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Synthesis, characterization and drug delivery application of self-assembling amphiphilic cyclodextrin

Mayank R. Patel^a, Dimitrios A. Lamprou^{b,c}, Pradeep R. Vavia^{a,}*

^a Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, University under Section 3 of UGC Act – 1956, Elite Status and Centre of Excellence – Govt. of Maharashtra, TEQIP Phase III Funded, Matunga (E), Mumbai - 400019, India.

^b Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE, United Kingdom.

^c School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, United Kingdom.

*Corresponding Author:

Prof. Pradeep R. Vavia,
Center for Novel Drug Delivery Systems,
Department of Pharmaceutical Sciences and Technology,
Institute of Chemical Technology,
N. P. Marg, Matunga (E),
Mumbai – 400 019. India.
Tel No.: +91 22 3361 2220
Fax No.: +91 22 2414 5614
Email: pr.vavia@ictmumbai.edu.in

Running head: Self-assembling amphiphilic-CD nanovesicles

aAbstract

The main aim of the research was to synthesize amphiphilic cyclodextrin (AMCD) by substituting C12 alkyl chain to a β -cyclodextrin (β CD) in a single step and to study its self-assembly in an aqueous medium. The drug delivery application of the AMCD was also evaluated by encapsulating tamoxifen citrate as a model hydrophobic drug. AMCD was able to self-assemble in aqueous media; forming nanovesicles of size <200 nm, capable of encapsulating Tamoxifen Citrate (TMX). Molecular docking and MD simulation studies revealed the interaction between TMX and AMCD which formed a stable complex. TEM and AFM studies showed that nanovesicles were perfectly spherical having a smooth surface and a theoretical AMCD bilayer thickness of ~ 7.2 nm as observed from SANS studies. XRD and DSC studies revealed that TMX was amorphized and molecularly dispersed in AMCD bilayer which was released slowly following Fickian diffusion. AMCD has excellent hemocompatibility as opposed to β CD and no genotoxicity. IC_{50} of TMX against MCF-7 cell lines was significantly reduced from $11.43 \mu\text{g/ml}$ to $7.96 \mu\text{g/ml}$ after encapsulation in nanovesicle because of nanovesicles being endocytosed by the MCF-7 cells. AMCD was well tolerated by IV route at a dose >2000 mg/kg in rats. Pharmacokinetic profile of TMX after encapsulation was improved giving 3-fold higher AUC; extended mean residence time is improving chances of nanovesicle to extravasate in tumor via EPR effect.

Keywords: Amphilipic cyclodextrin; nanovesicles; bilayer; molecular modelling; cytotoxicity

INTRODUCTION

In recent decades, nanotechnology has been put to an application in the drug delivery arena very profoundly (1,2). There are numbers of nanotechnology-based products which are getting approved worldwide. To name a few, nanosuspensions, nanocrystals, polymeric nanoparticles, lipidic nanoparticles, SMEDDS, microemulsion, nano-emulsions, liposomes are nano-systems which are clinically approved. However, there are many hurdles in exploring the full potential of nanotechnology in drug delivery. Most of the systems require complex processing to achieve nano size, which poses difficulty in scaling up the process and maintaining the reproducibility (3,4). Few systems are fairly simple and scalable, such as SMEDDS that require a very high amount of surfactants which limits its applications. Liposomes are one of the most successful nano-systems to date. The popularity of liposomes is due to its self-assembling nature and capability to encapsulate both hydrophobic and hydrophilic drugs. Nevertheless, liposomes suffer serious limitations due to their chemical instability, low drug loading capacity, drug leakage, and complex processing. All these reasons encouraged scientists to look for various other self-assembling systems for drug delivery applications.

Cyclodextrins (CD) are cyclic oligosaccharides formed by (α -1,4)-linked α -D-glucopyranose units. They are obtained by enzymatic degradation of starch, with α , β , and γ been the three native types of cyclodextrin with 6, 7, and 8 α -D-glucopyranose units, respectively (5). CDs have been used for decades in the pharmaceutical industry to enhance the solubility, stability, and bioavailability of the active pharmaceutical ingredients (6–8). Native CD has more hydrophilic exterior and a less hydrophobic, less-polar cavity (the dielectric constant of the cavity is close to the 40% ethanol/water mixture) (9) with advantages like forming molecular inclusion with hydrophobic drugs. Several modifications are reported by various scientist in which they crosslinked cyclodextrin with the help of bifunctional crosslinkers and form a network of nanopores; this material is coined as cyclodextrin nanosponges (10). Application of nanosponges were reported by many groups for delivery of actives such as Camptothecin (11), Resveratrol (12), Erlotinib (13), Tamoxifen (14) **etc.** However, it is problematic for hydrophilic CD molecule and such crosslinked CD nanosponges to interact with the biological membrane, which is lipophilic in nature (7,15). A novel class of modified CD called amphiphilic cyclodextrins (AMCD) in which hydroxyl (-OH) groups of cyclodextrin was substituted with hydrophobic alkyl chains of different lengths had been reported (16–18). These type of chemical modifications can achieve many goals at once, such as: altering their water solubility, reducing the nephrotoxicity and hemolysis encountered on intravenous (IV) administration, facilitate the interaction with biological membranes, self-assembles to form different nanostructure like nanosphere, nanoparticle, and nanocapsules depending on the method of preparation and their components to encapsulate drug molecule (15). Many scientists have reported series of molecules obtained by grafting different substitutions selectively on either primary or secondary face of cyclodextrins via ether, ester or amide linkage (19–22) and studied their application as a drug carrier. Drug leakage is a major disadvantage of many nanocarriers, whereas amphiphilic cyclodextrin can form an inclusion complex with drug molecules and stabilize them in a self-assembled nanocarrier solving a problem of drug leakage.

In the present research, we synthesized AMCD by randomly alkylating hydroxyl groups of beta-cyclodextrin (β CD) with C12 alkyl chain. Based on the biochemical and nutritional evidence C12 acid has distinctive properties that are

not shared with other medium and long long-chain fatty acids. Lauric acid is better managed by the body and is quickly metabolized by the body as compared to other saturated fatty acids. Literature also suggests Lauric acid is less accumulated in body as bad cholesterol as compared to other saturated fatty acids. In fact, it improves the ratio of total cholesterol/HDL, which is good for the body (23). Having distinct benefits over other medium and long-chain fatty acids, authors have selected Lauric acid for the substitution. Author is focusing on bouquet type of amphiphilic cyclodextrin with approximately 7 alkyl chain on both primary and on the secondary rim of β CD and for this Lauric acid/ β CD ratio was selected as 15. The synthesized AMCD was thoroughly characterized, and the safety profile was established by various *in vitro* and *in vivo* tests. The morphology of self-assembled nanovesicles was evaluated using the various advanced microscopic technique (e.g., AFM, TEM, SANS). Tamoxifen citrate (TMX) being class BCS class II moiety having poor solubility in water, and having structure perfect for cyclodextrin complexation; less than 5 condensed rings and a molecular weight between 100-400 Dalton (24) is selected as a model drug. TMX is available in a tablet form for oral administration. However, it has severe dose dependent side effects such as endometrial hyperplasia, liver cancer, increase blood clotting, retinopathy etc. Thus, many scientists have proposed a colloidal delivery system for sustain release of TMX via parenteral route for passive targeting to the tumor via EPR effect such as PLGA based nanoparticles (25), Solid lipid nanoparticles (26), microemulsion (27), sustain release liposomes(28) etc. Interaction of TMX with synthesized AMCD was assessed by in-silico molecular docking and MD simulation studies. In vitro cytotoxicity and *in vivo* pharmacokinetics of TMX was assessed to understand the changes, if any, due to encapsulation in nanovesicles. In addition, flow cytometry and confocal study were conducted to evaluate the uptake of nanovesicles in MCF-7 cell line.

