Application of a novel pH-responsive gemini surfactant for delivery of curcumin molecules

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Abstract

During the last few decades, extensive studies have been conducted to elucidate the anti-cancer effects of curcumin. Despite promising results indicating curcumin could impede cancer cells ability thrive and proliferation, clinical applications of it have been limited. This limitation is mainly due to low solubility, poor bioavailability, rapid metabolism, and deficient absorption. To improve the physiochemical properties of curcumin, we have synthesized a novel biodegradable gemini surfactant in which curcumin molecules were entrapped. Gemini surfactant-curcumin nanocapsules were prepared using nanoprecipitation method and characterized by several techniques including, DLS, TEM, AFM, FTIR, DSC and XRD. The in vitro MTT assay, cellular uptake, and apoptosis assay were performed using MDA-MB-231 cell line. The gemini surfactant molecules were able to form vesicles in aqueous solution with a narrow size distribution (PDI ≅ 0.3). An encapsulation efficiency of 87.45 ± 2.3% and the drug loading content of 4.98 ± 0.12% were acquired. Curcumin molecules were dispersed in the hydrophobic shell of the vesicles, and sustained release profile was observed. Due to the increased cellular uptake and sustained release profile, the gemini surfactant-curcumin nanocapsules exhibited higher cytotoxicity and enhanced apoptosis in MDA-MB-231 cells compared to free curcumin. The results indicate that gemini surfactant-curcumin complex shows considerable promise as an anti-breast cancer drug.

Abbreviations

GS Gemini surfactant
CC Curcumin
GS-CC Gemini surfactant- curcumin
DHDQA Dihydroxy diquaternary ammonium
IPDI Isophorone diisocyanate
SASA solvent accessible surface area

Introduction

Cancer is defined as a swift formation of abnormal cells that multiply beyond their usual boundaries and spread across the body, hence invade other organs [1]. Cancer is one of the leading causes of mortality around the world. Therefore, the strategy of cancer treatment has received a lot of attention in recent decades.
Chemotherapy is still one of the principal approaches in treating different types of cancers. However, the application of chemical substances is limited by several factors, including the requirement for high concentrations of the cytotoxic drug, lack of selectivity, drug resistance, and undesired side effects [2, 3]. Consequently, the use of natural and herbal remedies, which in comparison to synthetic agents are mainly nontoxic and inexpensive, is frequently considered [4]. Curcumin is one of these natural, anti-cancer drugs which has manifested significant anti-inflammatory, anticancer and antioxidant effects both in vitro and in vivo [4, 5]. Curcumin is the main polyphenolic curcuminoid compound found in turmeric. Turmeric is derived from the rhizomes of Curcuma longa, a member of the ginger family that is traditionally used in many Asian countries as a spice, food colouring agent, nutritional supplement and herbal medicine [6, 7]. Curcumin is responsible for the light yellow colour of turmeric and is often considered as its most biologically active constituent [7]. Despite many medicinal properties of curcumin, its use has not yet become widespread. This is mainly due to its low aqueous solubility, poor absorption, low bioavailability, extensive metabolism and rapid elimination [5, 6, 8, 9].

The employment of nano-drug delivery systems has allured the attention of many researchers to conquer the physiochemical limitations of curcumin [10]. Lipid-based and polymer-based nanocarriers are among the most favoured nano-systems approved as carriers of anti-cancer drugs to be implemented in clinical trials [6, 11]. Tahmasebi Mirgani et al have reported curcumin nano-formulation based on dendrosomes (branched-polymeric micelles) that can down-regulate pluriotency genes in U87MG glioblastoma cells [12]. Furthermore, Erfani et al [13] have revealed that diblock copolymer-based micelles can be considered for delivery of curcumin molecules to the cancer cells [13].

Here, an mPEG urethane gemini surfactant was synthesised and its viability as a curcumin delivery vehicle was examined. This amphiphilic gemini surfactant (GS) is biodegradable, made up of mPEG2000, urethane, quaternary amine, and a hydrocarbon linker [14]. The gemini surfactant molecules can self-assemble and form vesicles in aqueous solutions in which the mPEG homopolymers stay in contact with water molecules forming a PEG brush on vesicle surface [15]. The existence of PEG molecules on the vesicle surface induces ‘stealth’ characteristics which avert the interaction with blood components, including opsonins [15–17]. The GS vesicles are capable of solubilising the curcumin in aqueous solutions and shielding the curcumin molecules against degradation.

In this study, the GS nanocapsules were synthesised and the chemical structure was verified by Nuclear magnetic resonance (NMR) and Gel permeation chromatography (GPC). Then, the curcumin was encapsulated within GS particles, and the encapsulation efficiency and drug loading content were investigated. Different characteristics of nanocapsules were studied, such as the solubility of curcumin, stability of gemini surfactant-curcumin (GS-CC) particles, in vitro release kinetics, cellular uptake, MTT assay and apoptosis.

Methods and materials

Materials

Human breast cancer cell line, MDA-MB-231, was acquired [7] and cultured in RPMI 1640 Medium (Gibco®, USA). The medium was complemented with 10% (v/v) FBS (Fetal Bovine Serum), 1% L-glutamine and 1% penicillin/streptomycin.

