Synthesis of calix (4) resorcinarene based amphiphilic macrocycle as an efficient nanocarrier for Amphotericin-B to enhance its oral bioavailability

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Abstract: The supramolecular based macrocyclic amphiphiles have attracted great attention in the field of drug delivery due to their unique self-assembling nature. Therefore, these macrocycles are used as nanocarriers for the delivery of poor water soluble drugs, and also for those which have lower permeability, and cannot cross the barrier to reach the desired site. Herein, we design and synthesized a new supramolecular amphiphilic macrocycle to overcome these problems. The macrocycle was synthesized in two steps. In first step 4-hydroxybenzaldehyde was treated with 1-bromotetradecane to obtain a derivatized product which was then treated with resorcinol to cyclize and get calix(4)resorcinarene based supramolecular amphiphilic macrocycle. The synthesized macrocycle and intermediate products were characterized by Mass spectrometry, IR and ^{1}H-NMR spectroscopic techniques. The amphiphile was screened for biocompatibility studies, vesicles formation, and then Amphotericin-B was loaded in the supramolecular amphiphile based vesicles and was characterized for shape, size, homogeneity, surface charge, drug entrapment, *in-vitro* release profile and stability through atomic force microscopy (AFM), Zetasizer, HPLC and FT-IR. Amp-B loaded macrocycle based vesicles were investigated for in-vivo bioavailability in rabbits. The synthesized macrocycle was nontoxic in cancer cells, hemo-compatible and safe in mice. The drug-loaded macrocycle based vesicles appeared spherical, nano-ranged, and homogeneous in size with negative surface charge and were able to load an increased amount of drug. The vesicles are stable upon storage and when incubated with gastric simulated fluid. Amp-B increased oral bioavailability was achieved when delivered in synthesized macrocycle based vesicles. These results point out that the synthesized supramolecular amphiphile is an efficient nanocarrier to enhance the oral bioavailability of lipophilic drugs.

Keywords: Synthesis; Amphiphilic Macrocycle; Biocompatibility; Amphotericin-B; drug delivery; pharmacokinetics

1. Introduction

Bio adhesives are synthetic and natural materials which possess the capability to adhere to biological substances. To retain the dosage form at the absorbing epithelial membrane, such materials are embedded which enhance the drug release pattern and also decrease the frequency of dose as compared to the conventional dose as well [1, 2]. For the last decade, bio adhesive properties of large materials have been evaluated. Similarly, the synthetic polymer like that Carbopol and polycarbophil present wonderful adhesive properties when they are tested *in-vitro* as well as *in-vivo* [3, 4] . Thus, the *in-vivo* properties of these adhesive materials cannot be replicated [5] , which shows that some

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bio adhesive drug delivery systems have great accessibility on the commercial scale [6, 7]. Moreover, the bio adhesives have great capability to reduce the toxic effect of other excipients present in the formulation. Also, they have excellent potential to enhance the solubility of less soluble drugs and result in increasing the oral bioavailability, when these drugs are taken through the oral route. The most effective and popular system to deliver the less soluble and low permeable drugs to deliver the target site by enhancing the solubility and permeability are the lipophilic components which have polar and non polar characters within the molecule and form an inert lipid vehicles (lipid bilayer membrane) [8], such as oils [9], surfactants [10], self-emulsifying nano-formulations [11], emulsions, liposomes[12], polymeric micelles[13], novel amphiphilic Dendrimers[14], amphiphilic peptides based drug delivery system[15], metal organic frame work (MOFs) [16], and niosomes [17]. The amphiphilic polymers have been widely used for the potential delivery of anticancer drugs due to their easy biodegradability and biocompatibility. Cyclodextrin polymers and star shaped polymers have eminent properties to form micelles when they come in contact with aqueous media. The polymeric micelles have greater capability to entrap the larger amount of both hydrophilic and hydrophobic drugs and enhance the oral bioavailability and depict a sustained drug release phenomena[13, 18]. Besides, these polymers, some other biopolymers which are derived from nature or tissues (e.g., hyaluronic acid, gelatin, chitosan, and alginate) are used to make hydrogels through intent intermolecular interactions. Therefore, these hydrogels formed from biopolymers are used in the local drug delivery system due to their wonderful biodegradability and biocompatible nature [19-21]. Among all these delivery systems, the most effective and prominent drug delivery approach is drug delivery through amphiphilic supramolecular self-assembly acting as nanocarriers and efficient solubilizer for lipophilic drugs encapsulating in their inner cores. The supramolecular self-assembly system enhances the solubility of poorly water soluble drugs and also increases the oral bioavailability, after an oral administration. The niosomal vesicular supramolecular amphiphilic drug delivery systems also have excellent potential to deliver a lager fraction of drugs to the target site, reducing their toxic effect as well [22] . Therefore, it is widely used for the treatment of most diseases, such as for the treatment of tumors present in the brain [23], visual diseases [24, 25], skin diseases [26] , joint diseases [27], and similarly wound healing diseases [28]. B-CD is a macromolecule which is widely used in the field of the pharmaceutical industries due to its biocompatibility, biodegradability and self-assembly to encapsulate a greater amount of drugs due to having large surface area and cage like cavity in its structure. Besides that, B-CD has a unique capability to form host guest supramolecular crossed linked hydrogels through a non-covalent interaction and via hydrogen bonding [29]. So, these supramolecular crossed linked hydrogels formed from B-CD have excellent potential to entrap large amount of drug and acts as a drug reservoir to the target site in the tissue and release the drug in a control and sustain manner [30-32]. Though, the feeble involuntary potency powerfully limits their biomedical application [33]. Hence, the recently synthesized nanocarriers have great and efficient abilities to enhance the oral bioavailability of poorly water soluble drugs, reducing their side effects. Therefore, they have got therapeutic advantages. Therefore, scientists are working to develop new formulations which have unique physicochemical characters as well[34, 35].