MATERIALS AND METHODS

Materials

Tamoxifen citrate was obtained from ArEx Laboratories (Mumbai). β CD was obtained from Roquette, India. Lauroyl chloride was purchased from (Merck-Sigma Aldrich, USA). Triethylamine (TEA), dry N, N-dimethylformamide (Dry DMF), dichloromethane (DCM), and other HPLC grade solvents were purchased from Merck, India. Kolliphor P 407 was procured from BASF, India. Mannitol (Perlitol™) was obtained from Signet Corporation, India. Dialysis membrane of the molecular weight cut off 12-14 kDa was purchased from Hi-media, India. MCF-7 cells were procured from ATCC, and HDMEC cells were procured from PromoCell GmbH. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from Invitrogen. Freshly prepared double distilled water and buffers filtered through 0.22 μ m membrane filter (Merck Millipore, India) was used where ever required.

Synthesis and characterization of Amphiphilic Cyclodextrin (AMCD)

Synthesis

Synthesis scheme is presented in figure 1. Briefly, β CD, due to high water content was dried at 60 °C in a vacuum oven for 24 hrs before use. Special dry DMF with water content <0.01% was stored under nitrogen with molecular sieves (1 Å). 1 mole of dry β CD was solubilized in DMF under nitrogen. Further, 15 moles of triethylamine was

added, and the solution was kept for stirring in an ice bath. When the solution became clear, 15 moles of Lauroyl chloride was added dropwise in the solution under constant stirring in an ice bath. Next, the temperature of the reaction was raised to 30 °C and stirred for 24 h in a nitrogen environment. The reaction was monitored by thin layer chromatography (TLC) (DCM: Methanol 50:50, R_f = 0.7). After 24 h, the product was precipitated in ice-cold water (pH 2) to neutralize any unreacted triethylamine. The product was filtered and washed thrice with ice-cold water (pH 2) to remove triethylamine traces. Finally, the product was solubilized in DCM and filtered to remove unreacted βCD. AMCD was recovered by vacuum drying.

Spectroscopic Characterization

AMCD was characterized using Fourier Transformed Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and ¹H and ¹³C Nuclear Magnetic Resonance (NMR) spectroscopy. Molecular weight and degree of substitution were evaluated using Matrix Assisted Laser Desorption Ionization coupled with Time of Flight detector (MALDI-TOF) and elemental analysis.

FTIR spectra of βCD and AMCD were recorded on Shimadzu FT-IR spectrophotometer using KBr discs. The instrument was operated under dry air purge, and the scans were collected at scanning speed of 2 mm sec⁻¹ with a resolution of 4 cm⁻¹ and an interval of 1 cm⁻¹ over the region of 4000-400 cm⁻¹.

The DSC curves were recorded on Differential Scanning calorimeter (DSC1, Mettler Toledo). The thermal behavior was studied by heating samples (~10 mg) in sealed aluminum pans, using the empty aluminum pan as a reference, over a temperature range of 0-100 °C for AMCD and 25-250 °C for TMX, AMCD, Poloxamer P- 407, mannitol and freeze-dried nanovesicles. The scan was recorded with a heating rate of 10 °C/min in a nitrogen atmosphere, and data evaluated using the Star® Evaluation Software.

¹H-NMR and ¹³C-NMR of AMCD and βCD were recorded using 500 MHz Agilent NMR spectrometer in DMSO-D₆. The molecular weight of AMCD was recorded using MALDI-ToF mass spectrometer (Kratos/Shimadzu, Axima-CFR). Elemental analysis of AMCD was performed using 2400 Series II CHNS/O elemental analysis system.

X-ray powder diffraction (XRPD) of TMX, physical mixture (TMX, AMCD, Poloxamer P- 407 and Mannitol) and freeze-dried nanovesicles were analyzed. X-ray diffractograms were recorded using X-Ray Diffractometer (Rigaku, Japan). The scan was obtained in the range (2θ) of 5 to 70 degrees with a scanning rate of 1 degree (2θ) per minute.

Preparation of tamoxifen citrate (TMX) loaded AMCD nanovesicles

Blank AMCD nanovesicles were prepared by modified ethanol injection method. Briefly, AMCD was dissolved in ethanol, and the solution was injected in an aqueous solution containing 0.5 % w/v Kolliphor P407 at 60 °C rapidly. The dispersion was stirred at 60 °C to evaporate the ethanol and get the blank nanovesicles. To prepare TMX loaded Nanovesicles, TMX and AMCD both were dissolved in ethanol and the rest of the method was similar as of the blank nanovesicles. Physical stability of the prepared nanovesicles was studied at room temperature and at 2-8 °C. Instability would be defined as any precipitation, drug crystallization, or turbidity.

Molecular modeling studies of TMX and AMCD interaction

Structure collection: TMX structure was drawn and optimized using Ligprep module. Finally, the geometry optimization was carried out using the OPLS2005 force field, and structure of AMCD was generated by manually attaching C12 alkyl chains to the β CD backbone. The geometry of AMCD structure was optimized OPLS2005 force field in Macro model suite (version 11.1, Schrödinger).

Molecular Docking: The Glide module was used for generating AMCD inclusion complexes. The grid was generated using the Glide Grid Generation panel in Glide. For generating AMCD binary supramolecular inclusion complex, TMX was docked with standard precision (SP) mode on AMCD (29).

MD simulation studies: Stability of AMCD: TMX complex was studied via MD simulation performed on the Desmond module (version 11.1, Schrödinger). The inclusion complex of AMCD-TMX was immersed in simulation box (orthorhombic 10 Å) with TIP4P explicit water molecules. The system was minimized and subjected to 10 ns MD simulation at 300 K temperature and 1.013 bar pressure with default settings. Generated MD trajectory was analyzed by event analysis tool.

Physicochemical Characterization of nano-vesicles:

Particle size and Zeta Potential determination:

The particle size and zeta potential (ζ) were analyzed by dynamic light scattering (DLS) using Zeta Sizer Nano ZS (Malvern Instrument Ltd., UK). Measurements were made at 25 °C with a fixed angle of 137° in a dilution of 1:10 using Milli-Q water.

Determination of morphology of nanovesicles

TEM analysis was employed to evaluate the shape and size of the nanovesicles. 10 μ l of the sample was placed on a grid and coated with uranyl acetate. After drying, the TEM image was recorded on TEM with accelerating voltage of 5KV on Phillips instrument.