Fetal Bovine Serum (FBS), L-glutamine and penicillin/streptomycin were purchased from Thermo Fisher Scientific (USA). The Annexin-V-FLUOS staining kit was purchased from Roche Holding AG (Basel, Switzerland).

Curcumin and pyrene were purchased from EMD Millipore (Billerica, MA, USA). Poly (ethylene glycol) methyl ether (M<sub>w</sub> 2,000) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 1, 4-dibromobutane, 2-(dimethylamino)-ethanol, isophorone disocyanate, isopropyl alcohol, and acetone were obtained from Merck & Co (New York, USA).

Methods

General procedure for synthesis of gemini surfactant [14]

-Synthesis of dihydroxy diquaternary ammonium salt

A combination of 1.78 g of 2-dimethylamino ethanol (0.02 mole) and 2.15 g of 1,4-dibromobutane (0.01 mole) were mixed in 30 ml isopropyl alcohol and heated at 80 °C for 2 h, to synthesise dihydroxy diquaternary ammonium (DHDQA) salt (Supplementary Figure 1a, available online at stacks.iop.org/MRX/7/065403/mmedia). The outcome was precipitated in diethyl ether, filtered out and dried in vacuum oven at 40 °C for 4 h. An average production yield of 94% was achieved in most cases.
The GS-CC nanocapsules were characterized using various techniques including, Dynamic Light Scattering (DLS), Molecular characterization

The urethane reaction was conducted in acetone containing, dihydroxy diquaternary ammonium (0.1 mole L^{-1}), mPEG2000 (0.2 mole L^{-1}) and isophorone diisocyanate (0.2 mole L^{-1}) at 55 °C for 4 h. The resulted mPEG2000 urethane gemini surfactant was purified by precipitation in diethyl ether, dried in vacuum oven at 40 °C for 4 h, reaching a yield of ~92% which is illustrated in Supplementary Figure 1b.

Gemini surfactant characterization

The structure of gemini surfactant (GS) was verified using 1H NMR (Bruker, 500 MHz, USA) in chloroform (CDCl3). The molecular weight and polydispersity of gemini surfactant were quantified by GPC using HPLC system (YL9100, Younglin Instrument Co. Ltd, Korea) [18]. The GS sample was dissolved in tetrahydrofuran (THF) at the concentration of 0.005 g L^{-1}.

Determination of CMC

The critical micelle concentration (CMC) of gemini surfactant was determined bed on the intensity of excitation spectra of pyrene, the most hydrophobic probe [19–21]. The pyrene solution in acetone was prepared at a concentration of 6 × 10^{-6} M and divided into ten glass tubes to evaporate the acetone. Afterwards, various concentrations of gemini surfactant dissolved in PBS (0.01 M, pH 7.4) were added to each tube, vortexed vigorously and shaken overnight at 37 °C. The final concentration of pyrene in each tube was 6 × 10^{-7} M. Using PerkinElmer fluorimeter (USA), the samples were excited at 300–350 nm and emission at the wavelength of 390 nm was recorded [13]. The widths of the excitation and emission slits were set at 5 nm and 2.5 nm respectively.

Preparation of gemini surfactant-curcumin (GS-CC) nanocapsules

The vesicles of gemini surfactant-curcumin (GS-CC) were self-assembled through nanoprecipitation method [22]. Different amounts of curcumin and 100 mg of gemini surfactant were co-dissolved in 3 ml of methanol, and diluted twice in water under mild stirring conditions. The solution was then sonicated (Ultrasonic UP200H, Hielscher Ultrasonics GmbH, Germany) at an amplitude of 80%, for 1 min to reach an evenly dispersed solution. The solution was passed through a 0.22 μm syringe filter to remove excessive uacked curcumin and possible bacterial contaminations. The methanol solvent was subsequently evaporated using a rotary evaporator, and the remaining GS-CC vesicles were freeze-dried and kept at 4 °C for further experiments.

Encapsulation efficiency (EE) and drug loading (DL)

Lyophilized GS-CC vesicles were dissolved in methanol (10:1, w/v) and shaken vigorously for 5 min. The solution was sonicated by probe sonicator with an amplitude of 80% for 5 min and centrifuged at 18000 × g for 20 min. Consequently, the entrapped curcumin molecules were released and the gemini surfactant molecules were precipitated. The amounts of curcumin were measured spectrophotometrically at 425 nm by NanoDrop (NanoDrop 1000, Thermo Fisher Scientific, USA). The EE and DL were calculated according to the equations (1) and (2), respectively [13, 22].

\[
EE\% = \frac{\text{Amount of Encapsulated Curcumin}}{\text{Amount of Fed Curcumin}} \times 100
\]

\[
DL\% = \frac{\text{Curcumin}}{\text{Gemini Surfactant} + \text{Curcumin}} \times 100
\]

Molecular characterization

The GS-CC nanocapsules were characterized using various techniques including, Dynamic Light Scattering (DLS), Transmission Electron Microscope (TEM), Atomic Force Microscopy (AFM), Fourier Transform Infra-Red spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and x-ray Diffraction (XRD).