For the cure of Leishmaniasis protozoa and fungal healing, the amphotericin-B is widely used and it is also the gold standard medication for the treatment of fungal infections [36-38]. The main problems with this drug, is its low aqueous solubility and permeability, instability in the acidic gastric environment and insufficient oral bioavailability. Due to these factors, it is placed in BCS Class IV group [39]. Moreover, the already present formulation of AmphB causes nephrotoxicity and unpredictable systemic oral bioavailability [40]. Therefore, it is required to prepare a more efficient and cost effective formulation of this drug through the investigation of a new nanocarrier system which has excellent capability to overcome this problem and results in enhancing the oral bioavailability, larger solubility and high stability in the gastric environment as well. Herein,

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we express the chemical synthesis of a novel calix (4) resorcinarene-based amphiphilic supramolecular macrocycle with four long carbon chains bearing the length of eighteen carbons each and its potential usage as a model medicine for oral delivery of Amphoterin-B.

2. Method and material

2.1. Materials

The HPLC grade solvents were used. Acetone, 4-ydroxybenzaldehyde, 1-bromooctadecane, K_2CO_3, Resorcinol, sulphuric acid, acetic acid, Polylysine (PLL), Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma- Aldrich, Germany. Cholesterol and Tween 80 were purchased from BDH, UK, and Merck, Germany, respectively. The antifungal and antileishmial drug Amphotericin-B was purchased from sigma Aldrich, USA.

2.2. Synthesis

In 100 mL round bottom flask took 610 mg of 4-hydroxybenzaldehyde (5 mmol) in 20 mL acetone and 690mg K_2CO_3 (5mmol), reflux at 80 ^{0}C for 40 minutes and then added 5 mmol 1-bromooctadecane and left the reaction mixture to proceed for 12 hrs at that temperature and monitor the reaction through TLC, the solvent system was ethyl acetate and n-hexane (1: 9 % volume /volume). After the completion of reaction, stopped the reaction, cooled down at room temperature, removed the salt adding the water, extract with dcm and rotator evaporate and purify through column using pure n-hexane. The pure white solid product was obtained with 90 % yield. In the second step for the synthesis of macrocycle, took 40 mL acetic acid in the round bottom flask, added 440 mg of resorcinol (4 mmol), 1497 mg (4 mmol) of compound **1** was added and 250 µL of acetic acid were added as a catalyst, heat at 80 ^{0}C for 24 hrs. The brown ppt was formed, and then stopped the reaction cooled down at room temperature, filtered and dried. The product was obtained as a brown solid with 92 % yield.