AFM analysis was used to confirm the shape of nanovesicles. AFM images were acquired in the air under ambient conditions using a Bruker Multimode 8 Scanning Probe Microscope (Digital Instruments, Santa Barbara, CA, USA) operated on Peak Force QNM mode. 10 μ l of the nanovesicle suspension was placed on a freshly cleaved mica sheet (1.5 cm $\times$ 1.5 cm; G250-2 Mica sheets 1" $\times$ 1" $\times$ 0.006"; Agar Scientific Ltd., Essex, UK) and air-dried for ~1 hr. The AFM measurements were obtained using ScanAsyst-air probes (Bruker) with V-shaped silicon nitride cantilevers with spring constants in the range of 0.25–0.4 N m⁻¹. AFM scans were acquired at a resolution of 512 $\times$ 512 pixels at a scan rate of 1 Hz and produced topographic images of the samples in which the brightness of features increases as a function of height. (30). AFM images were collected from random spot surface sampling.

The SANS measurements were performed on SANS instrument operating at Dhruva reactor, Bhabha Atomic Research Centre (BARC), Mumbai, India. The incident neutron of a wavelength of 5.2 Å with approximately 15% wavelength resolution ($\Delta\lambda/\lambda$). Linear position sensitive detector (angular range 0.5–15°) was used to impart Q-range of 0.018 and 0.32 Å⁻¹. All the sample measurements were carried out at 37 °C (31).

Encapsulation efficiency (% EE)

The percent encapsulation efficiency of TMX was calculated after separating untrapped and entrapped TMX from the Nanovesicles using Nanosep® centrifugal device having Mol. Wt. The cut off of 30 KD. Briefly, Nanovesicle suspension was centrifuged at 15,000 rpm for 10 min. The filter was washed thrice with Phosphate buffer saline (32). After three filtrations and centrifugation cycles, filtrate was analyzed using High Performance Liquid Chromatography (HPLC) using Waters C-18 columns (3 µm, 4.6 × 250 mm, 100 Å) as a stationary phase and 1% (v/v) triethanolamine in deionized distilled water, which was added to acetonitrile at a ratio of 50:50 (v/v) and pH adjusted to 3 using 0.1 N HCl as a mobile phase at a flow rate of 1 mL/min. TMX was detected at λ_{max} 275 nm (Linearity $r^2 = 0.999$, Repeatability CV: 0.184 %, Specificity confirmed)(33).

In Vitro Drug Release

In vitro release of TMX from nanovesicles was performed using dialysis method. The nanovesicle suspension was placed in a dialysis membrane with 12,000 – 14,000 Dalton cut-off. Dialysis bag was placed in PBS buffer solutions. Two different pH buffers were used as release media; pH 7.4 (plasma pH) and pH 6.5 (intertumoral pH) to study the effect of pH on TMX release from nanovesicles. The study was carried out at 37 °C, and the release medium was stirred at 100 rpm. Samples were collected at predetermined time points, and the amount of TMX released was determined using the HPLC described elsewhere. At each time point, the sample volume was replaced with fresh release medium pre-equilibrated at 37 °C, maintaining sink conditions during release studies (32,34).

In vitro and in vivo evaluation of Nanovesicles

In vitro Hemolysis using isolated human red blood cells

Hemolysis study was performed using the method reported previously (14). Briefly, erythrocytes were harvested from heparinized human (**Human blood collected from the healthy volunteer after their consent by expert pathologist in a pathology lab**) by centrifugation. The supernatant plasma was discarded, and the sedimented erythrocyte cake washed thrice with PBS (pH 7.4). After the final wash, erythrocytes were resuspended in saline. Thereafter, 1.8 ml of the erythrocyte suspension was incubated with 0.2 ml of test samples (10 and 20 mM solution of β CD and AMCD) at 37 °C for 30 min in a shaker and then centrifuged at 2,800 rpm for 5 min. The supernatant was analyzed for hemoglobin content at 543 nm spectrophotometrically (10). PBS (pH 7.4) was used as the negative control with 0 % hemolysis, and distilled water was used as the positive control with 100 % hemolysis. The percent hemolysis was calculated using Eq. 1 (35).

$$\% \text{ Hemolysis} = \frac{(\text{UV absorbance of sample treated with RBC})}{(\text{UV absorbance of sample treated with distilled water})} \times 100 \quad (1)$$

Comet Assay

The comet assay was performed on HDMEC cells obtained from PromoCell GmbH to assess the potential genotoxicity of the synthesized AMCD. An alkaline comet assay was performed according to the directions provided by the manufacturer (comet Assay; Trevigen, Gaithersburg, MD(1), USA) (36,37). HDMEC cells were seeded into six-well

plates at a density of 5×10^3 cells/cm² and allowed to grow for 24 hours. Cells were incubated with 20 and 200 µg/ml of AMCD for 24 hrs. The tail moment and olive moment were calculated (50 determination for each sample) and used as a measure of the primary DNA damage. Untreated cells and cells exposed for 20 min to H₂O₂ (200 µM) were used as negative control positive control, respectively.

$$\text{Tail Moment} = \text{Tail length} \times \% \text{ DNA in the tail} \quad (2)$$

$$\text{Olive Moment} = (\text{Centre of the mass of tail} - \text{Centre of the mass of head}) \times \% \text{ DNA in the tail} \quad (3)$$

***In vitro* cell cytotoxicity studies**

In vitro, cytotoxicity was carried out using the protocol described by T. Wongpinyochit *et al.* (38). Briefly, the human breast cancer cell line MCF-7 was purchased from ATCC (Manassas, VA, U.S.A.). Cells were cultured in complete DMEM high glucose glutaMAXTM (4.5 g glucose, 10% v/v FBS, 1% penicillin-streptomycin, 10 µg/ mL insulin), grown at 37 °C in a humidified 5% CO₂ atmosphere and regularly subcultured every 2–3 days. Cells were seeded in 96-well plates at a density of 2×10^4 cells/cm² and allowed to adhere overnight. Next, TMX stock solution in DMSO, blank AMCD nanovesicles and TMX loaded AMCD nanovesicle were filter sterilized and cells were treated using varying concentrations (0-250 µg/ml) of (a) TMX solution, (b) Blank AMCD nanovesicles, or (c) TMX loaded AMCD nanovesicles. After a 72-h incubation period, cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was prepared at 5 mg/mL in PBS, and 20 µL was added to each well followed by a 5 h incubation at 37°C in 5% CO₂. Further, 100 µL of DMSO was added to dissolve the formazan product, and absorbance was measured at 570 nm. Untreated control cells represented 100% cell viability, and the half maximal inhibitory concentration (IC₅₀) was calculated for the treatment groups.

***In vitro* cell uptake studies**

Fluorescein loaded AMCD nanovesicles (FNV) were prepared and their uptake by MCF-7 cells was studied by flow cytometry and confocal microscopy.

For cell uptake studies, the cells were cultured using phenol red free complete culture medium. The cells were washed thrice with PBS and the culture medium was replaced with either (i) control DMEM or (ii) DMEM supplemented with equivalent 10 µg/ml of fluorescein as freely diffusible fluorescein and FNV. Cells were incubated for 1 h. The incubation was arrested by placing the cells on ice, removing all the medium and washing with ice-cold PBS three times. For flow cytometry, live cells were trypsinized and harvested into 5-ml Falcon tubes and for confocal microscopy, cells were used as it is.