- Particle size, zeta potential and poly dispersity

S technique was utilized to determine size and zeta potential of GS-CC nanocapsules. Void gemini surfactant (GS) and GS-CC nanocapsules were prepared freshly at a concentration of 1 mg ml^{-1} and two different pH levels (7.4 and 5). Measurements were performed by DLS (Zetasizer Nano ZS, Malvern Instruments, UK) using an argon laser beam at 633 nm and in 90° scattering angle [13].

- Fourier Transform Infra-Red (FTIR)

The occurrence of chemical interactions between curcumin and gemini surfactant was evaluated by FT-IR spectroscopy. Curcumin, void gemini surfactant, and GS-CC nanocapsules were crushed with KBr, and their
resulting disks were studied by FT-IR spectroscopy (Spectrum One FT-IR Spectrometer, PerkinElmer Japan Co. Ltd) [23].

-X-ray diffraction (XRD)
The crystal structures of samples were obtained through XRD (STOE STADIVARI, GmbH, Germany) analysis under CuKα radiation (wavelength, 1.5406 Å). The scan rate was 3° < 2θ < 40° and the scan angle was set to 2° min⁻¹.

-Differential scanning calorimetry (DSC)
Thermal Analysis of free curcumin, void GS vesicles and GS-CC nanocapsules was carried out using DSC (Mettler Toledo 823e, Switzerland). Each sample was sealed in an aluminium crucible immediately before measurement. The samples were heated from 0 °C to 200 °C with a temperature speed of 10 °C min⁻¹. The thermograms were acquired under nitrogen gas flow rate of 10 ml min⁻¹.

-Atomic force microscopy (AFM)
The morphology of GS-CC complex was studied using AFM (Autoprobe, CP-Research, Veeco Inc., USA) in contact mode. A drop of GS-CC nanocapsules was placed on a clean glass slide and dried under a gentle stream of air at room temperature. The sample was then mounted on the microscope scanner [23, 24].

-Transmission electron microscopy (TEM)
The morphology of GS-CC capsules was also observed by Transmission Electron Microscopy (Philips CM30 microscope, Netherlands). The vesicles were diluted with distilled water and mildly sonicated for 15 min. Then the sample was placed on a grid.

Photophysical properties
Curcumin exhibits auto-fluorescence activity at an excitation wavelength of 425 nm. Accordingly, the spectroscopic analysis was performed to study the possible repercussions of curcumin encapsulation [19]. The solutions of free curcumin and GS-CC nanocapsules in methanol: water (50:50, v/v) were prepared at a concentration of 100 μg ml⁻¹. The absorbance of samples was measured at 425 nm by NanoDrop, and the fluorescence emission spectra were recorded at 450–700 nm [23].

The stability
The alteration in the physical stability of GS-CC nanocapsules was tracked and appraised by the naked eye. The aqueous solution of GS-CC nanocapsules was kept at 4 °C for a period of nine months. The occurrence of any precipitation is due to the release/leakage of curcumin from its container and could be considered a weakness in the stability of delivery vehicle [22, 25].

Computer simulation
The structure of gemini surfactant was built in HyperChem software [26] (HyperChem Pro 7.5, HyperCube, Inc., USA) and was optimized at the level of molecular mechanic with OPLS method [22, 27]. Thereupon, a progression of molecular dynamics was run as follows: First, the structure was heated from 0 K to 600 K and simulated at 600 K for 100 ps. The obtained structure was then cooled to 300 K and the simulation was run for another 100 ps [22, 28]. On the other hand, the curcumin structure was obtained from PDB with the code of 4PMF [29]. Since the structures of both GS and CC molecules were available, it was possible to study their interactions, combined structure, and conformation with the help of AutoDock Vina.

In vitro release study
The release kinetics of curcumin from GS-CC nanocapsules was discerned by the direct dispersion method [25, 30]. The experiment was conducted at two pH levels (7.4 and 5), each for 72 h [31]. The lyophilized GS-CC nanocapsules were dissolved in phosphate buffer (100:15; w/v) and individually placed into Eppendorf tubes (500 μl each). The experiments were performed in triplicate [23]. The tubes were placed into a thermostable water bath shaker operating at 37 °C and 100 rpm. Since curcumin is completely insoluble in water, one set of micro tubes were taken out at predetermined time intervals, and centrifuged at 10,000 × g for 10 min to separate the released drug from the complex. The pellet curcumin was re-dissolved in 500 μl methanol and the amount of released curcumin was measured at 425 nm using a NanoDrop Spectrophotometer.
In vitro cytotoxicity
The cytotoxicity of free curcumin, GS and GS-CC nanocapsules was evaluated by MTT assay. The MDA-MB-231 cells were seeded at the density of 1 × 10^4 in 96-well plates and kept in an incubator at 37 °C and 5% CO₂. After 24 h, the cells were treated with various concentrations of free CC, GS, and GS-CC nanocapsules in PBS (100 μM, pH 7.4). The curcumin was dissolved in PBS with the aid of methanol (< 0.4% v/v) [13]. At definite time intervals, the media were removed and supplemented with fresh media containing MTT solution (5 mg ml⁻¹; w/v). The cells were incubated further for 4 h, and then their media was replaced by DMSO (150 μl). The absorbance of formazan was measured at 490 nm using an ELISA reader (ELx800 Absorbance Reader, BioTek Instruments, Inc., USA). The measurements were performed in quadruplicate, and the results are presented as mean ± SD.