2.3. Preparation of Amphotericin B-loaded niosomal vesicles

The niosomal vesicular formulation of Amphoterin-B in newly synthesized macrocyclic amphiphilic resorcinarene based supramolecule (R-C-18) were prepared via thin film hydration method. 30 mg newly synthesized amphiphilic supramolecular resorcinarene based macrocycle was taken and dissolved in chloroform and methanol (3:1 volume/volume). As a membrane stabilizer, 15 mg cholesterol was also mixed in the solution of nanocarrier. The Amphotericin-B 10mg was dissolved separately in methanol and tetrahydrofuran (Methanol/THF) mixed solvent system separately and then mixed it in the previously prepared mixture of nanocarrier and cholesterol. To completely mixed them by sonication in the ultrasonicator (LABSONIC L, Germany). To obtain the thin lipid film, the organic solvents were evaporated through the rotorary evaporator under the reduce pressure. Hence to form hydrated lipid bilayer, added distil water and shaking it for three minutes at 45 ºC . futher to acheive small size niosomal vesicular suspension, sonicated it in the ultrasonicator (LABSONIC L, Germany) at 30 ºC with five second off and on cycle for 5 minutes and stored the niosomal vesicular suspension of Amphotericin-B loaded in the nanocarrier (R-C-18) for further applications.

2.4. Shape, Size, size distribution and polydispersity index of drug loaded niosomal vesicles

The morphology of Amphotericin-B loaded niosomal suspension of calix (4) resorcinarene based amphiphilic supramolecular macrocycle (R-C-18) was investigated by the used of atomic force microscopy (AFM-5500, Agilent). The Amphotericin-B loaded vesicles of calix (4) resorcinarene based amphiphilic supramolecular macrocycle (R-C-18) were diluted with deionized water and one drop of diluted vesicular suspension put on mice slide and dried it. The surface morphology of drug loaded niosomal vesicular suspension of nanocarrier was mounted on microscopy and images were obtained. The av-

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erage size distribution, polydispersity index and zeta potential of Amphotericin-B loaded and unloaded in newly synthesized macrocyclic supramolecular amphiphile (R-C-18) was measured by the used dynamic light scattering (DLS) technique, Zetasizer (ZS-90, Malvern Instruments, UK).

2.5. HPLC method development and validation

The hplc method development, validation, optimization and drug extraction from plasma all are present in previous published articles [41, 42].

2.6. Determination of percent drug entrapment efficiency (% EE)

It is very important for pharmaceutical scientist to find out the percentage of drug entrapment capacity of the carrier. However, it is very important to determine the percent entrapment efficiency of hydrophobic drugs in novel synthesised amphiphilic supramolecular macrocycles. For this purpose, a HPLC quantification method for newly synthesised amphiphilic supramolecular macrocycles based niosomal suspension was investigated. The drug loaded niosomal suspension (2mL) of novel synthesized amphiphilic supramolecular macrocycle (resorcinarene based) containing 1mg/mL of amphotericin-B was taken in Eppendorf and then centrifuged (Universal 16, Hettich) at 12000 rpm for 25 minutes. The supernant containing the free drug was separated to remove the free drug and the pellet in which there was entrapped drug was washed thrice with BPS (pH 7.4) through the same process to remove the entire free drug from the pellet. Then to determine the % drug entrapment efficiency of newly synthesized amphiphilic macrocycle, the pellet was dissolved in MeOH: DMSO (1:1 v/v) solvent system mixed both completely and centrifuged it for 20 minutes at 12000 rpm. The supernant (100 µl) was taken and then diluted with mobile phase, vortex mixed and 50 µl was injected into the HPLC. The % drug entrapment efficiency was determined as following

Drug entrapment efficiency (% EE): Amount of amphotericin B entrapped/ total amount of amphotericin B in formulation × 100

2.7. In- vitro drug release study

To determine the in-vitro release behaviour of Amphotericin-B from niosomal vesicular formulation of calix (4) resorcinarene base macrocyclic supramolecular amphiphile, the dialysis membrane (MWCO 12 KDa) was used. The 2 mL of Amphotericin-B loaded vesicular formulation (known amount) was poured into the dialysis membrane and then this membrane was immersed in the 50 mL beakers having two different pH buffer solutions separately and put on shaker and set it with 100rpm. 2mL aliquot was withdrawn from the beaker after different time interval and added 2 mL fresh phosphate buffer solution in the beakers separately with respective pH. The aliquot was read spectroscopically to find out the amphotericin-B release phenomena.

2.9. Storage stability

It is very important to know the storage stability of amphotericin-B entrapped niosomal suspension of lately synthesized supramolecular amphiphilic resorcinarene based macrocycle. The drug loaded suspension (formulation) was investigated to 30 days. At pre-determined time intervals, samples (2 mL) was pick up and from the niosomal suspension, the percent drug retained stability was determined. The amount of drug (amphotericin B) was quantified by the method which was used for the determination of in-vitro drug release study.