Data were collected using a BD FACSCantoTM flow cytometry system using BD FACSDiva software v6.3.1 (Becton Dickinson, San Jose, CA). A minimum of >9000 events was recorded within the gate, which was set and verified for the live singlets. FITC filter (530/30 nm) was used to measure the geometric mean fluorescence. FlowJo v10.1 (Tree Star, San Carlos, CA) was used for data analysis.

Confocal imaging was acquired using live cells using a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Single confocal sections were acquired at an airy disc of 1. Image analysis was performed using ImageJ v1.51k 1.

Studies were also carried out at two temperatures, i.e. 37 °C and 4 °C to see if the energy-dependent uptake mechanisms are involved in the uptake of AMCD nanovesicles by MCF-7. Further, to study the uptake mechanism, cells were incubated with various endocytosis inhibitors for 1 h. Chlorpromazine (20 µM) was used clathrin-dependent endocytosis inhibitor. Methyl-β-cyclodextrin (mβCD-5 mM) and genistein (50 µM) were used to inhibit caveolae-dependent endocytosis. Following a 1h pre-treatment with each respective inhibitor, cells were thoroughly washed and incubated with FNV for 30 mins and analyzed using flow cytometry as mentioned above (39–41).

Acute Toxicity studies

The LD₅₀ value of AMCD was investigated in Swiss albino mice (Female, 3 months, 20-25 gms). The study was planned after the animal ethics committee approval (Protocol no: ICT/IAEC/2017/P32). Five mice were used per group, and a single IV dose of 50, 300, and 2,000 mg/kg of AMCD and saline as control were injected via the tail vein. Mice were observed for 14 days, and the number of animals surviving in each group was recorded. Blood plasma/serum analysis and histopathology of vital organs were performed after 14 days to look for any abnormalities and signs of toxicity.

Pharmacokinetic studies

Pharmacokinetic studies were performed on Male Wistar rat after obtaining Animal Ethics Committee Approval (Protocol approval no. MVC/IAEC/23/2016). The rats weighing 220-250 gm were divided into 2 different groups consisting of 6 animals each. Group I received TMX solution in normal saline containing 1% ethanol and Group II received TMX loaded AMCD nanovesicles, at a dose equivalent to 5 mg/kg of TMX via the tail vein (42). About 400 µl of blood was collected via retro-orbital bleeding at suitable time points in the heparinized blood collection tube. Samples were centrifuged at 5000 × g to separate plasma. Plasma samples were carefully separated and stored at 4 °C until analyzed using HPLC for TMX content using etravirine as an internal standard. SALLE (salting out assisted liquid-liquid extraction) method was used for extraction of TMX from plasma. Chromatographic conditions were the same as described earlier in 2.4.3.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software (version 6.01) wherever necessary. Haemolysis data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data of cytotoxicity studies and comet assay was analyzed by two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Results were considered statistically significant at p<0.05.

RESULTS

Synthesis and characterization of AMCD

β CD was converted to AMCD via nucleophilic substitution reaction. Lauroyl chloride was reacted with β CD in alkaline conditions, and Lauroyl ester of β CD was formed with HCl as a by-product which was neutralized by an excess of Triethylamine in a reaction mixture. The degree of esterification of β CD was proportional to moles of Lauroyl chloride reacted with 1 mole of β CD. In the present study, 15 moles of Lauroyl Chloride were reacted with 1 mole of β CD to achieve partial random esterification of β CD. Pale yellow product (yield = 62 %) was obtained. Figure Supplementary figure 1(A) shows the FTIR spectra of AMCD showing a characteristic peak at 1743.65 cm^{-1} corresponding to carbonyl (C=O) stretching of ester suggesting the formation of Lauroyl ester of β CD, wide absorption band at 3525 and 3332 cm^{-1} corresponding to -OH stretching of β CD, strong peaks at 2854 and 2924 cm^{-1} and variable peaks in region 1350 - 1480 cm^{-1} assigned to C-H stretching and bending vibrations of C-H in alkyl chains respectively suggesting the formation of Lauroyl ester of β CD. Similar results were reported by Memisoglu et al. for hexanoyl ester of β CD (43).

Further, DSC thermogram of AMCD represented in Supplementary figure 1 (B) showed a small endothermic melting peak at $54.68\text{ }^{\circ}\text{C}$ with no other peaks till $300\text{ }^{\circ}\text{C}$ indicating no sign of degradation.

MALDI-TOF analysis of AMCD using dithranol as a matrix substance showed a group of signals separated by mass unit equivalent to that of laurate chain. Results suggest the presence of polydisperse AMCD molecule with varying degree of substitution of 9-15 Laurate units to native β CD with a molecular range of $2,799.81 - 3,884.50\text{ [M + Na]}^{+}$. Average Degree of substitution from MALDI-TOF results is considered to be 12 ± 3 , suggesting 12 hydroxyl groups out of 21 hydroxyl groups of β CD was substituted with Laurate chain and average Mol. Wt. of AMCD was found to be 3322.3 g/mol . MALDI-TOF spectra of AMCD is shown in Supplementary figure 1 (C).

Elemental analysis of AMCD was performed. The results showed that the compound has an average of $66.905 \pm 0.035\%$ Carbon and $10.125 \pm 0.134\%$ Hydrogen. As seen in Supplementary figure 1(D) there is a linear increase in calculated log % carbon and log % hydrogen with an increase in log Ds (Ds in AMCD is a number of lauroyl chain attached to a single β CD molecule). Thus, by extrapolating value of log %C ($\log_{10} 66.905 = 1.855$) and log %H ($\log_{10} 10.125 = 1.005$) log Ds of 1.08 and 1.096 respectively are obtained, which are equivalent to Ds of 12.021 and 12.473. The calculated results also support the finding of MALDI-TOF, which gave average Ds as 12.

^1H NMR and ^{13}C NMR of AMCD were recorded in CDCl_3 at ambient temperature and peaks were assigned assisted by HSQC spectra (Supplementary figure 2, 3 and 4). As seen in ^1H -NMR of AMCD, there is a downfield shift in H_2 , H_3 , and H_6 due to the attachment of Lauric acid chains on all three hydroxyl groups (2-OH, 3-OH, and 6-OH) of native β CD. Amongst the three, 6-OH is highly reactive, and thus all the 6-OH have been substituted and hence the 6-OH peak in ^1H -NMR is missing, whereas smaller peaks of 2-OH and 3-OH at δ ppm 5.45 and 5.28 are retained which illustrate some of the 2-OH and 3-OH of native β CD are unsubstituted. Due to the substitution of Lauric acid chain on 6-OH, two hydrogen atoms (H_{6a} and H_{6b}) at C6 gave two distinct signals at 4.5 and 4.12 δ ppm respectively which

otherwise gives a peak at 3.64 δ ppm in native β CD. Supplementary figure 2, ^{13}C NMR spectra of AMCD shows a peak at 173.22 δ ppm corresponding to carbonyl carbon suggesting the formation of the ester bond. Additional peaks at 14.26, 25.04, 29.81 and 34.11 δ ppm due to the lauric acid chain suggests the successful substitution of lauric acid chain on native β CD.

