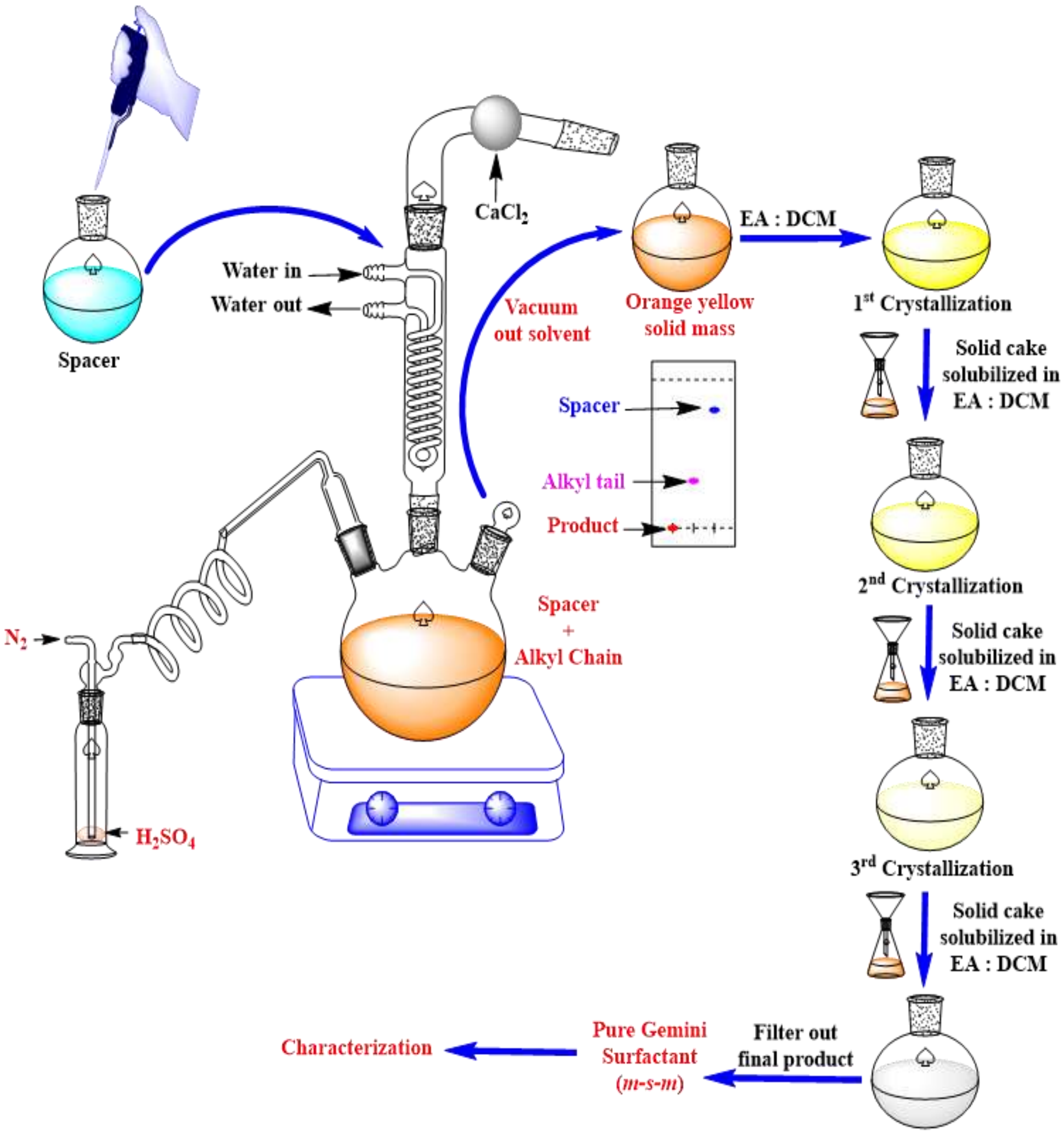
Chapter 2

Materials and Methodologies

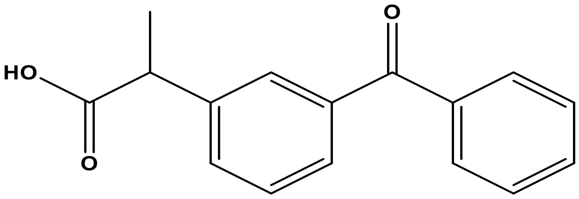


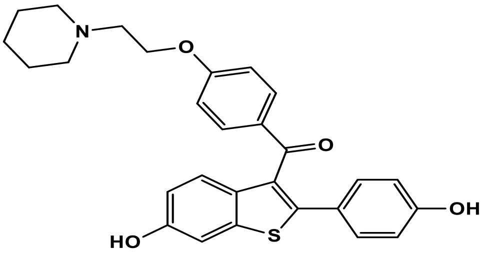
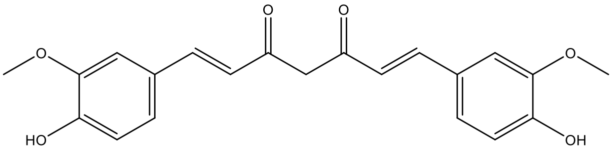
2.1. Materials

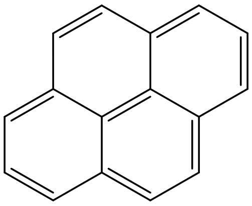
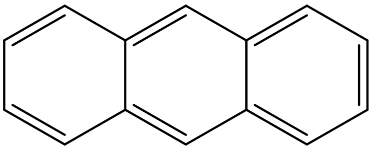
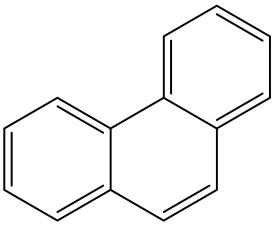
Materials (including reagents, solvents, and additives) used for various studies together with their make and purity, are compiled in Table 1. The general route of synthesis of ionic gemini surfactants is given in section 2.1.1.

Reagents Used	**Purity**	**Source**
1,4-dibromo butane	99%	Sigma Aldrich, USA
Ethylene glycol	≥ 99%	Sigma Aldrich, USA
Ethylene diamine	98%	Loba Chemie, India
Choloroacetyl chloride (CAC)	98%	Loba Chemie, India
N, N-decyldimethyl amine	> 95%	TCI Chemicals
Triethyl amine	99%	Spectrochem, India
1-dodecanol	98%	SISCO, India
Pyrophosphoric acid	≥ 90%	Sigma Aldrich, USA
Methanolic solution of tetramethyl ammonium hydroxide	25%	Sigma Aldrich, USA
Sodium metal	99.8%	Jyoti Chemicals, India
Sodium hydroxide	99.5%	Merck
Concentrated hydrochloric acid	35.4%	Loba Chemie, India

Solvents		
Dichloromethane (DCM)	≥ 99.5%	Merck
Toluene	99.5%	Spectrochem
Acetonitrile	99.5%	Spectrochem
Diethyl ether	≥ 99%	Merck
Chloroform	99%	Loba Chemie
Methanol	≥ 99%	Merck
Dry ethylacetate	99.5%	Spectrochem
Absolute alcohol	99.5%	Obtained in the laboratory by distillation and dehydration
Acetone	99%	Loba Chemie
De-ionized double distilled water	κ ~1 µS·cm-1	Freshly prepared in laboratory

Drug		
Ketoprofen  (RS)-2-(3-benzoylphenyl) propanoic acid	> 97%	Zydus cadila

Raloxifene hydrochloride  [6-hydroxy-2-(4-hydroxyphenyl)-benzothiophen-3-yl]-[4-[2-(1-piperidyl) ethoxy] phenyl]-methanone	> 97%	Zydus cadila
Curcumin  (1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione	>97%	TCI Chemicals
Dilaysis bag	-	HiMedia, India
Phosphate buffered saline, powder, pH 7.4	-	Sigma Aldrich, USA
Dimethyl Sulfoxide	Anhydrous, ≥99.9%	Sigma Aldrich, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)	98%	Sigma Aldrich, USA
Adenocarcinoma (MCF-7) human breast cancer cell	-	NCCS, Pune, India
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	>97%	TCI Chemicals

