab, Cancer Biology Division, Bio innovation Centre, Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India, 695014*
[b.] *Research Scholar, Dept of Biotechnology, Faculty of Applied Sciences, University of Kerala, Trivandrum Kerala, India 695581*
[c.] *Post Graduate & Research Department of Chemistry, Sir Syed College (Affiliated to Kannur University), Taliparamba, Kannur, Kerala, India-670142.*

[*] To whom correspondence should be addressed email: gsvinod@rgcb.res.in

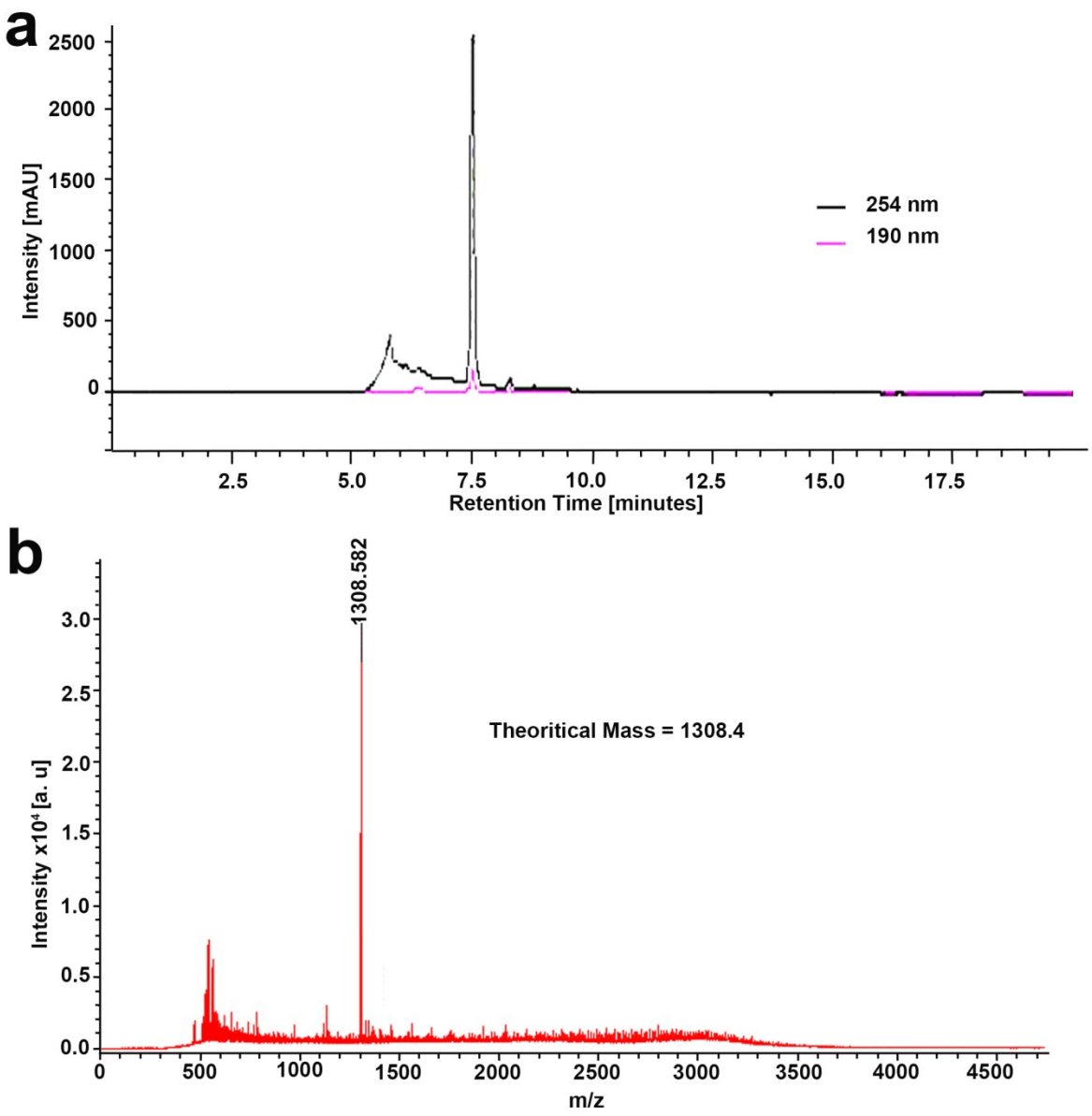

Figure.S1. Characterization of TGN peptide. (**a**) HPLC chromatogram of TGN peptide analyzed at dual wavelength. (**b**) MALDI-TOF spectral profile of TGN peptide showing the observed mass of 1308.582.