Preparation and optimization of self-assembling nanovesicles

Synthesized AMCD has the capability of self-assembling to form a nanovesicles in an aqueous medium. At first, no surfactant was used in the preparation of nanovesicles. The nanovesicles which were prepared without any surfactant gave a particle size of 256.3 ± 11.2 nm, but they were stable for only 2 days at R.T. and for 5 days at 2-8 $^{\circ}\text{C}$. Hence, different stabilizers were investigated in order to increase the stability of nanovesicles. Different stabilizer and their different concentrations were used to optimize nanovesicles are depicted in table 1.

Kolliphor P407 was very effective than Tween 80 and TPGS (d- α -Tocopheryl polyethylene glycol 1000 succinate) in reducing the particle size as well as in stabilizing the nanovesicles which are formed by self-assembling of AMCD. Polymeric stabilizers are more effective in stabilizing the nanovesicles than the surfactant stabilizers. Poloxamer P407 helped to achieve the particle size of nanovesicles < 150 nm with very narrow PDI and a very good physical stability, hence 0.5 % w/v of poloxamer P407 was selected for final formulation.

Molecular modeling studies of TMX and AMCD interaction

In-silico molecular docking and MD simulation studies are very helpful in understanding the molecular interaction involved during the complex formation and the stability of the complex. In the present study, when TMX was docked on AMCD, the stable complex was formed as seen in figure 2(A) and 2(B). One of the benzene rings of (1,2-Diphenyl-1-butenyl) phenoxy side chain of TMX enters the cavity of AMCD through the secondary rim. The complex is stabilized through hydrogen bond between the nitrogen of 3° amine in TMX and 2-OH group of glucopyranose of AMCD and several aromatic hydrogen bonds between phenyl groups of TMX and AMCD. The binding efficiency indicated by the glide docking score for the TMX and AMCD was found to be -5.722 kcal/mol. The stability of the complex in water was assessed by running an MD simulation of 10 ns. MD simulation trajectory was analyzed for RMSD of AMCD, RMSD for TMX and RMSD of TMX with respect to AMCD which are presented in figure 2(C). Lower RMSD of TMX with respect to AMCD, when compared to RMSD of AMCD, represents the TMX is remain associated with AMCD and not getting diffused out of the complex suggesting the stability of the complex. RMSD of TMX is not significant, which suggests there are no conformational changes in TMX throughout the simulation period. Hydrogen bond analysis was also performed which suggested that there is at least 1 hydrogen bond present between TMX and AMCD (figure 2D) throughout the period which is stabilizing the complex with several other aromatic hydrogen bonds.

Physicochemical Characterization of nanovesicles

Evaluation of particle size, Zeta potential, and encapsulation efficiency

The particle size of blank self-assembled nanovesicle was found to be in the range of 96.12 ± 8.09 nm with $PDI < 0.1$ and that of optimized TMX loaded nanovesicles are in the range of 112.40 ± 2.99 with $PDI < 0.2$. Zeta (ζ) potential of both blank and drug-loaded nanovesicles was -10.65 ± 0.53 mV and -11.56 ± 0.36 mV respectively. The encapsulation efficiency of optimized formulation was found to be 94.02 ± 3.49 %.

Morphology of nanovesicles

TEM images of blank nanovesicles, as seen in figure 3 (A) showed a perfectly spherical nanovesicle size of ~ 100 nm. TEM of TMX loaded nano-vesicles in figure 3 (B) showed a slight increase in particle size due to drug loading.

AFM images of TMX-loaded nanovesicles in figure 3 (C) confirms the spherical shape of nanovesicles. Nanovesicles have a smooth surface with a particle size of around < 200 nm. The particle size of nanovesicles is little larger in AFM which can be attributed to the flattening or collapsing of nanovesicle on the grid surface as an experiment is conducted in air.

SANS data and corresponding best-fit curve using spherical shell model for AMCD nanovesicle are shown in figure 4 (A) (31,37,44). Deuterated water was used as a media to provide good contrast between the AMCD and the dispersion medium. Scattering curve showed a typical scattering profile exhibited by vesicular systems and absence of Braggs peaks confirm the unilamellarity of the vesicle formed by AMCD. The vesicle size after fitting the data in spherical core-shell model was found to be ~ 100 nm with a shell thickness of about 7.2 nm and a polydispersity index of 0.3.

The length of the AMCD molecule, when drawn in 3D, was found to be around $34.02 \text{ \AA}$ as seen in figure 4 (B) and the approximate shell thickness of AMCD nanovesicle given by SANS data fitting was found to be around 7.2 nm which is approximately double the length of AMCD molecule, and supporting the theory of AMCD assembling in bilayer which in turn forms a nanovesicles. It is evident from the results of TEM, AFM, DLS, and SANS that AMCD is capable of self-assembling into nanovesicles of perfectly spherical shape and smooth surface which has a particle size of ~ 100 nm with a narrow PDI.

In vitro drug release studies

AMCD nanovesicles, as seen in figure 5 showed a sustained release of TMX over a period of 48 hrs. Approximately 75 % of the drug was released over 48 hrs in both the dissolution media i.e. pH 6.5 (Intratumoral pH) and pH 7.4 Phosphate buffer saline (blood plasma pH). Initial burst release of TMX within 1 hr from the system was found to be 16.9 % and 19.6 % for pH 6.5 and pH 7.4 respectively. **Previously, Bilensoy et. al. reported 40 % burst release of paclitaxel from 6-O-CAPRO- β CD based nanoparticles (45). AMCD helped in reduction of burst release and maintain the prolong release of drug for more than 24 hrs.** Release data when fitted using different release models, drug release at both pH was found to fit Korsmeyer-Peppas model according to equation $F = 19.811 * t^{0.361}$

($r^2 = 0.9750$) and $F = 22.376 * t^{0.315}$ ($r^2 = 0.9654$) for pH 6.5 and 7.4 respectively. n value of 0.361 and 0.315, which was less than 0.5 in both the pH media indicated that TMX release from AMCD nanovesicles following a fickian diffusion (46,47). Initial burst release can be associated with a drug bound to the surface rather than incorporated in the bilayer formed by AMCD which is released quickly while remaining drug is released slowly over a period of 48 hrs. There was no significant difference between the release at pH 6.5 and pH 7.4 and thus it can be assumed that TMX release will not be hampered due to the pH change in tumor environment and system will continue to release the drug as expected.

Differential Scanning Calorimetry studies

DSC thermogram of free TMX showed a sharp endothermic peak at 149.32 °C as seen in figure 6 (A), which corresponds to its melting point. This is in accordance with the previously reported results by Reddy et al. (42). Other excipients of nanovesicles such as AMCD, poloxamer P 407 and mannitol showed endothermic peaks at 54.68 °C, 58.5 °C and 168.34 °C respectively. In DSC thermogram of freeze dried TMX loaded nanovesicle, the peaks of excipients are retained whereas, the peak of TMX is missing suggesting that TMX is molecularly dispersed in AMCD bilayer and thus because of the amorphization of TMX, nanovesicle showed no characteristic melting peaks of TMX at around 149 °C.