2.10. Biocompatibility studies

2.10.1. Blood haemolysis assay:

The aim of newly synthesized amphiphilic supramolecular macrocycle was to use in the field of drug delivery as a nanocarrier. Therefore, these compounds were screened for their blood haemolysis assay. For this purpose, the fresh human blood was taken and

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was centrifuged for 10 minutes at 700 rpm at 4 °C to separate the RBCs from the fresh human plasma. After the removal of plasma from red blood cell, washed the RBCs thrice times with phosphate buffer solution (PBS, pH 7.4) and then centrifuged as before used the procedure. RBCs were suspended in PBS in 1:10 ratio (RBCs: PBS, w/v). In the different concentration (62.5-1000 mg/mL) of the test sample (4 mL) about (200 µL) of RBCs suspension was added. The commercially available surfactant (Tween 80) was used as a reference standard.

The samples were incubated at 37 ºC for 4 h. After 4 hrs incubation, the suspension was centrifuged to split the non-lysed RBCs and supernatants were analysed spectrophotometrically at 540 nm for the release of haemoglobin.

The following equation was used to determine the % haemolysis.

Homolysis (%) = (Abt - Ab0)/(Ab100 - Ab0) × 100

Ab0, Ab100 and Abt indicate the absorbance of the 0%, 100 % and test sample respectively

2.10.2. Cells culture study

The newly synthesized compound (amphiphilic supramolecular resorcinarene based macrocycle) was screened through different cell lines. The NIH/3T3 cell line was culture containing FBS 10 % in DMEM and in the humidified atmosphere containing 5 % CO_2 was antibiotics (50 units/mL streptomycin and 50 units/mL penicillin). The cytotoxicity of synthesized compound was investigated using MTT assay against NIH/3T3 cell line. So, in the 96 -well plates, the cells were seeded in a culture medium (200 µL) at a density of 8.0×10^3 cells/well. As the 24 hrs incubate, removed the original medium and the various concentrations (0.0625–1 mg/mL) of test sample which was fresh medium (200 µL) was added. The cells were incubated as a negative control in the media without test sample. Thus, further the cells were grown for 24 and 48 hrs. So, in each well, the solution of MTT (20 µL; 5 mg/mL) was added in PBS. The unreacted dye was removed after 4 hrs from the incubating cells. The formazan crystals which obtained were dissolved 200 µL per well dimethyl sulphoxide (DMSO) and the absorbance was observed at a wavelength of 570 nm in microplate readers (ELx808, BioTek, USA). As a standard reference and positive control Tween 80 and poly (L) lysine (PLL) were used.

The following formula was used to measure the % cell viability

% Cell viability: (Mean of absorbance value of test sample)/ (Mean of absorbance value of negative control) × 100

2.10.3. Biocompatibility and bioavailability studies using experimental animals

The rabbits which were used for study purchased from the DOW University of Health and sciences, Karachi, Pakistan. The animal studies were conducted for acute toxicity and bioavailability, with strict adherence to the principles of Institutional Animal Care and Use, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan.

2.10.4. Acute toxicity in-vivo

The Swiss albino mice (20-25 g) body weights male or female were used to investigate the acute animal's toxicity assay divided into two phases. Before used them for study, kept them for 12 hrs fasting. The mice were divided into four groups in the first phase and each group has four mice. The newly synthesized calix(4)resorcinarene based amphiphilic macrocyclic supramolecule (R-C-18) were injected intraperitoneally (i.p) at the dose of 0.1, 0.5, and 1.0 g/kg body weight and hence was investigated for transience. LD_{50} was determined experimentally. The mice was divided into two groups in the 2nd phase having four mice in each groups and same process was repeated for 2nd phase mice but here the amount of nanocarrier was injected intraperitoneally was 1.5-2.5 g/Kg body weight. After passing the 24 hrs of injected the dose to the mice, the number of mice died in each group was calculated to determine the lethal dose (LD50) accordingly [43, 44].

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2.11. Oral pharmacokinetic study:

To integrate the oral pharmacokinetic performance of Amphoterin-B loaded niosomal vesicular formulation of newly synthesized amphiphile (R-C-18), local species of rabbits (Oryctolagus Cuniculus) were used. The complete description is given in the supplementary file.

2.12. Statistical analysis

All the experiments were accepted in triplicate manner. The data of the study was express as Mean ±SEM. Two-way ANOVA followed by Boniferroni post-test was used for finding statistical significance. $P<0.05$ were considered statistically significant.