Cellular uptake analysis
The MDA-MB-231 cells were seeded using RPMI 1640 media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin at the density of 1 × 10^5 in 6-well plate and were incubated for 24 h (37°/5% CO₂). The cells were then incubated with 100 μM of void GS, free curcumin, and GS-CC, separately. After 3 h of incubation at 37 °C, the media was gently removed and cells were washed twice with PBS to omit excess nanocapsules or curcumin. The samples were examined by fluorescence microscopy at the excitation wavelength of curcumin (425 nm) using a blue filter (Inverted Microscope IX81, Olympus Corporation, Japan). Separately, after 3 h of incubating the cells with 50 μM of free CC, GS, and GS-CC nanocapsules, the cells were trypsinized and collected. The suspensions were centrifuged at 1500 rpm for 3 min at 4 °C. The pellets gently were rinsed twice with PBS and re-suspended in 500 μl PBS [32]. The cellular uptake of curcumin was measured at 488 nm by flow cytometry (BD FACSCanto™ II; BD Biosciences, San Jose, CA, USA). The results were evaluated using FCS express 5 software (De Novo Software, LA, USA).

Capoptosis assay
The flow cytometric assay was conducted using Annexin-V-FLUOS and PI staining kits (Hoffman-La Roche Ltd, Switzerland) to determine the apoptotic index for free curcumin, GS, and GS-CC treated cells. The cells were seeded at the density of 1 × 10⁶ in 6-well plate and incubated for 24 h at 37 °C and 5% CO₂. They were then treated with the desired concentration of curcumin, GS, and GS-CC and incubated for additional 24 h at 37 °C. In the next step, the cells were trypsinized and centrifuged at 1300 rpm, at 5 °C for 6 min. The pellets were rinsed with PBS and centrifuged again with the same procedure. The cell pellets were suspended in a solution containing Annexin, PI and Binding Buffer. The resulting samples were analysed by FCS express 5 software.

Statistical analysis
The experiments were performed in triplicate unless otherwise indicated. The data are reported as mean ± SD.

Results
Gemini surfactant characterization
The ¹H-NMR spectrum of mPEG2000 urethane gemini surfactant (GS) is shown in figure 1(a) [14]. The broad resonance peak at δ = 3.5–3.8 ppm was attributed to the methylene protons of the PEG group and also the protons of end methyl groups. The intense peak of 'e' was related to the CH₃ protons of dihydroxy diquaternary ammonium. The methylene protons in a spacer region were detected in signal ‘c’ at δ = 1.64 ppm, whereas a peak was shifted to δ = 2.8 ppm due to the bonding of CH₂ to the nitrogen atoms and illustrated in a peak ‘d’. The peak ‘j’ was related to methylene protons connected to the oxygen atoms which can be taken as a proof of the successful synthesis of DHDQA-IPDI (dihydroxy diquaternary ammonium-isophorone diisocyanate). The protons of the urethane group of IPDI were observed at δ = 7.2 ppm. Peaks ‘a’ and ‘b’ were associated with methyl and methylene groups of IPDI, respectively. GPC results revealed that the polydispersity index of gemini surfactant was 1.01 and the Mₙ value was 7.8 kg mole⁻¹ (figure 1(b)).

Determination of CMC
The shift in fluorescence excitation spectra of pyrene was monitored to obtain the CMC of gemini surfactant. Since pyrene has self-quenching characteristics in a polar solution, the sharp fluorescence peak is observed by locating the pyrene in a nonpolar environment. In this research, the fluorescence intensity of pyrene as a result of increasing the GS concentration was studied, and the fluorescent spectrum of pyrene alone in a polar environment was quantified as a control.

The transfer of pyrene from an aqueous solution to a less polar environment of GS vesicles caused a shift in the excitation spectrum from 333 to 336 nm (Supplementary Figure 2a). The intensity of I(336/333) is almost unaffected at the lower concentrations of gemini surfactant, while by increasing the concentration to the higher amounts, the
intensity increases exponentially. The CMC of vesicles was determined from a crossover point in Supplementary Figure 2b, and the CMC value was 15.09 μM (0.12 mg ml⁻¹). The CMC value of gemini surfactant is lower than that of low-molecular-weight surfactants (e.g., 2.3 mg ml⁻¹ for sodium dodecyl sulphate in water), which suggests that a GS carrier is thermodynamically more stable and might prolong circulation time in blood [13, 19].