X-Ray Diffraction analysis

The X-ray diffractogram of free TMX in figure 6 (B) showed several characteristics sharp peaks between at 11.72, 12.6, 13.94, 17.36, 17.92 and 21.54° (2 θ). Physical mixture retains peaks of TMX and other excipients whereas, peaks of TMX are either greatly diminished (12.72, 13.72, 21.1°) or entirely missing (11.72, 17.36, 17.92°) from the X-ray diffractogram of TMX loaded nanovesicles suggesting amorphization of TMX with entrapment of TMX within the nanovesicle. Results of XRD studies substantiate the results of DSC studies that TMX is molecularly dispersed in AMCD bilayer in nanovesicles.

***In vitro* and *in vivo* evaluation of Nanovesicles:**

***In vitro* Hemolysis study**

Starting material of AMCD i.e. β CD is well known for its hemolytic activity because of its ability to disrupt the cell membrane of RBC by extracting phospholipids and cholesterol from the cell membrane (48). Thus, it is of utmost importance to make sure the material is safe for parenteral administration. As seen in Supplementary figure 5 (A), β CD showed shown $\sim 3.3 \pm 0.32$ % and 29.64 ± 0.9 % hemolysis at 10 and 20 mM concentration respectively. Whereas, AMCD showed significantly lesser ($p < 0.0001$) hemolysis of 0.74 ± 0.07 % and 0.672 ± 0.11 % at equivalent concentration. There was no significant difference between the % hemolysis in negative control and that of 10 and 20 mM concentration of AMCD, which suggest the AMCD could be safe for parenteral administration. The Hemolytic potential of AMCD might be lost due to the self-assembling of AMCD molecules to form nanovesicles whereas, β CD which gets solubilized and could be damaging RBC cell membrane.

Comet Assay

Comet assay was performed to assess the genotoxicity of the AMCD in HDMEC cells by exposing cells to two different concentrations of AMCD 20 and 200 $\mu\text{g/ml}$. cells treated with H_2O_2 (200 nM) were used as a positive control. Treated cells were compared with untreated cells. Supplementary figure 5 (B) shows an image of HDMEC cell nuclei stained by SYBR® Gold dye after electrophoresis to separate intact DNA from damaged fragments. Cells treated with H_2O_2 showed degradation of DNA and the fragment of the damaged DNA formed a tail similar to a comet whereas the untreated cells nuclei appeared to be perfectly spherical and no tail was observed in cells (49). AMCD treated cells at both the concentrations was comparable to untreated cells and did not show any damage to DNA with no tail being observed around the nuclei. Results were further clear when the tail and olive moment was calculated. Values of both tail and olive moment showed no significant difference between untreated cells and AMCD treated cells at both the concentrations whereas positive control showed significantly higher tail and olive moment as seen in Supplementary figure 5 (C). This implies that the synthesized AMCD showed no damaging effect on the DNA of cells.

In vitro cell cytotoxicity studies

In vitro cytotoxicity of freely diffusible TMX solution, blank AMCD nanovesicles, and TMX loaded AMCD nanovesicles were assessed in MCF-7 cells after incubating cell with increasing concentrations of TMX (0-250 $\mu\text{g/mL}$) for 72 hours. As shown in figure 7 the growth inhibitory effect of TMX was evident both in freely diffusible form and when encapsulated in AMCD nanovesicles. Blank AMCD did not show any significant cytotoxic effect ($p > 0.05$) when compared to control. This suggested that the AMCD formulation was highly biocompatible. IC_{50} was calculated by nonlinear regression curve fitting. IC_{50} of freely diffusible TMX solution was found to be 11.43 ± 0.955 $\mu\text{g/ml}$ and that of TMX loaded AMCD nanovesicles was 7.796 ± 0.247 $\mu\text{g/ml}$. TMX showed concentration-dependent growth inhibition. At concentrations >20 $\mu\text{g/ml}$ both freely diffusible TMX solution and TMX loaded AMCD nanovesicles showed no significant difference in % inhibition of growth, however, below 20 $\mu\text{g/ml}$ the encapsulated TMX showed significantly enhanced growth inhibitory effects than the freely diffusible TMX solution. This can be explained by the fact that the TMX loaded in the nanovesicles would have been taken up by the cells and further, the slow release of TMX within the cells over the period of 72 h showed higher % inhibition at lower concentrations.

In vitro cell uptake studies

Cellular uptake of freely diffusible fluorescein and FNV was quantitatively measured using flow cytometry. As seen in figure 8 (A), there was a significant ($p < 0.05$) increase in uptake of FNV than that of freely diffusible fluorescein. This suggests that the internalization of nanovesicles by the MCF-7 cells was prominent than the freely diffusible fluorescein and thus the cell-associated fluorescence was significantly enhanced for FNV as compared to freely diffusible fluorescein. Further, when cells were incubated at 37 °C and 4 °C to study the energy dependency of nanovesicle uptake, it suggested that the uptake of nanovesicles was significantly higher ($p < 0.001$) at 37 °C as compared to at 4 °C, whereas uptake of freely diffusible fluorescein was not significantly affected at 4 °C. This indicates that cellular uptake of AMCD nanovesicles was through energy-dependent endocytosis. Pre-treatment of cells with caveolae-dependent cell uptake inhibitors i.e. genistein and M β CD, significantly affected the uptake of FNV

by ~15 and 40 % respectively as seen in figure 8 (B) whereas, chlorpromazine which is a clathrin-mediated endocytosis inhibitor showed no significant reduction in cell-associated fluorescence when compared to control, suggesting that clathrin-mediated endocytosis is minor uptake mechanism for AMCD nanovesicles and caveolae-dependent cell internalization pathway plays a major role in internalization of AMCD nanovesicles (50,51).

Confocal imaging was performed to confirm that the nanovesicles were actually internalized in the cells and not just associated with the surface of a cell which could also give the false positive results in FACS analysis. MCF-7 cells were incubated with FNV for 3 hr and cells were washed and imaged live. Figure 9 shows a confocal image of MCF-7 cells in bright field and in the green channel. As it is clearly seen from the image, FNV is internalized in the cells and not just associated with the surface, thus confirming the results of FACS.

Acute toxicity study

An acute toxicity study was performed to assess the median lethal dose (LD₅₀) of AMCD. An acute toxicity study was conducted by giving 3 doses of 50, 300 and 2000 mg/kg of AMCD to five mice and observing them for 14 days for mortality. Mice injected with all the doses of AMCD were alive after 14 days, suggesting LD₅₀ of AMCD can be safely assumed to be > 2000 mg/kg. Mice which were given AMCD (2000 mg/kg dose) were sacrificed after 14 days and histopathology of their vital organs showed no abnormalities or any other signs of any toxicity (Supplementary figure 6A). Blood hematology parameters of that animal were not significantly different than the control animals (Supplementary figure 6B). Similar results were observed with serum biochemistry which remained unchanged and no significant difference was seen in serum chemistry of control animals and animals which were given a dose of AMCD at 2000 mg/kg (Supplementary figure 6C). Acute toxicity studies demonstrated that AMCD was well tolerated in mice at doses as high as 2000 mg/kg.