3. Results and discusion

EI-MS technique was used to determine the molecular mass of 4-(octadecyloxy) benzaldehyde. The observed mass was 375.1 m/z and the calculated mass 374.3 which satisfy the formula $C_{25}H_{42}O_2$. The proton nmr give triplet for methyl at 0.85 with 3H with coupling constant 6.8 Hz, multiplet at 1.25 with 30H of CH_2, methylene 2H at 1.76 ppm giving a triplet with coupling constant 8.0 Hz, at 3.99 ppm 2H of methylene proton appeared as triplet with coupling constant 6.4 Hz, the aromatic proton comes at 6.95 ppm with doublet giving 2H aromatic with coupling constant 8.8 Hz, at 7.78 ppm doublet with 2H of aromatic with coupling constant 8.4 Hz and at 9.85 ppm as a singlet with 1H of aldehyde. The function group was determined using FTIR, at 3517 cm^{-1} (-OH), at 2853 cm^{-1} (CH_2), at 1610 cm^{-1} (C=C), and at 1246 cm^{-1} (-O-) which confirmed the structure. The molecular mass of resorcinarene based amphiphilic molecule was measured by MALDI-TOF-MS and was 1867.4 m/z and the ^{1}HNMR showed triplet at 0.78 ppm of methyl group with coupling constant 7.2 Hz giving 12H,at 1.18 ppm (m, CH_2, 120H), at 1.67 ppm appeared as quartet with 8H of CH_2, at 2.80 ppm CH as a singlet with 4H, at 3.76 ppm appeared as sextet with 8H of CH_2, at 5.43 doublet with 4H of CH group, at 6.24 ppm appeared as doublet with 8H of CH with coupling constant 4.0 Hz, at 6.49 ppm showed doublet with 4H of aromatic ring with coupling constant 8.0 Hz, at 6.94 ppm appeared as doublet with 8H of C=C of aromatic with coupling constant 4.0 Hz, at 7.24 ppm as singlet with 8H of OH.

3.1. Shape, size, PDI, size distribution and zeta potential of drug loaded niosomal vesicles

The shape of Amphotericin-B loaded vesicles of newly synthesized calix (4) resorcinarene based supramolecular amphiphilic macrocycle (R-C-18) was determined with the used of atomic force microscopy (AFM). The images obtained from atomic force microscopy revealed spherical shaped as depicted in Figure 2. The mean diameter, polydispersity index and zeta potential of both Amphotericin-B loaded and unloaded vesicles of nanocarrier (R-C-18) were measured by the used of dynamic light scattering spectroscopy (DLS), zeta sizer as shown in Figure 3. The mean diameter of drug loaded niosomes of nanocarrier obtained was 335.5 ± 4.32 nm and the polydispersity index (PDI) measure was 0.02 ± 0.01 presenting that almost niosomes have uniform population. The value of zeta potential obtained for drug loaded niosomal vesicles of nanocarrier was -13.1 ± 2.35 mV which confirming the stability of niosomal formulation. Similarly, the unloaded niosomal formulation of nanocarrier indicates the value of mean diameter as 216.7±3.64 nm having smaller value of polydispersity index value 0.22 ±0.02 as shown in table **1**. The value of zeta potential measured for unloaded vesicles of R-C-18 was -6.77 ±1.12 as shown in table **1**. They also influence the overall pharmacokinetics and biodistribution of the loaded drugs in-vivo [45]. Nanocarriers with small size and narrow polydispersity index values have excellent absorption and stop quick drug discharge in physiological environments. Moreover, nanocarriers with negative zeta potential values have been observed technically appealing as the adjacent similarly charged vesicles repel each other, thus the particles will remain suspended in the dispersed medium and overall the formulation is stabilized [46, 47].