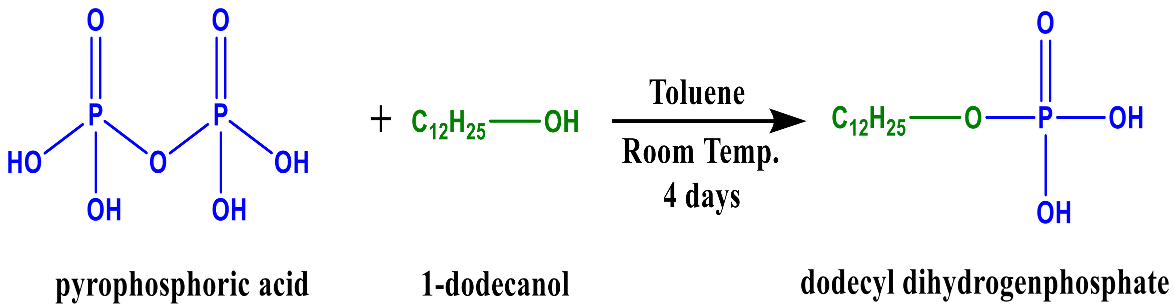
Polycyclic Aromatic Hydrocarbon (PAH)	Purity	Source
Pyrene 	> 99%	Sigma Aldrich, USA
Anthracene 	> 99%	Sigma Aldrich, USA
Phenanthrene 	> 99%	Sigma Aldrich, USA

2.1.1. Synthesis of Gemini Surfactants

Gemini surfactants, used in the study, have been synthesised and characterized based on the methodologies reported in the literature [1–3].

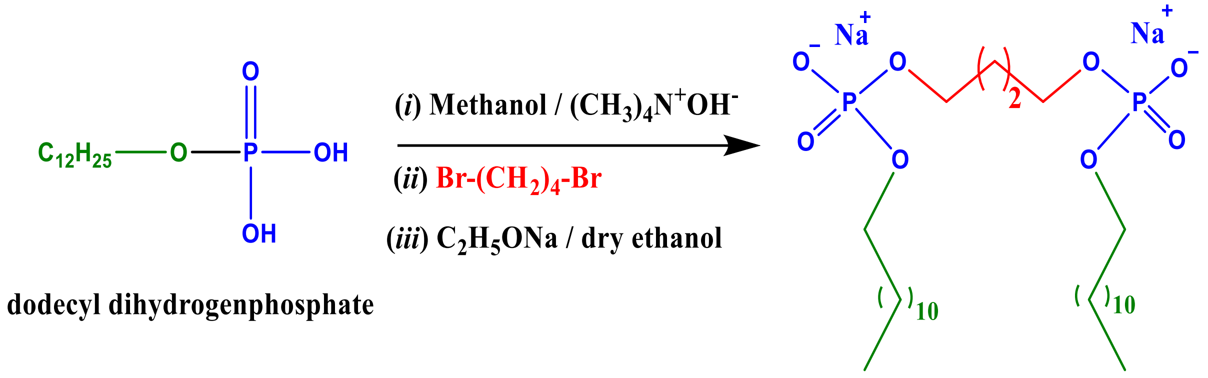
2.1.1.1. Synthesis of Anionic Gemini Surfactant

DDHP has been obtained by blending 1-dodecanol (14.91 g) and PPA (17.80 g) in 15 mL of toluene, followed by continuous stirring until a transparent mixture is resulted. This transparent mixture was kept for 96 h with proper stirring. The obtained yellowish mass mixed with 50 mL ether and followed washing with water(thrice) which results in two layers. The upper layer (ether content) was mixed with NaOH solution (1:1), followed by the rejection of ether layer. However, pH of the NaOH solution (containing product) was adjusted to acidic range by adding HCl solution. This acidic solution was extracted using ether, followed by discarding the water layer. The remaining content was washed twice with HCl. The product layer has been vacuumed to obtain DDPH as a semi-solid mass (Scheme 1).



Scheme 1. Synthesis of DDPH (dodecyl dihydrogenphosphate).

Above obtained DDPH (13.3 g) was mixed with a methanolic solution of TMAOH (0.1 mol) at ambient temperature. The methanol was removed (under reduced pressure) and the solid product was mixed with 1,4-dibromobutane, a spacer (25 mmol), in acetonitrile with 1-2 h of stirring at boiling temperature. The remaining solvent was removed (using a rota evaporator), and the resulting mass was acidified and extracted (thrice) with HCl solution and ether, respectively. The extracted layer was washed thrice with H2O. The brownish solid mass was obtained after the removal of ether and crystallised with a 40:60 ratio (v/v) of chloroform and ethyl acetate. This step resulted hydroxy form of 12-4-12A. This form was further treated with sodium ethoxide in anhydrous ethanol. Ethanol was removed, and the solid mass (12-4-12A) was vacuum dried and kept for characterization (Scheme 2).



12-4-12A

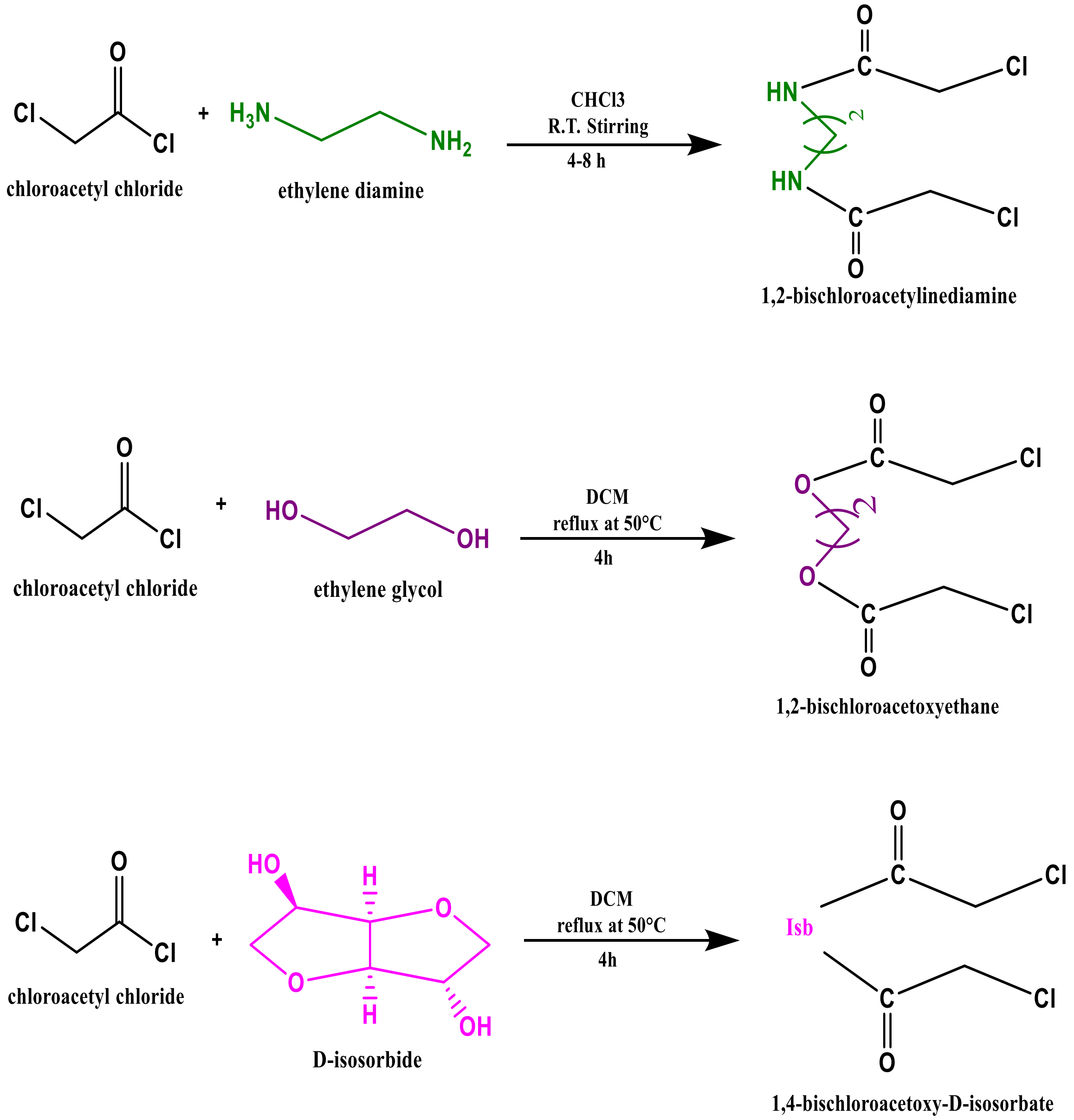
Scheme 2. Synthesis of 12-4-12A from DDPH.