Pharmacokinetics studies

Mean plasma concentration of TMX vs time after single bolus IV bolus injection of TMX solution and TNV is shown in figure 10. Data were analyzed using PKSolver 2.0. The best fit was achieved with two compartmental IV bolus model which was described by the following equation

$$C = \frac{D \cdot (K_{21} - \alpha)}{V_d(\beta - \alpha)} \cdot e^{-\alpha t} + \frac{D \cdot (K_{21} - \beta)}{V_d(\beta - \alpha)} \cdot e^{-\beta t} \quad (4)$$

where, $\alpha + \beta = K_{12} + K_{21} + K_{10}$, $\alpha \cdot \beta = K_{21} \cdot K_{10}$, C is concentration, D is dose, V_d is apparent volume of distribution, K₁₂ and K₂₁ represents distribution rate constant from the central compartment to the peripheral compartment and from peripheral compartment to the central compartment respectively. K₁₀ denotes rate constant associated with the elimination from the central compartment, α is rate constant associated with the distribution phase of the concentration-time curve, β is rate constant associated with the terminal phase of the concentration-time profile and t is time (52). The calculated pharmacokinetic parameters are presented in table 2.

TMX solution showed a rapid initial drop in plasma concentration as compared to TNV due to rapid distribution of TMX solution in tissues, followed by a slow elimination phase. Encapsulation of TMX and slow release from nanovesicles reduced the rapid decline in plasma concentration. The rate of both the phases i.e. distribution, as well as

elimination phase, was reduced and $t_{1/2}$ of both the phase was prolonged for TNV as compared to TMX solution. Clearance of TMX solution was faster as compared to TNV, thus an increase in AUC by approximate three folds was observed. The mean residence time of TNV was also extended to 33 h as compared to 22 h for TMX solution. These results clearly show the changes in pharmacokinetic parameters of TMX due to the encapsulation in nanovesicles. An increase in MRT ensures that nanovesicles are present in circulation for a longer period of time providing more opportunities to nanovesicle for entering tumor tissues through EPR effect and thus making therapy more targeting.

DISCUSSION

Synthesis of amphiphilic cyclodextrin via nucleophilic substitution reaction using acid chloride yielded randomly substituted AMCD. FTIR and NMR studies confirm the formation of the ester bond. NMR studies revealed that primarily all 7, 6-OH of β CD was substituted as it is highly reactive and few 2-OH and 3-OH were also substituted. MALDI and micro-elemental analysis confirmed the synthesis of AMCD with an average mol wt. of 3322.3 g/mol and average Ds of 12. AMCD was able to self-assemble to form a nanovesicles in aqueous media, however, due to low zeta potential the physical stability was not achieved. Kolliphor P407 being a polymeric stabilizer enhanced the physical stability of the nanovesicles, similar results were obtained by Lemos-Senna et. al. who studied the effect of different stabilizer on the stability of amphiphilic cyclodextrin nanospheres (20). Molecular docking and MD simulation gave fair idea about how AMCD and TMX will interact and the stability of the complex which was formed. Alkyl chains attached to the β CD moiety extended the cavity of β CD and increase the interaction of TMX with AMCD. Sherje et al. also reported formation of stable complex of Etodolac with HP β CD with similar glide score and complex was stabilized by various hydrogen and aromatic bonds as in present case (53). This stable complex will help retaining TMX in nanovesicle and will overcome the precipitation of drug which is a common problem for liposomal formulations (54). Many authors have used SANS to study the bilayer thickness in liposomes (37,55,56), here the same study was used to study the thickness of AMCD shell which combined with data from in-silico data helped to deduce the structure of AMCD nanovesicles. After establishing the morphology and stability of AMCD nanovesicles, they were evaluated for their potential as a drug carrier by encapsulating TMX. AMCD nanovesicles were able to encapsulate TMX with drug loading as high as 10 % w/w and released them slowly over the period of 48 hrs. DSC and XRD studies showed the reduction in characteristic crystalline nature of TMX after encapsulation in AMCD nanovesicles. A similar observation was made by Çirpanli et.al., and Bilensoy et.al., who studied the encapsulation of Camptothecin and Paclitaxel in amphiphilic cyclodextrin nanosphere respectively (45,57). Further, the safety of AMCD was established by *in vitro* hemolysis where it was found to exhibit significantly lower hemolytic potential as compared to the parent β CD. Literature suggests that increasing substitution on native β CD leads to a reduction in the hemolytic potential (58). Current research for the first-time established genotoxicity for amphiphilic cyclodextrins, comet assay suggested AMCD is non-genotoxic even at very high concentrations. *In vitro* cytotoxicity studies showed that nanocarrier itself was not cytotoxic but enhanced the cytotoxicity of TMX at low concentrations. This can be attributed to the slow release of drug over the period of time and active uptake of nanocarrier by the cells as seen by cell uptake studies. Unal et.al., reported similar findings where the cytotoxic effect of Camptothecin was significantly improved by encapsulating it in amphiphilic CD nanocarrier (59). Encapsulation in TMX in the AMCD nanovesicles

has greatly affected its pharmacokinetic parameters. Reduced elimination rate constant, clearance and increased elimination $t_{1/2}$, AUC and mean residence time suggests longer retention of TMX in plasma when encapsulated in AMCD nanovesicles. Similar results were reported by Li et. al., where encapsulation of TMX in liposomes extended the retention of TMX in plasma with an increase in AUC as seen in pharmacokinetic profile analyzed using two compartmental model (60).

CONCLUSION

Through the findings of the present study, it can be concluded that one step synthesis for randomly alkylated amphiphilic cyclodextrin is successful. Synthesized AMCD can form nanovesicles which are also able to encapsulate and slowly release TMX. Further, the safety of the AMCD was established by *in vitro* hemolysis, comet assay and acute toxicity studies making it a viable nanocarrier system for parenteral delivery. Nanovesicles appeared to be actively endocytosed by cells increasing the cytotoxicity of the encapsulated agent at lower doses. The pharmacokinetic study pointed out that elimination of encapsulated agent can be slowed down and mean residence time of carrier can be extended, giving it more opportunity to be targeted to the tumor via EPR effect.

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ABBREVIATIONS

^{13}C NMR, carbon-13 nuclear magnetic resonance; ^1H NMR, proton nuclear magnetic resonance; AFM, atomic force microscopy; AMCD, amphiphilic cyclodextrin; ATCC, American type culture collection; CD, Cyclodextrin; CO_2 , carbon dioxide; DCM, dichloromethane; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle medium; DMF, N, N-dimethylformamide; DMSO, dimethyl sulfoxide; DMSO-D_6 , deuterated dimethyl sulfoxide; Ds, degree of substitution; DSC, differential scanning calorimetry; FBS, fetal bovine serum; FNV, fluorescein loaded AMCD nanovesicles; FTIR, fourier-transform infrared spectroscopy; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum correlation; IC_{50} , half maximal inhibitory concentration; IV, intravenous; KBr, Potassium bromide; LD_{50} , median lethal dose; MALDI-TOF, matrix assisted laser desorption/ionization-time-of-flight; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; $\text{M}\beta\text{CD}$, methyl β cyclodextrin; PBS, Phosphate buffer saline; PES, polyether sulfone; PLGA, poly(lactic-co-glycolic acid); PM, physical mixture; R.T., room temperature; RBC, red blood cells; SANS, small-angle neutron scattering;

SMEDDS, Self-micro emulsifying drug delivery system; TEA, triethylamine; TEM, transmission electron microscopy; TLC, thin layer chromatography; TMX, tamoxifen citrate; UV, ultraviolet; β CD, β cyclodextrin

AUTHOR CONTRIBUTIONS

M.R.P. and P.R.V. conceived the study. M.R.P. manufactured and characterized AMCD nanovesicles as well as performed transmission microscopy and AFM studies. D.A.L. provided training for AFM and host for the majority of experiments at the University of Strathclyde. M.R.P. performed all molecular docking and MD simulation studies. M.R.P. and P.R.V. interpreted all the results of in-silico studies. *In vitro* and *in vivo* experiments were conducted by M.R.P. M.R.P. performed all animal studies. All authors (M.P., D.A.L, and P.R.V.) designed research, discussed the results, and/or advised on the analysis. M.R.P. and P.R.V wrote the manuscript with support from all the authors.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table caption

Table 1: Effect of different surfactant and their concentration on particle size and stability of nanovesicles.

