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Dedicated to Professor Bogdan C. Simionescu
on the occasion of his 70th anniversary

PEGYLATED SQUALENE: A BIOCOMPATIBLE POLYMER AS PRECURSOR FOR DRUG DELIVERY**

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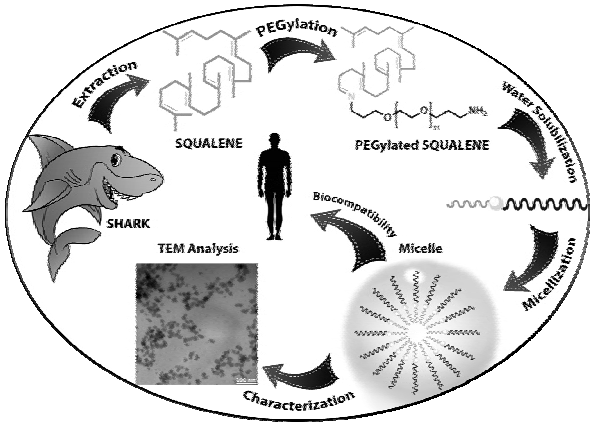
In recent decades great attention was given to lipid-based drug carriers due to their capability to increase pharmacokinetics, decrease toxicity, and upsurge the therapeutic index of the associated drugs, prompting the search for innovative nano-carriers to safely transport drugs or genes to specific cells. Herein, we describe a simple method to obtain PEGylated Squalene starting from squalene, which makes the product able to self-assemble in water, and a PEG unit which offers biocompatibility and water solubility, connected together *via* imine bonds. In aqueous media PEGylated Squalene adopts a core-shell structure forming micelles confirmed by transmission electron microscopy (TEM) and critical micelle concentration (CMC) measurements. Biocompatibility and cytotoxicity tested on NHDF (Normal Human Dermal Fibroblasts) cells revealed that PEGylated Squalene showed promising features suitable for a precursor for drug delivery.



INTRODUCTION

Delivery of hydrophobic drugs is still a major challenge for researchers despite the progress made in drug delivery systems in the past decades.[1,2] The use of micellar solutions of low molecular weight surfactants has been one of the common methods for the solubilization of hydrophobic drugs; however, such surfactants usually have high critical micelle concentration (CMC), consequently low thermodynamic and kinetic stabilities. In contrast, polymeric micelles show remarkable potential due to their large solubilization power, better loading capacity, higher stability in blood stream, therapeutic potential and longevity. In the aqueous system, the polymeric micelles possess an amphiphilic character, due to the presence in their structures of both hydrophilic ("water loving") and hydrophobic part ("water repelling"). The applications of polymeric micelles can be related to their unique core–shell architecture in which the hydrophobic part provides a space for the encapsulation of hydrophobic drugs, protein or DNA through physical or chemical binding

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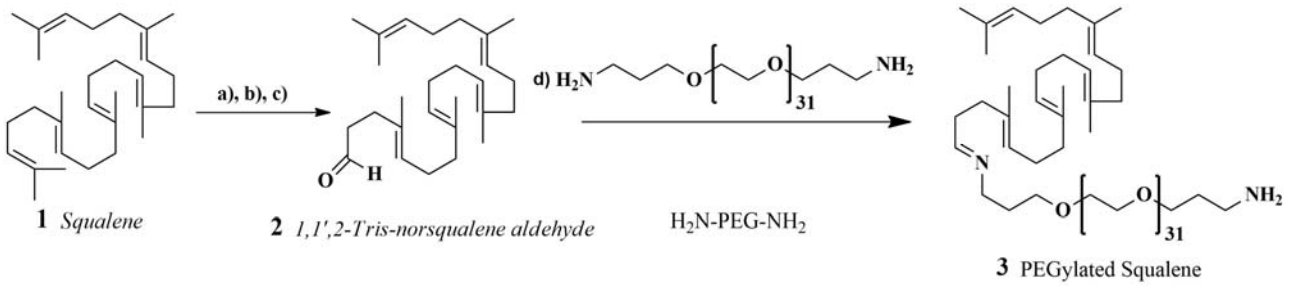
Squalene from squalene tail, known for its ability to self-assemble,[6,7] and poly(ethylene glycol) (PEG) unit that offers biocompatibility and water solubility, connected *via* imine bond. In aqueous media these molecules adopt a core-shell structure and showed good biocompatibility and low cytotoxicity on NHDF (Normal Human Dermal Fibroblasts) cells, offering promising features as precursor for drug delivery.