2.1.1.2. Synthesis of Cationic Gemini Surfactant

Cationic gemini surfactant synthesis part can be divided into two sub-parts: (a) synthesis of spacers and (b) synthesis of cationic geminis involving above synthesised spacers in part a.

(a). Synthesis of Spacers

Ethylene diamine, ethylene glycol and D-isosorbide are mixed with CAC (in stoichiometric quantities) to obtain various spacers as depicted in Scheme 3.



Scheme 3. Synthesis of various spacers.

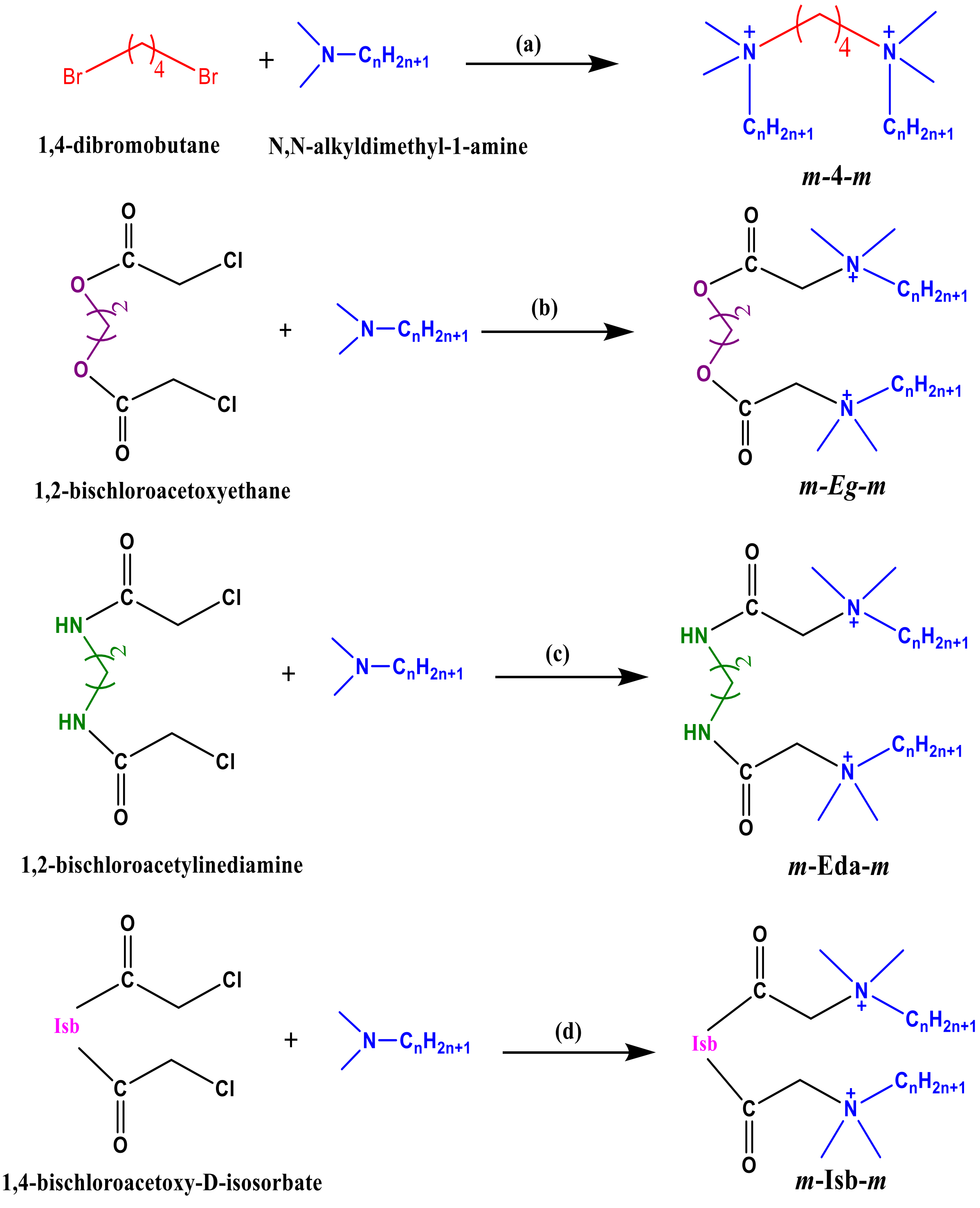
Ethylene diamine-based spacer (1,2-bischloroacetylinediamine) has been obtained by taking CAC (37 mmol) with 50 mL $CHCl_3$ followed by mixing it (drop-by-drop) with another cooled (0-5 C) mixture of ethylenediamine (180 mmol), triethylamine (540 mmol) and 50 mL $CHCl_3$ and thereafter the mixture was put for 6h stirring. CHCl3 has been vacuum evaporated to obtain solid product which was further recrystallized thrice with anhydrous ethanol resulting in pure 1,2-bischloroacetylinediamine.

1,2-bischloroacetoxyethane and 1,4-bischloroacetoxy-Disosorbate were synthesized by the reported method [4]. CAC (190 mmol) was taken in 50 mL DCM and gradually added into solution of 90 mmol ethylene glycol (or 70 mmol D-isosorbide) in 25 mL DCM under inert atmosphere (between 0-5 C). The resulted mixture was stirred for 4h at 50 C and then neutralized with aqueous sodium bicarbonate followed by washing with concentrated brine (NaCl). The solvent has been vacuumed evaporated and resulted mass was crystallized below 10 C. The procedure gives 1,2-bischloroacetoxyethane and 1,4-bischloroacetoxy-Disosorbate.

(b). Synthesis of Cationic Geminis

Cationic geminis were synthesized (Scheme 4) with both polymethylene and bio-based spacers (as synthesized in Scheme 3) by well-known reported procedures [5–9]. Polymethylene spacer based geminis were obtained by mixing 1,4-dibromobutane (20 mmol) with respective quaternary amines (41 mmol) in anhydrous ethylacetate-DCM mixed solvents followed by stirring in inert atmosphere at refluxing temperature (24h).

Similar procedure was adopted to obtain 1,2-bischloroacetoxyethane and 1,4-bischloroacetoxy-D-isosorbate based geminis under similar reaction conditions. However, for 1,2-bischloroacetylinediamine spacer anhydrous ethanol has been used as solvent. Completion of reaction was detected by absence of spacer spot (using TLC method). Crude geminis were obtained by vacuum evaporation and further used for recrystallization with ethylacetate-DCM mixed solvent. The recrystallized material of respective gemini appears as shiny white amorphous mass.



Scheme 4. Synthesis of cationic gemini surfactants: (a,b & d) ethylacetate : DCM (8:2), reflux in inert atmosphere, 24 h; (c) ethanol, reflux in inert atmosphere, 24h. (where m = 14,12 or 10)

2.1.2. *Characterization of Synthesized Gemini Surfactants*

Gemini surfactants synthesized in section 2.1.1. are characterized by usual techniques such as FT-IR, NMR, TGA etc. The characterization data were compared with earlier studies [10].

2.1.2.1. Characterization of 12-4-12A

12-4-12A has been characterized first with FT-IR spectroscopy and the spectrum is shown in Figure 1.