Table 2: Pharmacokinetic parameters of TMX solution and TNV after single IV *bolus* of 5 mg/kg per rat.

Table 1.

Stabilizer	concentration	Particle size (nm)	PDI	Stability at R. T (days)	Stability at 2-8°C (days)
No stabilizer	-	256.3 ± 11.2	0.366 ± 0.036	2	5
Tween 80	0.25 % w/v	173.2 ± 9.7	0.293 ± 0.047	3	7
	0.5 % w/v	153.6 ± 7.6	0.264 ± 0.037	3	7
TPGS	0.25 % w/v	185.6 ± 9.9	0.225 ± 0.054	2	6
	0.5 % w/v	154.7 ± 11	0.263 ± 0.063	3	7
Kolliphor P407	0.25 % w/v	112.0 ± 9.4	0.149 ± 0.026	15	>3 months
	0.5 % w/v	102.9 ± 7.7	0.095 ± 0.007	20	>3 months

Table 2

Parameter	Unit	TMX solution	TNV
k10	1/min	0.003164	0.00077
k12	1/min	0.060266	0.019385
k21	1/min	0.018592	0.036736
t1/2Alpha	min	8.52603	12.29216
t1/2Beta	min	958.0821	1381.804
C0	µg/ml	0.822446	0.73008
V	(mg)/(µg/ml)	1.519857	1.712141
CL	(mg)/(µg/ml)/min	0.004808	0.001318
V2	(mg)/(µg/ml)	4.926661	0.903501
CL2	(mg)/(µg/ml)/min	0.091595	0.033191
AUC 0-t	µg/ml*min	171.0271	489.9296
AUC 0-inf	µg/ml*min	259.9724	948.1578
MRT	min	1340.734	1984.033
Vss	mg/(µg/ml)	6.446519	2.615642

Figure Caption

Figure 1: Reaction scheme for the synthesis of AMCD. a) Dry DMF, triethylamine, 30 °C, 24 h, under nitrogen environment.

Figure 2 (A) Top view of docked pose for TMX and AMCD **(B)** Side view of docked pose of TMX and AMCD showing hydrogen bonds formed between TMX and AMCD **(C)** RMSD calculation for trajectory of TMX and AMCD MD simulation **(D)** Hydrogen bond analysis between TMX and AMCD during MD simulation period.

Figure 3 (A) TEM image of Blank nano-vesicles **(B)** TEM image of TMX-loaded nano-vesicles **(C)** AFM height image (3D) of TMX-loaded Nanovesicles (image size $1 \times 1 \mu\text{m}$) **(D)** AFM height image (2D) of TMX-loaded nanovesicle (image size $3 \times 3 \mu\text{m}$) **(E)** cross-section of single nanovesicle showing a smooth spherical particle of 180 nm.

Figure 4 (A) SANS data of AMCD nanovesicles with a corresponding best fit curve using spherical core-shell model **(B)** 3D structure of AMCD molecule with molecular length measurement **(C)** Schematic representation of the proposed structure of self-assembled nanovesicle of AMCD showing the arrangement of AMCD molecules to form a bilayer of thickness $\sim 7.2 \text{ nm}$.

Figure 5 The release profile of TMX from AMCD nanovesicles in pH 6.5 and pH 7.4 Phosphate buffer at 37 °C using the dialysis method. Data expressed as mean $\pm$ SD, n=3.

Figure 6 (A) DSC thermogram of TMX, AMCD, Poloxamer P 407, Mannitol and freeze dried TNV **(B)** X-ray Diffractogram of TMX, Poloxamer p 407, Mannitol, AMCD, Physical mixture and freeze dried TNV.

Figure 7 Cell viability of blank AMCD nanovesicles, freely diffusible TMX solution, and TMX loaded AMCD nanovesicles against MCF-7 cell line after 72 h incubation. ** signifies $p < 0.01$ and **** signifies $p < 0.0001$. Data represent the mean $\pm$ SD of 6 independent experiments.

Figure 8 cell uptake studies **(A)** cell-associated fluorescence after incubating cells with freely diffusible fluorescein and FNV at 37 °C and 4 °C **(B)** effect of endocytosis inhibitors on internalization of FNV in MCF-7 cells. Cells were pre-treated with Chlorpromazine, Genistein, and Methyl- β -CD for 1 h followed by washing and incubation with FNV for 1 h followed by flow cytometric analysis. * signifies $p < 0.05$ and *** signifies $p < 0.001$. Data represented as Mean $\pm$ SD (n = 4).

Figure 9 confocal images of MCF-7 cells incubated with FNV for 3 h and imaged live after washing **(A)** bright field image at 10X **(B)** Green channel image of the same area at 10X **(C)** composed image of bright field and green channel superimposed at 10X.

Figure 10 Mean plasma concentration of TMX vs Time profile for TMX solution (closed square) and for TNV (closed triangle). Data represented as Mean $\pm$ SD (n=6).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Supplementary figure 1 (A) FTIR spectra of AMCD showing a characteristic peak of ester (B) DSC thermogram of AMCD (C) MALDI-TOF spectra of AMCD showing group of signals corresponding to βCD-Laurate with 9-15 substitutions (D) Change in log % C and log % H with an increase in log Ds of AMCD.

Supplementary figure 2: ^1H -NMR spectrum of synthesized AMCD

Supplementary figure 3: ^{13}C -NMR spectrum of synthesized AMCD

Supplementary figure 4: 2D NMR (HSQC) for synthesized AMCD

Supplementary figure 5 **(A)** Hemolysis study on RBC by β CD and AMCD at 10 and 20 mM concentrations. **(B)** Fluorescence microscopy image (20X) of HDMEC cells **(C)** comet score in HDMEC cells. Data represented as Mean $\pm$ SD of individual duplicate experiments.

Supplementary figure 6 **(A)** Histopathology of vital organs of untreated mice and AMCD (2000 mg/kg) treated mice (after 14 days) **(B)** Haematological parameters of untreated and AMCD (2000 mg/kg) treated mice (after 14 days) **(C)** Serum Biochemistry of untreated and AMCD (2000 mg/kg) treated mice (after 14 days). Data for blood biochemistry are represented as Mean $\pm$ SD (n=5).