Encapsulation Efficiency (EE) and Drug loading (DL)
Curcumin was successfully loaded into gemini surfactant nanocapsules by the nano-precipitation method. Various concentrations of curcumin were loaded into 100 mg of nano-carrier to optimize encapsulation efficiency and drug loading content. The highest drug-loading content belongs to a sample with the ratio of 8:100 (CC: GS, w/w) but a sample with the ratio of 6:100 demonstrated more encapsulation efficiency. The sample with the ratio of 6:100 (CC: GS, w/w) had the encapsulation efficiency of 87.45 ± 2.3% and the drug loading amount of 4.98 ± 0.12%. Hence, considering EE and DL, the sample with the ratio of 6:100 (CC:GS; w/w) was chosen for further experiments. The results are represented in Supplementary Figure 3.

Molecular characterization
-Particle size, zeta potential and poly dispersity
The lyophilized GS-CC nanocapsules were dissolved in a phosphate buffer at two different pH levels (5 and 7.4), then the size and zeta potential of particles were measured by DLS. Also, freshly prepared GS vesicles were characterized by DLS. The data are represented in table 1 and Supplementary Figure 4.
The GS vesicles had a narrow size distribution with a mean hydrodynamic diameter of 181.06 ± 21.54 nm. Interestingly, the particle size in curcumin-loaded samples was decreased in compared to void vesicles, and a 40 nm-reduction of diameter was observed. Considering these data, GS particles may be assembled into tighter structures in the presence of curcumin. Even more important is the enlargement of particles at pH 5 occurred as a result of rapid swelling. As shown in table 1, the zeta potentials for GS, GS-CC pH 7.4 and GS-CC pH 5 were determined 0.265, 1.69 and 1.17 mV, respectively.

Fourier transform infrared (FTIR) spectroscopy
FTIR analysis of free CC, GS and GS-CC nanocapsules was performed to investigate the presence of curcumin in GS-CC nanocapsules. The FTIR spectra are shown in figure 2. Broad band at 3426 cm⁻¹ in free CC is an evidence of O-H stretch of phenolic groups [33]. In GS-CC nanocapsules a 3426 cm⁻¹ peak is shifted to 3421 cm⁻¹ and the peak is widening which indicates the improvement in hydrogen bonding [23, 33]. The O–H stretch of the carboxylic acid of gemini surfactant is manifested through a medium peak at 2888 cm⁻¹ in both the GS and GS-CC samples. The existence of the carboxylic acid group and C=O absorption can also be verified by the existence of a peak at 1720 cm⁻¹ [34]. A sharp peak at 1110 cm⁻¹ demonstrates the C–N stretch aliphatic amine in the void and drug-loaded surfactant. Two characteristic peaks of curcumin are observed at 1600 cm⁻¹ and 1628 cm⁻¹ as a result of the stretching vibration of C–C in the ring and C=C alkene group, respectively [23, 34]. Since the vibration of surfactant in the range of 1470–1720 cm⁻¹ is masked these two main bands of curcumin, we could not conclude the drug-surfactant complex formation. A peak at 1511 cm⁻¹ in free curcumin was observed due to C–H bending and C=O stretching vibrations [33]. A shift from 1511 cm⁻¹ to 1562 cm⁻¹ in GS-CC nanocapsules, could be considered as an evidence of complex formation.

XRD and DSC
The XRD diffraction patterns of GS, CC and GS-CC samples were obtained to study the crystallinity of the samples, and the graphs are displayed in figure 3(a). Free curcumin had sharp characteristic peaks at 2θ = 8.82°, 17.22°, 23.34°, 24.48° and 25.5° indicating the high crystalline structure of curcumin. The GS specific peaks were observed at 2θ = 19.14° and 23.34°. In the XRD pattern of curcumin-loaded GS, none of the curcumin characteristic peaks can be observed and furthermore the intensities of GS specific peaks were diminished.

Table 1. Properties of GS and GS-CC nanocapsule.

| Samples       | Particle size (d.nm) | Zeta potential (mV) | Polydispersity Index (PdI) |
|---------------|----------------------|---------------------|---------------------------|
| GS, pH 7.4    | 181.06 ± 21.54       | 0.265               | 0.293 ± 0.02              |
| GS-CC, pH 7.4 | 142.95 ± 27.75       | 1.69                | 0.386 ± 0.09              |
| GS-CC, pH 5   | 166.2 ± 30.9         | 1.17                | 0.397 ± 0.15              |

Figure 2. FTIR spectra of (a) free CC (b) void GS (c) GS-CC nanocapsules.
Moreover, the peak at $2\theta = 31.74^\circ$ in GS-CC nanocapsules appeared due to the formation of new crystalline structure. The absence of curcumin peaks in GS-CC vesicles is an evidence that curcumin crystals did not exist in GS-CC nanocapsules, and instead the curcumin existed in an amorphous state or a disordered crystalline phase in the matrix [35]. The sustained release of curcumin from the vesicle matrix happens as a result of this amorphous state or disordered crystalline phase of the curcumin [23]. Since large molecules are not able to diffuse from the nanoscopic pore of the vesicle, the change of the curcumin structure into the amorphous state prevents its release.