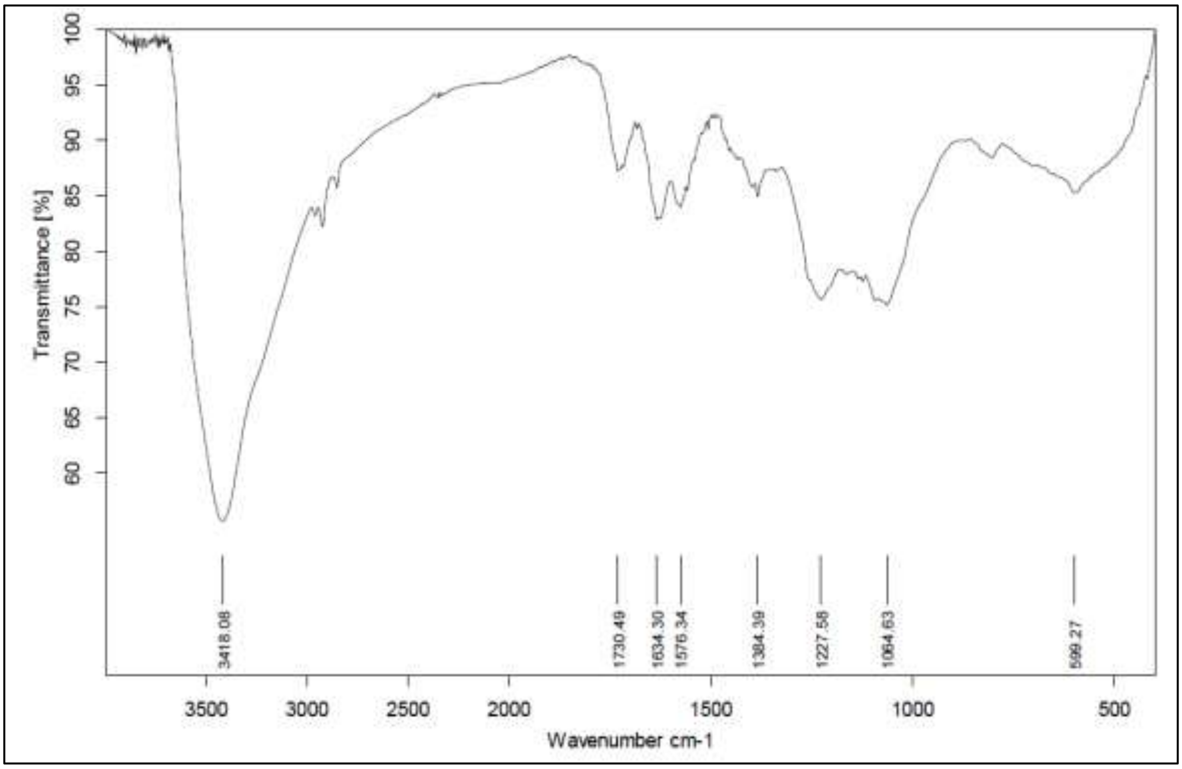


Figure 1. FT-IR spectra of 12-4-12A

12-4-12A was further characterized by ^{1}H-NMR (Figure 2). Signals (δ ppm) are found matching with the literature data [11].

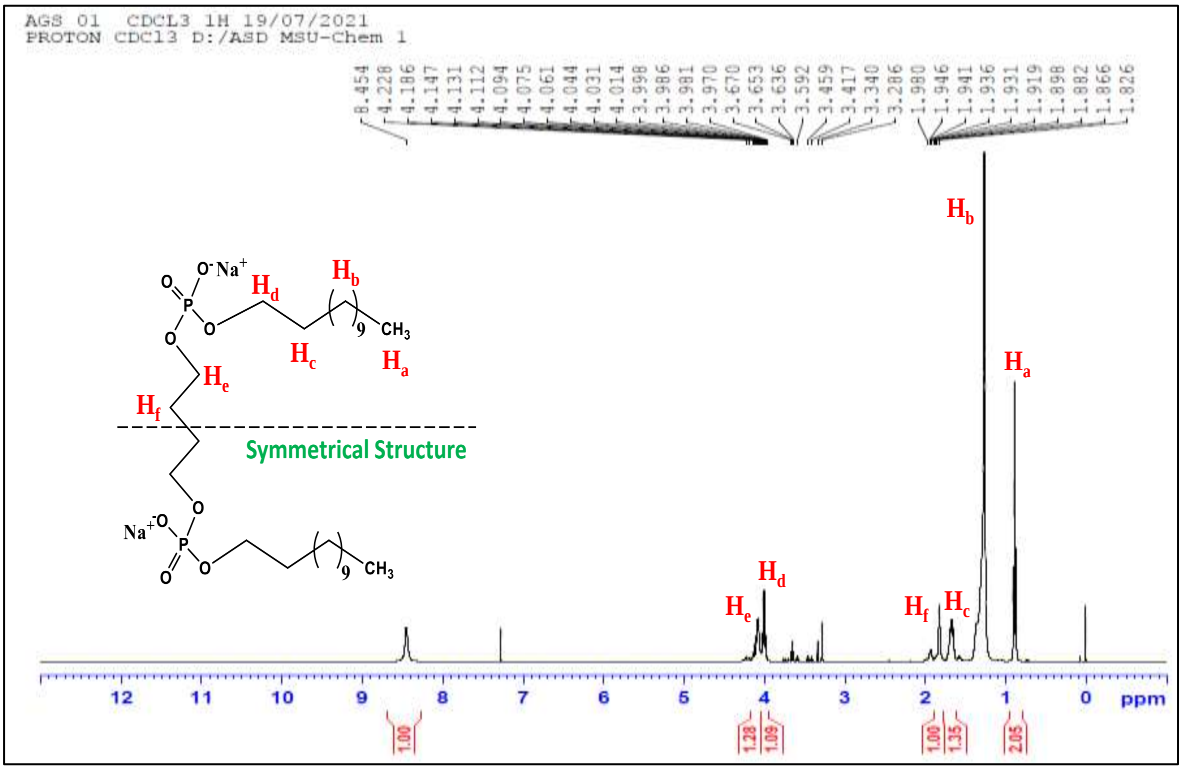


Figure 2. ^{1}H NMR spectra of 12-4-12A

2.1.2.2. Characterization of Cationic Gemini

Typical FT-IR and NMR spectra for various cationic gemini surfactants are provided in Figures (3-10), respectively. Spectroscopic data are well matched with corresponding geminis of higher homologous chain [12].