Materials and Methods

1. Materials Used

Stearic acid (SA) was purchased from Merck. Glycol chitosan (GC, MW- 586kDa), 1-Ethyl-3-(3-dimethylaminepropyl) carbodiimide (EDC), N-hydroxisuccinimide (NHS), Rhodamine-B, Rhodamine-123 and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) purchased from Sigma (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Gaithersberg, MD). All the organic solvents used were of high-performance liquid chromatography grade.

2. Methods

2.1. Synthesis of SAGC copolymer

The SAGC graft copolymer was synthesized via the reaction of carboxyl groups of SA with amine groups of GC.[1,2] Briefly GC (500mg, 0.88uM) was dissolved in distilled water (30ml) and EDC (129 mg) was added to it. SA (60.11mg, 211uM 23mol% of free amino group in GC) was dissolved separately in methanol (30ml) along with NHS (77.4mg). Both the reactants were mixed and kept for stirring under nitrogen for 24 hours at room temperature. The reaction mixture was dialyzed in 1:4 mixture of water: methanol for 24 hours, then in 4:1mixture of the same for 24 hours and later in distilled water for 48 hours. The product thus obtained was washed repeatedly with ether and water to remove organic and aqueous impurities followed by lyophilization.

2.2. Characterization of SAGC copolymer

2.2.1. Fourier transform-infrared (FT-IR) spectra

The copolymer formed was characterized using FT-IR (Perkin Elmer spectrum 65). For each sample, eight scans were collected and averaged to reduce the signal to noise ratio. The spectral range covered the mid-infrared region from 4000 to 600 cm^{-1}.

2.2.2. Proton-Nuclear Magnetic Resonance (^{1}H NMR) spectra

The ^{1}H-NMR spectrum of the polymer was acquired using a Bruker 500 MHz spectrometer (Bruker BioSpin, Billerica, MA) with $CDCl_3$ as a solvent.

2.2.3. Differential Scanning Calorimetry (DSC) analysis

DSC thermograms were obtained using an automatic thermal analyzer system (Pyris^{TM}DSC 6000; PerkinElmer, Waltham, MA). Samples were crimped in standard aluminum pans and heated from 0°C up to 300°C at a rate of 10°C/minute under constant nitrogen purging of 20 mL/minute. An empty pan, sealed similarly to the sample, was used as a reference.

2.3. TGN peptide synthesis

The TGN peptide with 12 amino acid sequence TGNYKALHPHNG was synthesized using solid phase peptide synthesis by the Fmoc strategy. TentaGel™–S–NH2 was used as the resin which was linked to HMPB linker using HOBT/HBTU/DIEA cocktail. After the completion of attachment of all the amino acids the peptide was cleaved using trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water at a ratio of 95:2.5:2.5 and precipitated using chilled diethyl ether followed by washing with the same for 10-15 times. The washed peptide was later lyophylized and stored at -20^{0}C. The purity of the peptide was confirmed by Shimadzu LC-AD HPLC system using binary mobile phase of water (0.1% TFA) and acetonitrile (0.1% TFA) at 1 mL per min was used, and the column eluents were monitored by UV absorbance at 190 and 254 nm. The molecular mass of the peptide was confirmed by using matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF).

2.4. Preparation and characterization of TGN conjugated SAGC

The TGN peptide was conjugated to the amino group of SAGC using EDC-NHS coupling reagent with the activation of COOH group.[3] Here 0.2mmol of TGN peptide was dissolved in PBS (pH=7.4). To this, 0.2 mol of EDC and 0.5 mol of NHS was added and stirred for about 15 minutes in room temperature. SAGC (0.1 mg/ml) was dispersed uniformly in PBS and then added to the peptide solution followed by stirring at room temperature for 4-6 hours.The product was collected by centrifugation at 10,000 rpm for 30 minutes and then washed repeatedly for 3 times to remove the excess EDC and NHS. The unconjugated TGN peptide was then estimated by UV spectroscopy of the collected supernatant at 280 nm.

2.5. Determination of primary amino groups

The primary amino groups available in SAGC for conjugation of TGN peptide as well as the confirmation of its conjugation was done using quantitative ninhydrin test.[4] The primary amino groups react with ninhydrin reagent to form a purple coloured product. The absorbance of this product was

measured at 570 nm. Standard curve was plotted using different concentrations of a standard amino acid glycine (0.1 to 1mg/ml). The test sample of TSAGC was prepared similarly. To all the standards and the test, 1ml of ninhydrin reagent was added, heated these solutions for 15 minutes in boiling water and then cooled. 1 ml ethanol was added to all the tubes and mixed thoroughly. The percentage of primary amino group was determined by measuring the absorbance at 570 nm.[4]