Figure 2 and 3 to be placed here

3.2. Percent drug entrapment efficiency (% EE) determination

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The encapsulated amount of drug in the nanocarriers reflects its kinetic drug release behaviour. Therefore, the entrapment efficiency of any drug in nanocarrier systems plays a vital role in the drug delivery systems [48]. Newly synthesized drug loaded resorcinarene based supramolecular amphiphilic niosomal vesicles were tested for their Amphotericin-B loading capability via HPLC. The supramolecular nanocarrier (R-C-18) was found highly competent in entrapping increased concentration of the drug i.e. 96.56 ±4.56 % as depicted in table **1**. This may be due to its unique macrocyclic structure which has a cage like cavity and also four-eighteen carbon chains which resulted in increasing the lipophilicity of the nanocarrier. Moreover, the presence of membrane stabilizer (cholesterol) in the niosomal vesicular formulation of Amphotericin-B. The greater lipophilic behaviour in the nanocarrier results higher the stability of vesicles and smaller the permeability of drug [49, 50]. In the case of this newly synthesized amphiphilic supramolecular macrocycle, improved lipophilicity is obtained in the course of the existence of larger alkyl chains. Increased lipophilic nature of the drug used i.e. Amphotericin-B can also be attributed to the increased EE% of the carrier as highly lipophilic drugs get well dissolved in the lipophilic membrane of the vesicles [51].

Table 1 to be placed here

3.3. In-vitro drug release study

To investigate the permeability of drug (Amphotericin-B) from the niosomal vesicular formulation of newly synthesized calix (4) resorcinarene based macrocyclic amphiphilic supramolecule, the dialysis membrane was used. The *in-vitro* release behaviour of the drug Amphotericin-B from drug loaded calix (4) resorcinarene based macrocyclic amphiphilic (R-C-18) based vesicles was assessed at different pH, 7.4 and 1.2. The drug was released in a sustain and slow manner from the niosomal vesicular suspension of newly synthesized calix(4)resorcinarene based macrocyclic amphiphile (R-C-18). At acidic pH, the release behaviour of Amphotericin-B from niosomal formulation was lower as compared to physiological pH (i.e 7.4). During first four hrs of study, near about fifty percent Amphotericin-B was discharged from niosomal vesicular formulation at physiological pH 7.4 and maximum amount 87.34 % of Amphotericin-B released was observed at 7^{th} hrs of the study. Similarly, at pH1.2, the fifty percent Amphotericin-B release behaviour was obtained at 8^{th} hrs of the study and also maximum drug released was achieved about 56.72 % at 8^{th} hrs respectively as shown in Figure 4. This amphotericin-B release behaviour from the calix (4) resorcinarene based macrocyclic amphiphilic (R-C-18) provides that the drug remain safe at lower pH environment. The smaller size of vesicles provided larger surface area which causes majority of the drug encapsulated in it to release faster in the media as compared to the greater size particles, where the entrapped drug release very slowly and very little from the inner layers [52]. The sustained drug release pattern from the newly synthesized R-C-18 based vesicles may be due to appropriate vesicles size and encapsulation of the drug deep in the bilayers as well as in innate macrocyclic cavity of the carrier.

3.4. Storage stability

The newly synthesized calix (4) resorcinarene based macrocyclic amphiphilic supramolecule (R-C-18) based drug loaded vesicles were found highly stable upon their 30 days storage. The formulation was able to retain 99.78 ±0.87, 97.54 ±2.99, 94.88 ±0.82 and 91.18 ±2.67 % drug after 1^{st}, 10^{th}, 20^{th} and 30^{th} days respectively. These results confirm stability of the R-C-18 based drug loaded formulation and its storage stability in the drug loaded niosomal vesicular formulation.

3.6. Blood haemolysis

The ultimate aim of the newly synthesized calix (4) resorcinarene based macrocyclic amphiphilic supramolecule (R-C-18) was to use it for drug delivery application as a nanocarrier, it was therefore imperative to screen this compound for in vitro blood haemolysis. Nanotechnology based nanocarriers, devices and gold and silver nanoparticles

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are promising as a choice to conventional therapeutics; therefore, their biocompatible evaluation is very necessary to assess them for blood hemolysis assay before clinical development[53]. For this purpose, fresh human blood was taken and separated the plasma to obtain RBCs and prepared different concentrations of newly synthesized amphiphilic supramolecular macrocycle ranging from 62.5-1000 µg/mL and the behaviour was compared with Tween80. The negligible hemolysis was noticed for it, even at highest concentration 1000 µg/mL as compared to the Tween80 as depicted in Figure 5. It has been previously reported that macrocycles have a key role in improving solubility of various drugs like testosterone [54] and anthelmintic drugs [55] and have excellent biocompatibility properties [56]. The synthesized amphiphile (R-C-18) was found to be compatible and present less hemolysis as compared to the commercial surfactant Tween 80.