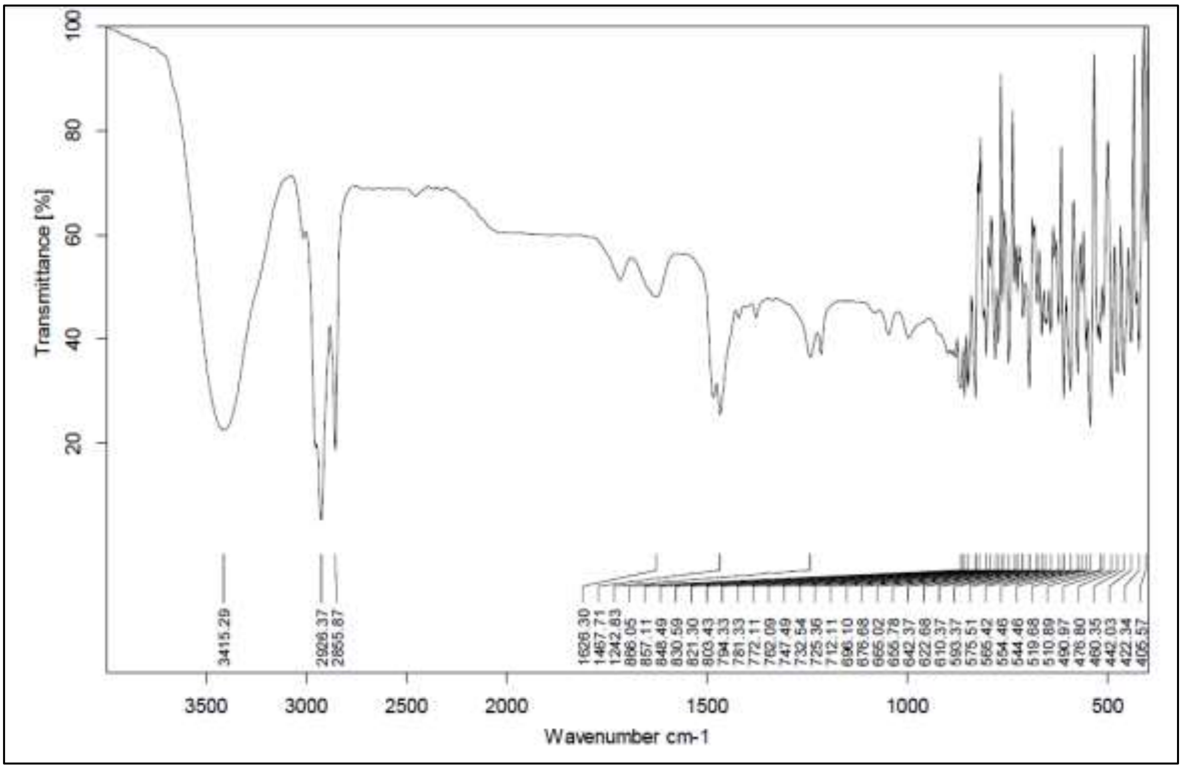


Figure 3. FT-IR spectra of 10-4-10

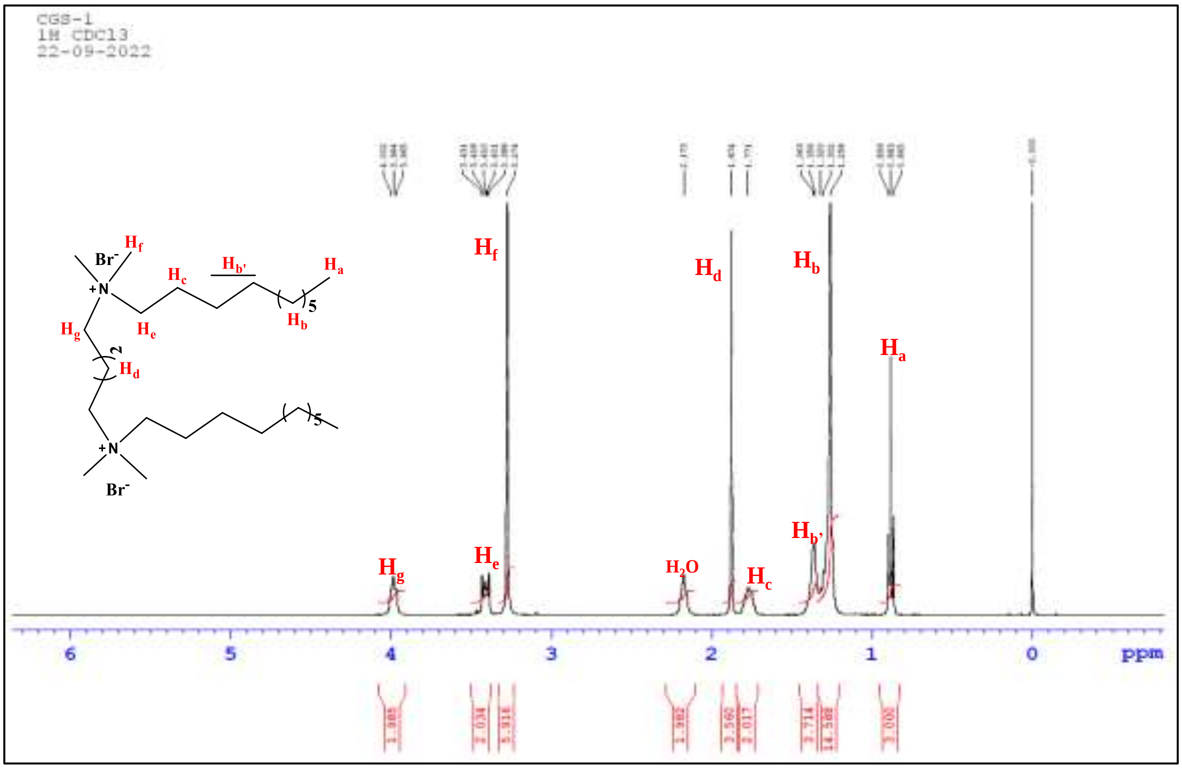


Figure 4. ^{1}H-NMR of 10-4-10

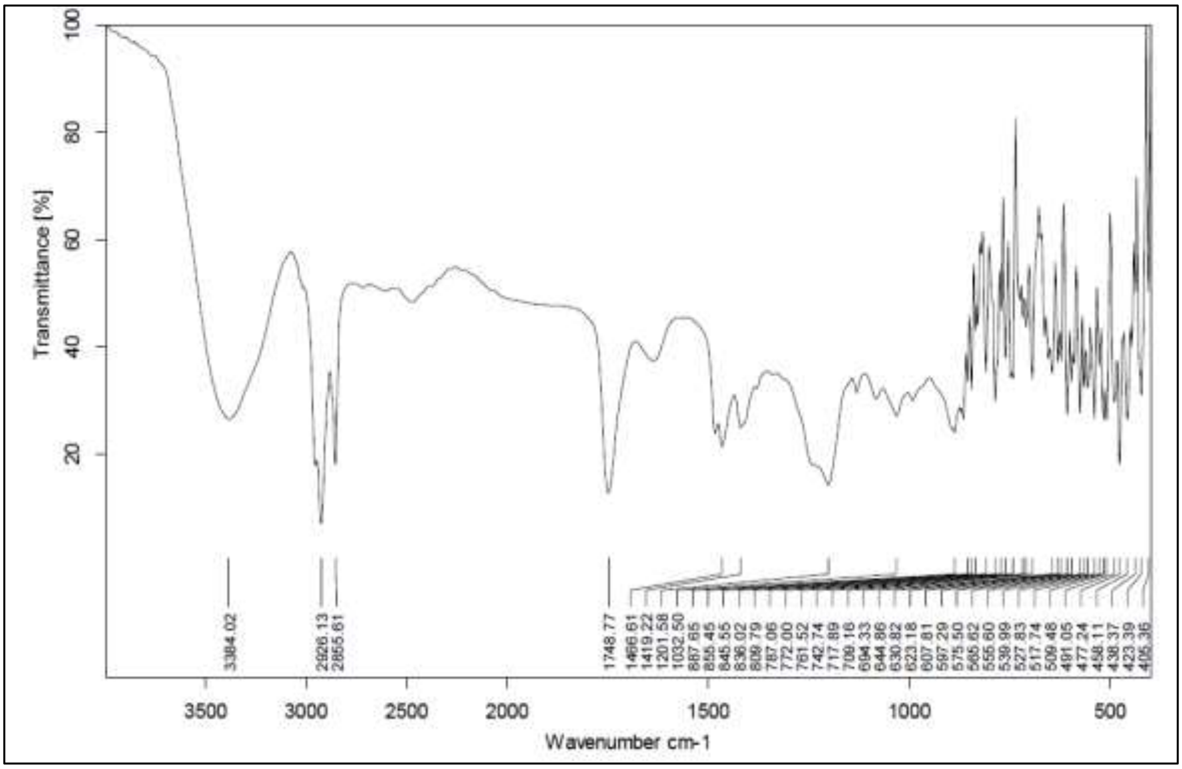


Figure 5. FT-IR spectra of 10-Eg-10

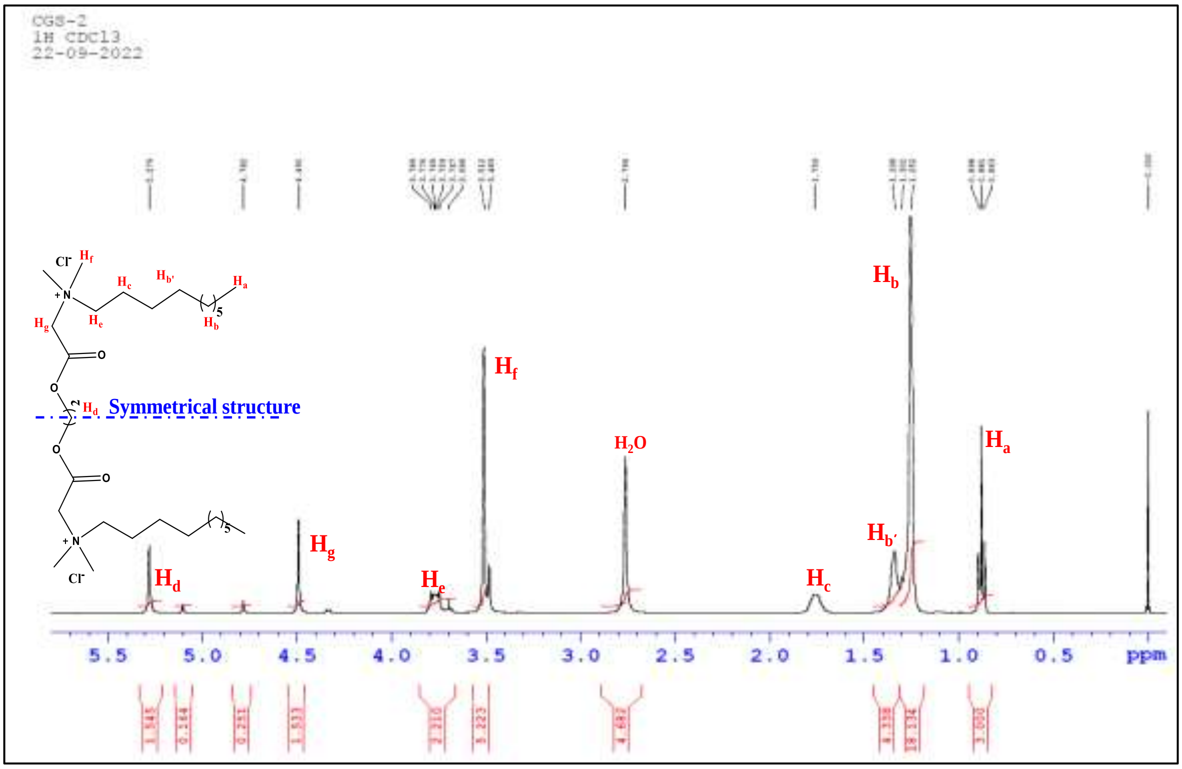


Figure 6. ^{1}H-NMR spectra of 10-Eg-10

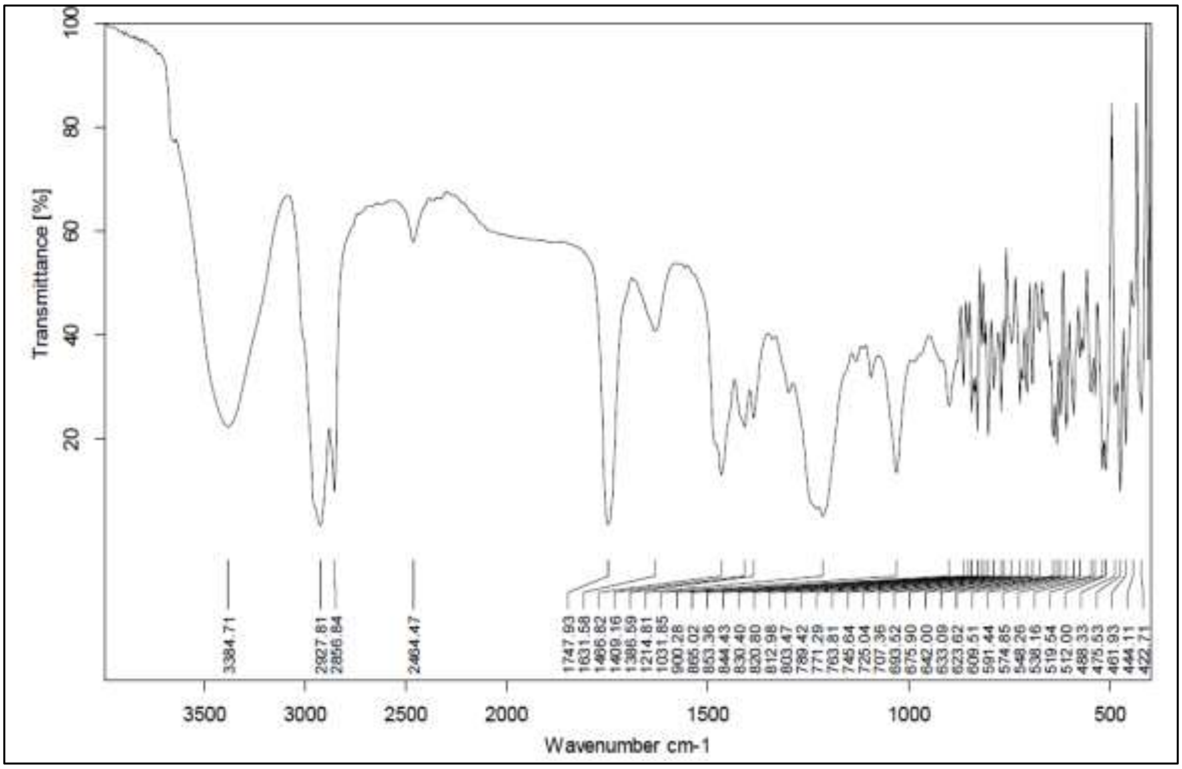


Figure 7. FT-IR spectra of 10-Isb-10

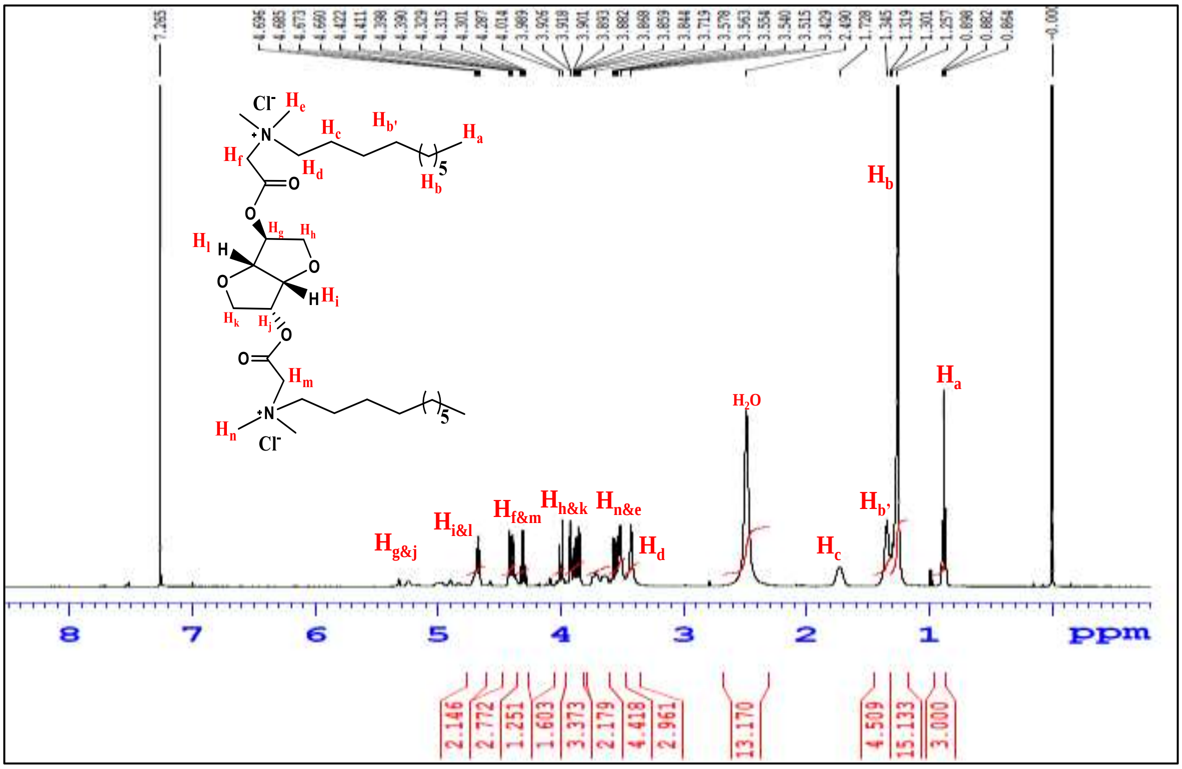


Figure 8. ^{1}H NMR spectra of 10-Isb-10

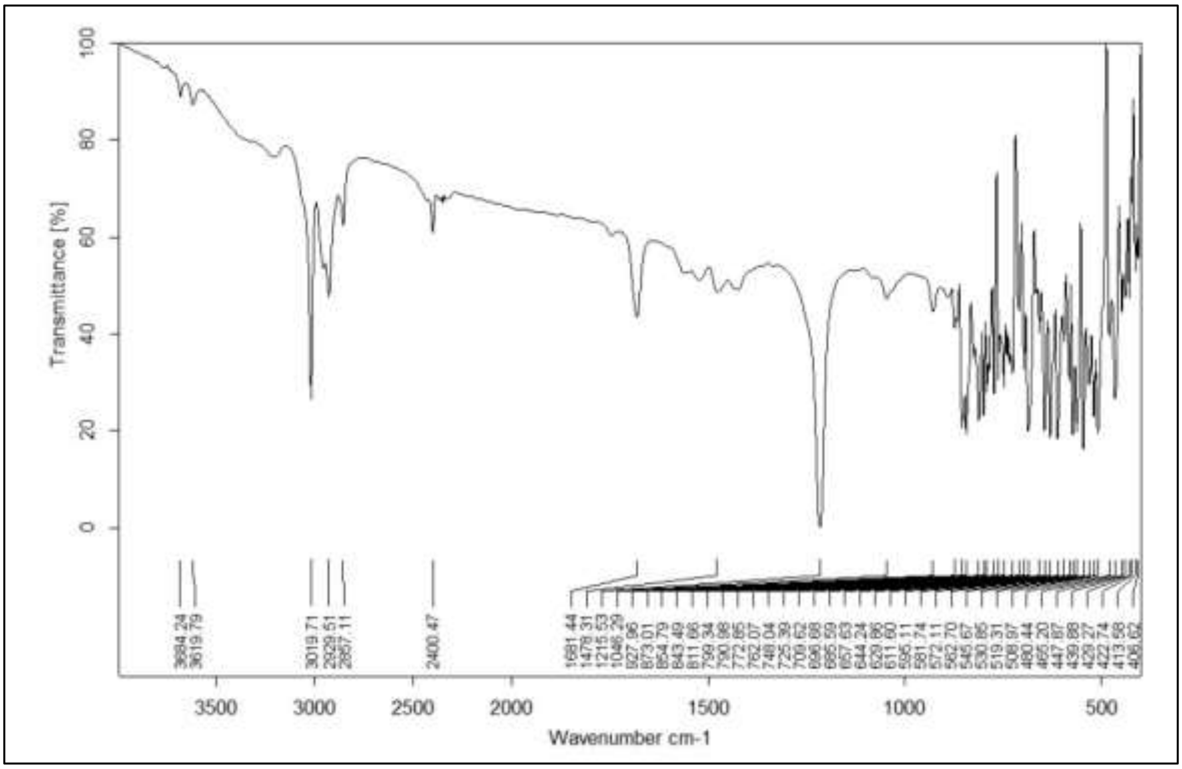


Figure 9. FT-IR spectra of 10-Eda-10

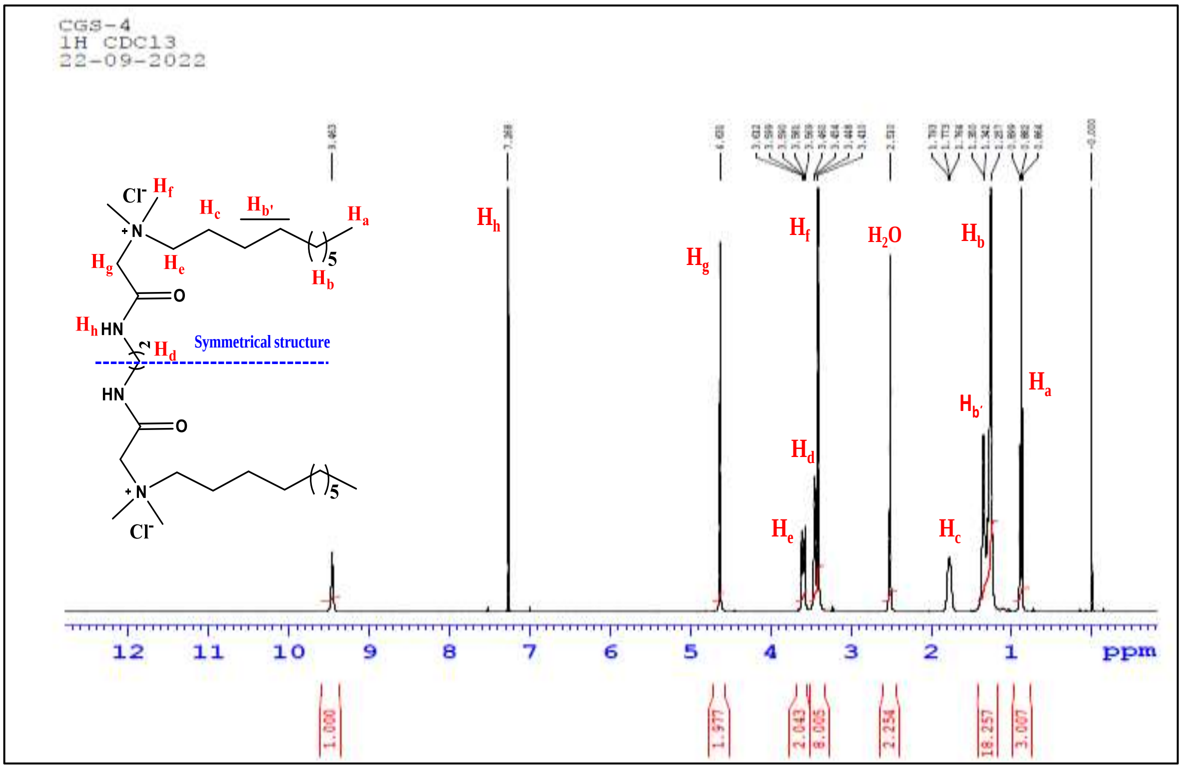


Figure 10. ^{1}H NMR spectra of 10-Eda-10

2.1.3. Thermal Stability of Cationic Geminis