Moreover, the crystallinity as well as physical state of drug and surfactant was examined by the DSC thermal analysis technique (figure 3(b)). Free curcumin manifested an endothermic melting peak at 187.5 °C due to its crystalline structure [36]. The surfactant vesicles showed an endothermic peak at 56.7 °C. Therefore, the disappearance of the curcumin characteristic peak in the GS-CC nanocapsule sample is a proof of the incorporation of the drug into the disordered-crystalline or amorphous phase [37]. It can be inferred that the drug loading process did not affect the surfactant structure, since both the GS and GS-CC samples had almost the same thermal decomposition values [37].

- Atomic force microscopy (AFM)

The curcumin-loaded gemini surfactant vesicles were analysed and imaged using contact mode AFM. The lyophilized GS-CC vesicles were re-dissolved at two different concentrations (above and below CMC) on a glass slide. As shown in figure 4, the vesicles tended to aggregate in the concentration above CMC and formed larger vesicles with an average width of 750 nm. The median height obtained by AFM was about 135 nm, slightly smaller than that measured by DLS which is ascribed to the difference in the mode of measurement of the two techniques [38].

Upon diluting the sample, small aggregates of vesicles were observed with an average width of ~220 nm and an average height of ~30 nm. Therefore, the average diameter to height ratio is 7.3 which is in accordance with the values reported by the other researchers [39–41]. The ratio of 7.3 indicates the formation of spherical 'cap' structures by surface confined vesicles [41]. The attractive interaction between surface and substrate and also the pressure caused by the tip of the AFM instrument, resulted in deformation and spreading of the vesicles [40, 41]. Moreover, the flattened structure of the vesicles was revealed in the cross-sectional profile with the roughness ($R_{\text{q}}$, root-mean-square roughness) of 7 nm. The GS vesicles are mechanically stable enough not to fracture which may also lead to longer circulation time in the bloodstream [42].
- **Transmission electron microscopy (TEM)**

The Transmission Electron Microscopy technique was applied to confirm the morphology of a single vesicle. The picture is illustrated in figure 5, and the core/shell spherical structure was revealed. The hydrophobic membrane can be seen in light grey, whereas the inner core, which possibly corresponds to the existence of water, is displayed in dark grey.

- **Photophysical properties**

The photophysical properties of encapsulated curcumin in comparison to free curcumin were studied (figure 6). The methanol: water (50:50, v/v) solutions of both GS-CC and free CC were prepared. The absorption spectrum of free CC showed a sharp peak at 425 nm and also the GS-CC spectrum displayed similar trends with less absorptivity. As a result of fluorescence excitation at a wavelength of 425 nm, free CC sample showed a fluorescence emission peak at 530.5 nm. The fluorescence emission spectrum of GS-CC samples had two noteworthy features, the peak was shifted to 529.5 nm and its intensity was increased. The increase in the emission intensity of curcumin along with the observed blue shift of the maximum wavelength, indicate that the entrapment of the curcumin by the hydrophobic core of the vesicles successfully occurred, and these novel nano-carriers did not impact the photophysical properties of curcumin. It is known that the linearity relation

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**Figure 4.** AFM contact-mode image of GS-CC nanocapsules in aqueous solution (a) At concentration 10 times lower than CMC. (b) At concentration above CMC. (c) Cross section of vesicles along the red line presented in part (a).
between fluorescence intensity and concentration is true for concentrations up to $10^{-6}M$ [43]. In free CC sample, the concentration of curcumin molecules is too high, hence the incident beam could not pass through the sample and generate sufficient excitation [43].
The stability

The re-dissolved lyophilized GS-CC nanocapsules at a concentration of 1.7 mg ml\(^{-1}\) were kept at 4 °C for a period of nine months and the presence of any aggregates were counted as instability. As it is presented in figure 7, the solution is transparent and no lumps could be detected by naked eyes.

Computer simulation

A simulation of the GS molecule was built and optimized in the HyperChem software. The GS simulated molecule was heated from 0 k to 600 k, and then held at that temperature for 100 ps. The initially optimized conformation had a curve-like structure, but during the heating stage, the surfactant changed to a globular shape (figures 8(b), (c))\(^{[28]}\). Since water molecules were used as the solvent during the whole process of simulation, the mPEG molecules favoured exposing themselves to the solvent and increasing their solvent accessible surface area (SASA). On the other hand, the isophorone diisocyanates (IPDIs) tended to decrease their SASA. Therefore, the mPEG molecules tried to cover the IPDIs.

To investigate the possible location of curcumin in the GS vesicles, conformations of mPEG and the remainder of the GS molecules (DHDQA + IPDIs) were simulated, and the binding affinity between curcumin and mPEG or the remainder was assessed. The structure of both mPEG and the remainder is illustrated in figures 8(d), (e). As was expected, the binding affinity between curcumin and the remainder was intense with the value of \(-16.31\) kJ mole\(^{-1}\) and the binding affinity between curcumin and mPEG was \(-12.97\) kJ mole\(^{-1}\) which is the same with the value reported by Gou et al\(^{[22]}\). Also, it is concluded that curcumin is mainly located in the hydrophobic part of the GS molecule because the binding affinity between the GS and curcumin is close to that of the remainder and curcumin \((-17.99\) kJ mole\(^{-1}\) versus \(-16.31\) kJ mole\(^{-1}\)). At the end of the MD simulation, the SASA value for the GS surfactant was 3197.69 Å\(^{2}\) whilst the value reduced to 3112.86 Å\(^{2}\) after docking. It is obvious that the GS adapted a conformation to protect the curcumin molecules from the polar solvent.