3.7. Cytotoxicity study

It is necessary to use the newly synthesized nanocarrier for their pharmaceutical applications that previously screened them for their cytotoxicity assay. Therefore, the newly synthesized calix (4) resorcinarene based supramolecular macrocyclic amphiphile (R-C-18) was screened out for its cytotoxicity assay against NIH/3T3. The cells were incubated for 24 and 48 hrs after the addition of synthesized calix (4) resorcinarene based supramolecular macrocyclic amphiphile (R-C-18) with different concentration ranges from 62.5-1000 µg/mL as shown in Figure 6 (A & B). It was observed that the synthesized nanocarrier showed very small cytoxicity even at higher concentration 1000 µg/mL (63.76 ±1.68 %) after 24 hrs incubation as compared to the standard Tween80 and the value obtained at the same concentration for Tween80 was 63.76 ±1.68 % after 24 hrs incubation as shown in Figure 6 (A). Similarly, the cell viability results obtained for the newly synthesized amphiphile (R-C-18) after 48 hrs was 58.67 ±1.44 % at the highest concentration (1000 µg/mL) and whereas for Tween80 at the same concentration (1000 µg/mL), it was 52.11 ±1.4 2% as shown in Figure 6 (B). The nominal cytotoxicity of the novel synthetic supramolecular amphiphilic macrocycle may be related to many diverse factors such as saturation in structure, as well as appropriate lipophilicity achieved through suitable carbon chain length [51]. This peculiar feature of the nanocarrier confirmed that it possesses excellent biodegradability, biocompatibility and non-toxic characters which make it efficient for pharmaceutical applications [57]. The cytotoxicity study confirms the safety and biocompatibility of the synthesized supramolecular amphiphilic macrocycle and thus its employment in designing an effective vesicular drug delivery system can be justified.

3.8. Acute toxicity

To find out the advance (more) safety and biocompatibility of the newly synthesized calix (4) resorcinarene based supramolecular amphiphile, the acute toxicity assay was carried out using the laboratory animals (mice). Because, before to deliver it for clinical trial, acute animal toxicity assay is very necessary for confirming and identifying biomaterials cause potentially death in animals [57]. When this newly synthesized macrocyclic supramolecular amphiphile was subjected for acute toxicity in the mice, it was found to be quite safe and the animals (mice) remain alive even at greater amount of nanocarrier was given up to 2.8 g/kg body weight as well.

3.9. Oral bioavailability study

To investigate the pharmacokinetic behaviour (oral bioavailability) of the poorly water soluble and less permeable drug, Amphotericin-B was studied by preparing two different formulations. The one formulation was prepared by dissolving the Amphotericin-B in DMSO, whereas the 2nd niosomal vesicular Amphotericin-B suspension was prepared with nanocarrier (R-C-18) through the thin film hydration method and both solutions were orally given to the rabbits. After injection of both formulations via oral administration to the rabbits, the blood from the rabbits was collected from them via the air

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marginal vein at various intervals of times, then centrifuged it and separated the plasma from the blood. The drug (Amphotericin-B) was concentration quantified through high pressure liquid chromatography (HPLC). The value of C_{max} was observed for drug loaded formulation of synthesized nanocarrier (R-C-18) was 0.325 ± 0.018 µg/mL, and whereas for the simple Amphoterin-B solution, the maximum concentration (C_{max}) in plasma was obtained 0.218 ± 0.016 µg/mL as shown in table 2. The value of C_{max} for the Amphotericin-B loaded vesicular formulation of nanocarrier was 1.5 times greater than that of the simple Amphotericin-B solution. The maximum concentration of Amphotericin-B in the plasma achieved at that time (Tmax = 8 hrs) was greater as compared to the maximum concentration of Amphotericin-B from the simple Amphotericin-B solutions in the plasma at maximum time (Tmax = 2 hrs) as depicted in table 2. The clearance (CL) of Amphoterin-B solution in the plasma was achieved as 1.158 ± 0.106 L/h.kg, whereas, the clearance (CL) for Amphotericin-B loaded niosomal vesicular suspension of newly synthesized macrocyclic supramolecular amphiphile was CL 0.957 ±0.417 L/h.kg. Hence, the mean residence time (MRT) for Amphotericin-B loaded niosomal vesicular formulation of R-C-18 was 17.463 ± 4.123 h, which was larger as compared to the simple solution of Amphotericin-B (MRT = 15.953 ± 0.941 h).