2.6. Determination of Critical Micelle Concentration (CMC)

The CMC of the nanomicelle was determined using fluorescence spectroscopy with pyrene as the hydrophobic fluorescent probe. The ratio of fluorescence emission intensities (I_1/I_3) is determined which depends on the polarity of the medium. The value of I_1/I_3 is directly proportional to the polarity of the micelle. The decrease in the intensity ratio is a result of micelle formation by the polymer with a precise hydrophobic core which accommodates the pyrene molecule.[5] A solution of pyrene was prepared with a concentration of $12x10^{-5}$ M in acetone. The different volumes of pyrene solution were added into a series of volumetric flasks to get a final pyrene concentration of $6x10^{-7}$M. The acetone was allowed to evaporate completely. Polymer solutions were made in these flasks at various concentrations (10^{-4} to 1g/L). The flasks were kept in a thermostated water bath at 25^0C overnight in the dark to equilibrate the partitioning of pyrene between water and the micelles.

Fluorescence intensities were measured using FP-777 spectrofluorometer (JASCO International, Tokyo, Japan) by excitation at 310 nm and emissions scanning from 350 nm to 440 nm. The ratio of emission intensities was calculated from I_1 (383 nm) and I_3 (404 nm). The ratios of intensities I_1/I_3 were plotted against logarithmic polymer concentration (log C). The CMC values were obtained from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at a low concentration.[6,7]

2.7. Preparation of model drug loaded Nanoparticle

Curcumin entrapped TSAGC (TSAGC-Cur) was prepared by dialysis method. Briefly 10mg of TSAGC was dissolved in 2ml DMSO and 5mg of curcumin was dissolved separately in 1ml DMSO. Both were mixed together and kept for stirring overnight at room temperature. This mixture was then dialyzed against distilled water using a membrane with MWCO 12000-14000 (SERVAPOR,SERVA) for 48 hours. After dialysis, the solution was centrifuged at 10000g for 30 minutes to remove insoluble curcumin.[8]

2.8. Determination of encapsulation efficiency

Encapsulation efficiency of curcumin in the TSAGC was determined using UV-Visible spectroscopy. Briefly TSAGC-Cur lyophilized particles (1mg) were dissolved in 1ml of DMSO to form a clear solution. The concentration of curcumin was found by measuring the absorbance at 435 nm.

2.9. Particle size analysis

The particle size of the nanomicelles was measured by dynamic light scattering (DLS) using a Delsa™ Nano particle size analyzer (Beckman Coulter, Inc, Fullerton, CA) instrument at ambient temperature. The samples were analyzed without prior filtration.

2.10. Zeta potential analysis

The surface charge of the TSAGC-Cur was determined using zeta potential measurement using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) instrument at ambient temperature. The 1mg/ml concentration of nanomicelle in water was used for analysis.

2.11. TEM analysis

Morphological studies of the nanomicelles were characterized by TEM (1101-JEM-1011; JEOL Ltd, Tokyo, Japan). The nanomicelles were dispersed in Milli-Q®water (Millipore Corporation, Billerica, MA), and dropped on a copper-coated formvar microscopy grid. The sample on the grid surface was allowed to dry at room temperature in a desiccator containing calcium chloride and measurements were taken. TEM pictures were taken using Digital MicrographTM (GatanInc, Pleasanton, CA) and Soft Imaging Viewer software (Olympus Soft Imaging Solutions GmbH, Munster, Germany).

2.12. *In-vitro* drug-release studies

The release of curcumin from the TSAGC nanoparticle was studied by performing in vitro drug-release studies. A weighed 10 mg amount of the curcumin containing micelles was dispersed in 1 mL freshly prepared phosphate-buffered saline (PBS) solutions of pH 7.4 and placed in a dialysis membrane (MWCO= 12000-14000). The dialysis tubes were immersed in 30 ml PBS and gently shaken with 100 rpm at 37^0C in a shaker incubator. At different time points, 1 ml medium was removed and replaced with the same quantity of fresh medium. The release study was carried out for a period of 3 days. The concentration of curcumin released was found out from the absorbance in

Lambda 25 ultraviolet-visible spectrophotometer (PerkinElmer) at 430nm.

Percentage of Curcumin released = [Concentration of released drug at each time point / Concentration of entrapped drug within the nanoparticle]

2.13. *In-vitro* cell studies

2.13.1. Cell culture

U87MG cells were obtained from National Centre for Cell Science (NCCS), Pune, India and were maintained in DMEM medium supplemented with 10%v/v fetal bovine serum and a 1% antibiotic antimycotic cocktail at 37^0C in an incubator under 5% CO_2.