In vitro release study

The release profile of curcumin was examined via the direct dispersion method at pH 7.4 and 5\(^{[31]}\). The release profile was investigated for a period of 3 days. The amounts of drug released over the entire period of study at pH 5 and 7.4 were only 24% and 15%, respectively. As depicted in figure 9, the initial burst release was observed in the first 4 h, which was mainly due to dissociation of surface-absorbed curcumin from the hydrophobic shell of the vesicles\(^{[23, 31]}\). Following the fast release period, the diffusion-controlled release was noticeable. Since the drug was completely incorporated into the hydrophobic shell of the vesicles, sustained release of curcumin occurred.

In the acidic environment, the amine group of gemini surfactant was protonated, so the vesicle matrix swelled up and as a result, the release rate in pH 5 is higher than that in a neutral medium\(^{[31, 44]}\). The tumour microenvironment is acidic due to increased glucose metabolism, hypoxia, and deficient blood perfusion\(^{[45, 46]}\). Thus, the encapsulated curcumin could be released more rapidly at the site of cancerous cells which also reduced the exposure of normal tissue to the anti-cancer drug\(^{[47]}\).

In vitro cytotoxicity

The MTT assay was accomplished to test the fatality of our drug delivery system. The viability of MDA-MB-231 cells versus various concentrations of GS, CC and GS-CC is graphed in Supplementary Figure 5. The GS

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![Figure 7. Stability study of GS-CC nanocapsules after nine months’ storage (concentration of 1.7 mg ml\(^{-1}\)).](image)
Figure 8. Computer simulation studies of gemini surfactant and curcumin. (a) The conformation of GS-CC complex. (b) Optimized structure of GS molecule in HyperChem. (c) The structure of GS molecule after MD simulation. (d) The conformation of mPEG. (e) The conformation of the remainder. Curcumin is represented in yellow stick rendering. The GS is colored by polarity. Atom coloring: grey, carbon; red, oxygen; blue, nitrogen.

Figure 9. In vitro release kinetics of GS-CC nanocapsules (at two different pH levels). Reproduced with permission from [14].
nanocapsules did not prohibit cell growth even after 48 h, and the cell viability was $95 \pm 1.79\%$ at a concentration of 70 $\mu$M. The half maximal inhibitory concentration (IC$_{50}$) of free curcumin and the GS-CC nanocapsules were quantified at about 62 $\mu$M and 28 $\mu$M, respectively, following 48 h of treatment. The IC$_{50}$ value for the GS-CC nanocapsules after 24 h treatment was about 42 $\mu$M.

Notably, free curcumin at the concentration of 10 $\mu$M exhibited cell viability of 90.45 $\pm$ 1.4% and 99.8 $\pm$ 0.9% after 24 h and 48 h of treatment, respectively. The reduction of curcumin toxicity could occur as a result of curcumin metabolism in physiological pH [23]. It is evident that in the case of the GS-CC nanocapsules, increased exposure time leads to a decrease in the IC$_{50}$ value. Furthermore, the amount of curcumin required to kill 50% of cells was reduced when curcumin was entrapped within the vesicles, which is mainly due to enhanced bioavailability and cellular uptake.

Cellular uptake analysis
The cellular internalization of curcumin, owing to its intrinsic fluorescence activity, was quantitatively analysed using flow cytometry technique. The MDA-MB-231 cells were exposed to GS vesicles, free CC and GS-CC nanocapsules with concentrations mentioned in the method section.

The results revealed that encapsulation of curcumin inside gemini surfactant vesicles amplified the uptake of curcumin. The median values for free CC and GS-CC nanocapsules were 15.12 and 36.52, respectively. Thus, curcumin entrapment in a proper delivery vehicle could improve the cellular internalization by a factor of 2.4 at a concentration of 50 $\mu$M, presented in figure 10(a). Curcumin uptake is a concentration-dependent phenomenon, but in this study, the relation between curcumin uptake and concentration was not investigated [16, 23, 44].

Additionally, the fluorescence microscopic image was captured following 3 h of incubation with void GS, free CC and GS-CC nanocapsules. Strong fluorescence intensity was observed in the cells treated with GS-CC nanocapsules, which is the evidence of more efficient internalization of curcumin (figure 10(b)).

Cell apoptosis assay
In recent years, many studies have revealed that curcumin could induce apoptosis pathways in various cancerous cells [48–52]. The MDA-MB-231 cells were double stained with FITC-Annexin V and PI to explore the role of curcumin in apoptotic cell death.

The cells were treated with 60 $\mu$M of GS, CC and GS-CC nanocapsules for 24 h and the results are presented in figure 11. As shown, there was no significant difference in the number of live cells in control, and void GS treated cells. The existence of early and late apoptosis was clarified in the cell population treated with free CC and GS-CC nanocapsules. The GS-CC treated cells demonstrated a late apoptosis value of 79.6%, while this value was about 16.4% in free CC treated cells. Thus, the programmed cell death in a cell population treated with GS-CC nanocapsules was enhanced by a factor of 4.85 compared to those treated with free CC.