The values of MRT, Cmax and Tmax obtained for niosomal vesicles of Amphotericin-B in newly synthesized calix(4)resorcinarene based macrocyclic supramolecular amphiphile (R-C-18) was greater as compared to the nano-formulation of simple Amphotericin-B. The greater values of Tmax and C_{max} for drug loaded niosomal vesicular formulation of newly synthesized nanocarrier could be due to various factors which were subjected to enhance its oral bioavailability and pharmacokinetics parameters, such as nano size of vesicles which results to enhance the surface area to load greater amount of drugs, similarly the shape of vesicles also play key role and also the lipophilicity of nanocarriers play vital role in enhancing the T_{max} and C_{max} values of any niosomal vesicular formulations [59]. Moreover, the release behaviour of Amphotericin-B from niosomal vesicular suspension of a newly synthesized macrocyclic supramolecular amphiphile (R-C-18) showed the slow manner which resulted in the decrease in its clearance and enhancing the time (residence time) to remain long time in the blood as compared to the Amphotericin-B solution. In addition, the larger value of T_{max} for the niosomal vesicular formulation of amphotericin-B loaded in newly synthesized macrocyclic amphiphile (R-C-18) confirmed its sustained release behaviours as well. This sustained release character of Amphotericin-B from the vesicular formulations of the newly synthesized nanocarrier (R-C-18) could be due to the lipophilic nature of nanocarrier, which consisted of four long eighteen carbon chains which resulted in the enhancement of lipophilicity. Moreover, the inner cavity (cage like) in the structure of a nanocarrier imparts it a larger place to encapsulate a greater amount of drug and acts as a drug reservoir.

4. Conclusion

The various factors which cause impedance in delivery of many drugs and result in low oral bioavailability include enzymatic degradation first pass gastric mechanism, poor water solubility and low membrane permeability. Therefore, the drug delivery system consisting of niosomal vesicles has been proved to be an excellent nanocarrier system which has greater advantages over the other drug delivery systems due to its unique physical and chemical constancy, and also due to its modified surface properties. To address all the above problems, we have designed a new approach to synthesise a novel nanocarrier based on calix(4)resorcinarene macrocyclic supramolecular amphiphile (R-C-18) which is biocompatible and biodegradable. The newly synthesized macrocyclic amphiphile (R-C-18) has an excellent and eminent character to encapsulate the highly hydrophobic drug Amphotericin-B (96.56 ±4.56 %) through a thin film hydration method. The larger amount of drug entrapment efficiency of the novel nanocarrier (R-C-18) is due to its lipophilicity and inner cavity in its structure. The niosomal vesicles of the newly synthesized calix(4)resorcinarene based amphiphilic macrocycles show a spherical shape which is confirmed from atomic force microscopy, and average size dis-

tribution 335.5 ±4.32 nm, polydispersity index 0.02 ±0.01 which demonstrate the particle size of nano-suspension prepared from calix(4)resorcinarene based amphiphilic macrocycle is uniform. Similarly, zeta potential -13.1 ±2.35 mV of the drug-loaded niosomal vesicle of Amphotericin-B confirmed its stability, whereas sustained drug release behaviour from the dialysis membrane (in-vitro drug release study) presents good physicochemical properties as well. Similarly, the niosomal vesicular formulation of amphotericin-B demonstrates greater stability over 30 days storage at 4 °C and doesn't show any degradation in simulated gastric fluid. The newly synthesized amphiphilic macrocycle doesn't show acute toxicity when tested in mice with more than 2.8 g/kg body weight. The nanocarrier exhibits good biocompatibility when screened out against NIH/3T3 and fresh human blood hemolysis assay. Similarly, excellent pharmacokinetic results have been obtained when given nano-suspension of Amphotericin-B loaded in nanocarrier (R-C-18) and nano-formulation of Amphotericin-B alone to the rabbits. When quantified in blood plasma, the Amphotericin-B loaded niosomal vesicular formulation of R-C-18 shows Cmax 0.325 ± 0.018 µg/mL at maximum time (Tmax.) 8 hrs as compared to Amphotericin-B formulation which gives Cmax 0.218 ± 0.016 µg/mL at Tmax 2 hrs. Therapeutic concentration of 0.3 µg/mL was also achieved with a 2 mg/kg dose of Amp-B with RSA vesicles. [58]. These results demonstrate that the newly synthesized calix(4)resorcinarene based macrocyclic supramolecular amphiphile is an excellent nanocarrier for the safe and efficient delivery of, lipophilic drug which has low oral bioavailability and permeability such as Amphotericin-B to enhancing its permeability and oral bioavailability when injected orally to the animals (rabbits).

Conflicts of interest All authors declare, they have no conflicts of interest.

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