TGA analysis has been used to check about the thermal stability of cationic geminis. The initial small loss in weight is indicative of hygroscopic nature of the material (Figure 11). Data depict two temperatures of stability showing sharp degradation and indicative of fair purity of the material.

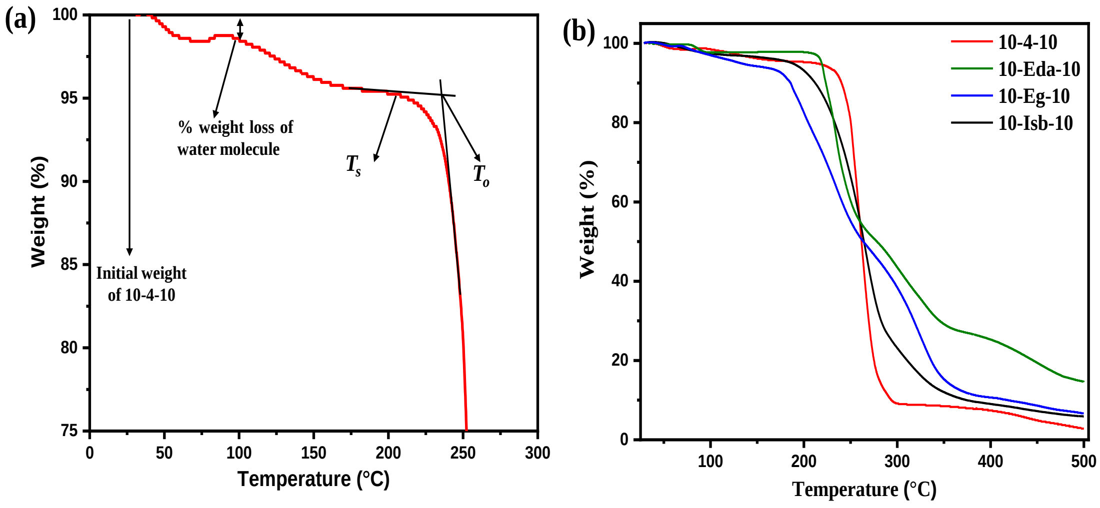


Figure 10. (a) Expanded TGA plot showing loss of water from cationic gemini (10-4-10) and depcting $T_{s\ and}$ T_o **(b)** Thermal degradation curve of cationic geminis by TGA.

The onset temperature (T_o) is the inflection point obtain from the baseline weight after the water loss responsible for hydration and the tangent on the weight *vs* temperature plot, where breaking of the molecule takes place. The starting decomposition temperature has been denoted by $T_{s.}$ T_o and T_s are compiled in Table 1. This behaviour is well matched with the one reported in literature [13–15].

Table 1. Starting (T_s) and onset (T_o) degradation temperature of cationic geminis obtained from TGA plots.

Gemini Surfactant	T_o (°C)	T_s (°C)	Start to onset ratio ($T_{s/o}$)
10-4-10	250	146	0.584
10-Eg-10	190	140	0.737
10-Isb-10	230	135	0.587
10-Eda-10	225	186	0.827

2.2. Methodologies

2.2.1. Conductometric Measurements

A conductivity probe (EUTECH Cyberscan CON510, cell constant 1 cm^{-1}) was utilized to monitor the electrical conductivity of a blend of charged aqueous surfactants of varying concentrations. The specific conductance (κ) was measured at different [surfactant(s)] using a calibrated detector. A 0.5 mL standard surfactant solution was mixed with a fixed water content (thermostat at 303 ± 0.1 K).