As a consequence, encapsulating curcumin inside gemini surfactant vesicles could be judged as a safe and efficient anti-cancer drug delivery system.

Discussion
Vesicles are one of the supramolecular structures that have many fascinating features. Vesicles have the potential to carry both hydrophobic and hydrophilic agents; they can entrap hydrophobic molecules within the membrane. The in vitro and in vivo stability of vesicles is greater than micelles [42]. In this study, mPEG2000 urethane gemini surfactant (GS) was synthesized. The existence of mPEG molecules on the surface of vesicles could enhance biocompatibility and the circulation time in blood.

Following the synthesis of GS vesicles, curcumin was loaded into the nanocapsules. Then, the interaction of curcumin with GS vesicles and its integrity was confirmed by FTIR, DSC and XRD experiments. The encapsulation efficiency as well as impact of surfactant on photophysical properties of curcumin were investigated by spectroscopic studies. The results demonstrated an encapsulation efficiency of about 87% and did not reveal a significant impact on the photophysical properties of curcumin.

The fluorescence emission of GS-CC was blue-shifted and its intensity was increased, when compared to free CC, clarifying the entrapment of curcumin in the membrane of GS vesicles. Sahu et al have previously reported 40 nm blue-shift in the fluorescence spectrum as a consequence of the encapsulation of curcumin in bovine casein micelles [53]. The higher shifted value verified entrapment of curcumin in the core of micelles. In our delivery system, the curcumin was entrapped within the membrane of vesicles, so the lower shifted value (1 nm) was observed.

One of the major challenges in the therapeutic application of curcumin is the low solubility of curcumin in aqueous solutions and its instability. In this research, the GS-CC nanocapsules were kept at 4 °C for more than
nine months and no precipitates were observed. Thus, our formulation improved the solubility of curcumin.

The release kinetics of GS-CC nanocapsules were investigated at both acidic and neutral pH. A biphasic release scheme was noticed, at either pH 5 or 7.4. The initial burst release pattern was followed by sustained release; the conserved pattern is mainly due to the entrapment of curcumin in the hydrophobic membrane of nanocapsules. As a result of the existence of urethane bonds, a faster release rate was observed at pH 5. At an acidic pH, the urethane bonds were protonated and the whole structure swelled so the entrapped curcumin could be released more quickly.

The toxicity of GS vesicles was examined, and MTT results revealed the nontoxicity of the drug carrier up to 100 μM concentrations. Also, MTT outcomes verified that the IC50 of GS-CC nanocapsules was lower than that of free CC. Such behaviour may have occurred because of the various uptake profiles of curcumin [23]. The modes of drug internalization play a fundamental role in the anti-proliferative effect of the drug. Free curcumin diffuses across the cell membrane. Therefore, as the cytoplasm become saturated, further diffusion of the drug will become impossible. Hence, all properties of curcumin can be seen within a short period of its existence inside the cytoplasm, while GS-CC nanocapsules enter cells through endocytosis which leads to having sufficient curcumin reservoirs inside cells. Then, curcumin can be released in a sustainable manner [35]. Moreover, it is

Figure 10. (a) Flow cytometric analysis of void GS (●●●●), free CC (—) and GS-CC nanocapsules (■■■■)[14]. (b) Fluorescence microscopic image of cellular uptake.
well known that the stability and intracellular preservation of drugs are directly associated with its anti-proliferative effect [23].

Previous studies have revealed that curcumin can induce apoptosis pathway in cancer cells. Our results were also showed the same for MDA-MB-231 cell line. The percentage of cells that were in the apoptosis stage was about 16.4% for the cells treated with free curcumin, and about 79.6% for the ones treated with GS-CC nanocapsules. In the GS-CC treated cells, due to better uptake, more curcumin was accumulated inside cells. Therefore, it can be released in a timely manner so a larger number of cells will undergo apoptosis.

**Conclusion**

In this research, the mPEG2000 urethane gemini surfactant (GS) were synthesized and completely characterized for the first time. The GS molecules formed nanocapsules in aqueous solutions. Moreover, curcumin as a hydrophobic anticancer agent was encapsulated in this novel nanocarrier. The straightforward encapsulation process had some benefits, including high encapsulation efficiency, pH sensitivity, narrow size distribution, improved bioavailability, well solubility and high stability.

The *in vitro* outcomes confirmed that encapsulated curcumin was more efficient in sustained release and cellular internalization, as compared to free curcumin. Also, the GS-CC nanocapsules showed a higher value of apoptosis. The GS-CC nanocapsules did not show any toxicity towards MDA-MB-231 cell line. Accordingly, the GS-CC nanocapsules are a proper delivery vehicle candidate for hydrophobic molecules, with the potential to
convey hydrophilic substances. The pH-sensitive gemini surfactant could be regarded as a promising strategy for delivering anti-cancer agents. Furthermore, these nanocapsules can be functionalized for targeted delivery, although further studies need to be performed to obtain in vivo results.

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