2.13.2. Cell uptake studies

U87MG cells were seeded on sterile cover-slips in a 12 well plate ($5x10^4$ cells) and incubated for 24 hours for cell attachment. After 24 hours the media was removed and the cells were treated with Rhodamine-Btagged, TSAGC and SAGC nanomicelles respectively (20 μM in media) and incubated for 2 hours. Then the cells were treated with DAPI for 5 minutes and fixed using 4% paraformaldehyde (10 min, Room Temperature). The cells were washed twice with PBS to remove the paraformaldehyde and mounted on a clean glass slide using fluoromount. Finally, the mounted cells were imaged using a confocal laser scanning microscope (Leica SP8 Spectral Confocal microscope, Leica Microsystems, Germany).

2.13.3. MTT assay

To perform MTT assay, cells were seeded into 96 well plates (5000 cells/ well) and incubated under standard conditions for 24 hours to reach 60 -70% confluence. Cells were treated with 5 to 100μM free curcumin (CUR), Free TSAGC and TSAGC+Cur for 24, 48 and 72 hours respectively. After incubation with the formulations for the given time points, the cells were again incubated for an additional 4hours with 10μL MTT (5mg/ml in PBS, pH=7.4). Then media was removed and 100μl isopropyl alcohol was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a Bio-Rad microplate reader, model 680 (Bio-Rad laboratories, Inc, Hercules, CA). Percentage inhibition of cell growth was calculated using the formulae:

[(Average OD of control-Average OD of Test)/(Average OD of control)x100]

2.14. *In vivo* studies

2.14.1. Animals

Male Wistar rats, seven to eight weeks of age weighing 200-250g were used for the study. The rats were kept under standard conditions. Nine rats were used for the study, which was further divided into 3 groups of three members each. One member in each group was selected as a control. All animal experiments were carried out with the approval of the Institutional animal ethics committee (IAEC) in Rajiv Gandhi Center for Biotechnology (RGCB) under the protocol no. IAEC/213/GSV/2013. Animal experiments were conducted by strictly following the rules and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.14.2. Brain uptake

Rhodamine-123 was used as the fluorescent dye for studying the brain uptake of TSAGC nanomicelles. TSAGC nanomicelles with Rhodamine-123 incorporated nanomicelles (both SAGC and TSAGC), was injected through the intravenous route at a dose of 0.5mg/kg. The control group was administered vehicle (PBS) alone. After one hour, the animals were sacrificed to remove the brain and fixed in 4% paraformaldehyde for 48 hours and then placed in 15% PBS sucrose solution for 24 hours until subsidence, then in 30% sucrose for 48hours until subsidence. Afterward excised brains were frozen at -80^{0}C in OCT embedding medium. Frozen sections of 10 µm were prepared with a cryotome cryostat (Leica CM 1950) and stained with DAPI (1µg/ml) for 5 mins at room temperature. The slides were then rinsed twice with PBS (pH=7.4) and dried. The sections were then observed under confocal microscopy (Leica SP8 Spectral Confocal microscope, Leica Microsystems, Germany).

3. Statistics

All the measurements were done in triplicate, and the results were expressed as arithmetic mean ± standard error of the mean. For cytotoxicity evaluation, the mean of the untreated samples was taken as zero for the normalization of the data. Statistical difference and level of significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) were calculated using Graph PadInstat 3.

References

(1) H.Y. Nam, S.M. Kwon, H. Chung, S.Y.Lee, S.H.Kwon, H. Jeon, Y. Kim, J.H. Park, J. Kim and S. Her, *J. Control. Release.* 2009, **135,** 259–267.

(2) P.K.Gupta, A.K. Jaiswal, V. Kumar, A. Verma, P. Dwivedi, A. Dube and P.R. Mishra, *Mol. Pharm.,* 2014, **11,** 951–963.

(3) L. Pan, Q. He, J. Liu, Y. Chen, M. Ma, L. Zhang and J. Shi, *J. Am. Chem. Soc.,* 2012, **134,** 5722–5725.

(4) E. Curotto and F. Aros, *Anal. Biochem.,* 1993, **211**, 240–241.
(5) P. Alexandridis and T.A.Hatton, *Colloids Surf. Physicochem., Eng. Asp.* 1995, **96**, 1–46.
(6) X. Hu, S. Liu, X. Chen, G. Mo, Z. Xie and X. Jing, *Biomacromolecules,* 2008, **9**, 553–560.
(7) Y.Q. Yang, L.S. Zheng, X.D. Guo, Y. Qian and L.J. Zhang, *Biomacromolecules* 2010, **12**, 116–122.
(8) J.H. Kim, Y.S. Kim, S. Kim, J.H. Park, K. Kim, K. Choi, H. Chung, S.Y. Jeong, R.W. Park and I.S. Kim, *J. Control. Release* 2006, **111**, 228–234.