2.2.2. Steady-state Fluorescence Measurements

Fluorescence data were acquired (at 303 K) by employing a spectrofluorometer (RF-6000) and a probe (pyrene, 2 mM). The test sample has been retained inside a 10 mm × 10 mm quartz specimen holder that has been temperature-controlled using a cooling bath (PCI Analytics, India). The excitation was kept at a steady 337 nm throughout the acquisition of the observations, and the excitation and emission slit dimensions were, respectively, 1.5 and 3 nm. Spectra were recorded at a scanning rate of 60 nm per minute, spanning the range between 350-410 nm.

2.2.3. DLS/ Zeta (ζ) – Potential Measurements

Malvern Zeta Sizer (Nano ZS ZEN3600) has been used to record hydrodynamic diameter (D_h) and ζ at 303 K. It was designed with a laser pointer with a wavelength of 532 nm and PMT for collecting morphological information. Immersing electrode specimens are placed in a chamber in which there is 0.5 mL of liquid (filtered through a 0.22 μm nylon filter). The final value is an average of the five cycles of the decay process.

2.2.4. Transmission Electron Microscopy

TEM micrographs of the microstructure of micelles are acquired by using a microscope designed by JEOL (JEL 2100) and functioning at 120 kV of voltage. A tiny volume of the test solution was sprayed on a carbon-coated Cu grid, which was then dried at room temperature followed by treatment fresh uranyl acetate spray and dried again.

2.2.5. Solubilization Investigations

The solubility of various hydrophobic molecules in aqueous blended solution was evaluated spectrophotometrically by administering an excess of insoluble material and equilibrating the mixtures for 48 hours at 303 K. Prior to spectrophotometry (Shimadzu UV-1900), the mixtures were centrifuged to remove excess insoluble material [16].

2.2.6. *In-vitro* drug release and kinetics

The drug release behaviour from a blend of geminis in an alcoholic phosphate buffer (pH 7.4, 37 °C) was examined employing a dialysis bag procedure [17]. 3 mL of drug in mixed geminis was trapped in a dialysis membrane and immersed in a PBS solution (pH = 7.4) at 310

K. At predetermined time intervals, 1 ml of aliquots of sample were collected from the released mixture, and sink conditions were kept up by refilling that volume with fresh dissolution media. The aforesaid bag released [drug] was quantified by a UV-visible spectrophotometer.

2.2.7. *In-vitro* antioxidant activity

The oxidant effects of drug and drug dissolved in mixed geminis at different concentrations were evaluated using a fixed concentration of DPPH [18]. After such a thorough mixing, the samples were incubated for a period of 15 minutes in the dark, and the scavenging rate was subsequently assessed by determining the absorbance at 517 nm using UV-visible spectroscopy [19]. All of the trials were performed in triplicate.

2.2.8. *In-vitro* cell proliferation studies

A tetrazolium salt MTT assay was used to analyse the cytotoxic effects of optimal drug, mixed geminis (excluding drug), and drug entrapped in blended geminis on breast cancer cells [20]. In 96-well plates, 4 cells per well are grown in air containing 5% carbon dioxide at physiological temperature for 24 h. After cell incubation the resultant systems are interacted with free drug, mixed geminis, or drug solubilized in mixed geminis with different concentrations. Thereafter, 100 μL of MTT (5 mg/mL) being loaded to every well and kept for three hours at 37 °C. The control mixture has been separated followed by addition of 100 μL DMSO to every well to solubilized formazan dye crystals. A spectrophotometrically measured absorbance (Spectamax M2^{e}, molecular device, USA) at 570 nm was utilised to quantify the cell proliferation percentage.

References:

[1] A.K. Nelson, A.D.F. Toy, The preparation of long-chain monoalkyl phosphates from pyrophosphoric acid and alcohols, Inorg. Chem. 2 (1963) 775–777.

[2] R.A. BAUMAN, The preparation of mixed dialkyl phosphates, (1974).

[3] S. De, V.K. Aswal, P.S. Goyal, S. Bhattacharya, Characterization of new gemini surfactant micelles with phosphate headgroups by SANS and fluorescence spectroscopy, Chem. Phys. Lett. 303 (1999) 295–303.

[4] Z. Gao, S. Tai, Q. Zhang, Y. Zhao, B. Lü, Y. Ge, L. Huang, X. Tang, Synthesis and surface activity of biquaternary ammonium salt gemini surfactants with ester bond, Wuhan Univ. J. Nat. Sci. 13 (2008) 227–231.

[5] M.J. Rosen, Gemini: a new generation of surfactants, J. Chemtech. (1993) 30.

[6] F.M. Menger, J.S. Keiper, Gemini surfactants, Angew. Chemie Int. Ed. 39 (2000) 1906–1920.

[7] F.M. Menger, C.A. Littau, Gemini-surfactants: synthesis and properties, J. Am. Chem. Soc. 113 (1991) 1451–1452. https://doi.org/10.1021/ja00004a077.

[8] R. Zana, Dimeric and oligomeric surfactants. Behavior at interfaces and in aqueous solution: a review, Adv. Colloid Interface Sci. 97 (2002) 205–253. https://doi.org/https://doi.org/10.1016/S0001-8686(01)00069-0.

[9] Kabir-ud-Din, U.S. Siddiqui, G. Ghosh, Growth of gemini surfactant micelles under the influence of additives: DLS studies, J. Dispers. Sci. Technol. 30 (2009) 1310–1319.

[10] K. Parikh, B. Mistry, S. Jana, S. Gupta, R. V Devkar, S. Kumar, Physico-biochemical studies on cationic gemini surfactants: role of spacer, J. Mol. Liq. 206 (2015) 19–28.

[11] S.K. Yadav, K. Parikh, S. Kumar, Solubilization potentials of single and mixed oppositely charged gemini surfactants: A case of polycyclic aromatic hydrocarbons, Colloids Surfaces A Physicochem. Eng. Asp. 514 (2017) 47–55. https://doi.org/https://doi.org/10.1016/j.colsurfa.2016.11.042.

[12] S. Kumar, K. Parikh, Influence of Spacer on Association Behavior and Thermodynamic Parameters of Dimeric Cationic Surfactants, J. Surfactants Deterg. 16 (2013) 739–749. https://doi.org/10.1007/s11743-013-1467-z.

[13] N. Templeton, Cationic liposomes as in vivo delivery vehicles, Curr. Med. Chem. 10 (2003) 1279–1287.

[14] L. Tavano, M.R. Infante, M.A. Riya, A. Pinazo, M.P. Vinardell, M. Mitjans, M.A.

Manresa, L. Pérez, Role of aggregate size in the hemolytic and antimicrobial activity of colloidal solutions based on single and gemini surfactants from arginine, Soft Matter. 9 (2013) 306–319.

[15] M. El Achouri, S. Kertit, H.M. Gouttaya, B. Nciri, Y. Bensouda, L. Perez, M.R. Infante, K. Elkacemi, Corrosion inhibition of iron in 1 M HCl by some gemini surfactants in the series of alkanediyl-α, ω-bis-(dimethyl tetradecyl ammonium bromide), Prog. Org. Coatings. 43 (2001) 267–273.

[16] R.A. Friedel, M. Orchin, Ultraviolet spectra of aromatic compounds, Wiley New York, 1951.

[17] S.A. Pillai, U. Sheth, A. Bahadur, V.K. Aswal, P. Bahadur, Salt induced micellar growth in aqueous solutions of a star block copolymer Tetronic® 1304: Investigating the role in solubilizing, release and cytotoxicity of model drugs, J. Mol. Liq. 224 (2016) 303–310. https://doi.org/https://doi.org/10.1016/j.molliq.2016.09.091.

[18] B. Gabriele, A. Fazio, M. Carchedi, P. Plastina, In vitro antioxidant activity of extracts of Sybaris liquorice roots from Southern Italy, Nat. Prod. Res. 26 (2012) 2176–2181.

[19] Y.-J. Xie, Q.-L. Wang, M. Adu-Frimpong, J. Liu, K.-Y. Zhang, X.-M. Xu, J.-N. Yu, Preparation and evaluation of isoliquiritigenin-loaded F127/P123 polymeric micelles, Drug Dev. Ind. Pharm. (2019).

[20] B.H. Pursuwani, B.S. Bhatt, F.U. Vaidya, C. Pathak, M.N. Patel, Tetrazolo [1, 5-a] quinoline moiety-based Os (IV) complexes: DNA binding/cleavage, bacteriostatic and photocytotoxicity assay, J. Biomol. Struct. Dyn. 39 (2021) 2894–2903.