RESULTS AND DISCUSSION

Synthesis of PEGylated Squalene

First, synthesis of 1,1',2-tris-nor-squalene aldehyde **2** (Scheme 1) was accomplished in three steps, starting from squalene **1**, using literature known procedure.[8,9,10] Compound **2** has subsequently reacted with diaminated PEG in the 1:1 molar ratio to achieve PEGylated Squalene **3**, as previously described.[11,12] All procedures were adapted and optimized in order to reach better yields.

PEGylated Squalene **3** proved to be stable in aqueous solution for a long period of time. Monitoring for 10 days the degradation of the compound **3**, by ^{1}H-NMR technique, only 0.2% of 1,1',2-tris-nor-squalene aldehyde **2** was observed, as hydrolysis products; in ^{1}H-NMR spectrum (Fig. 1) the small increase of carbonyl signal at 9.3 ppm (D_2O) in aqueous solution of **3** indicates the presence in reduced amount of degradation product, confirming its high stability in water, explained by self-assembly process into micelles, sterically hindering the imine bond.



Scheme 1 – Synthesis of PEGylated Squalene 3, a) NBS, THF, 0°C, 1.5h; b) K_2CO_3, MeOH, r.t., 2h; c) HIO_4x2H_2O, H_2O, dioxane, r.t., 2h; d) H_2N-PEG-NH_2, CH_3CN, 24h, r.t.

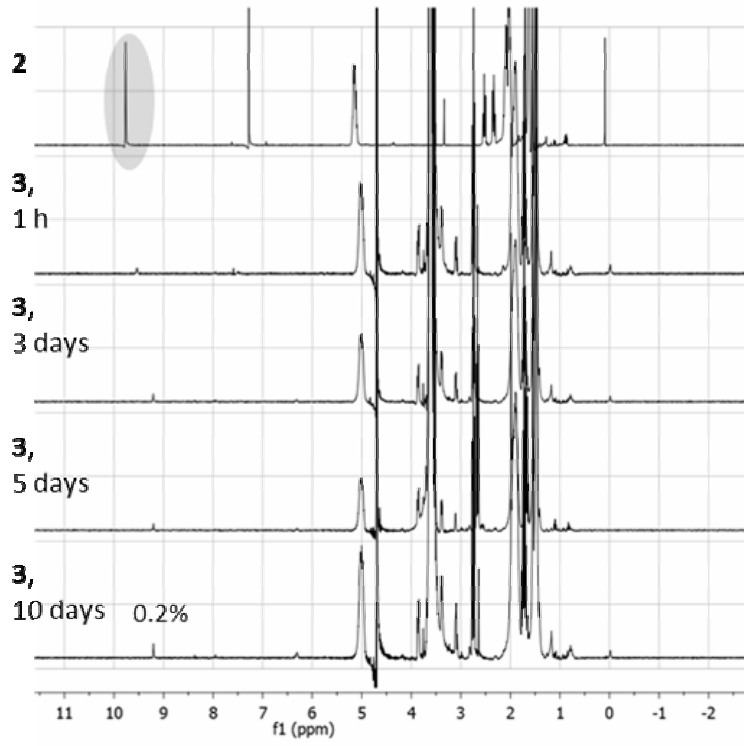


Fig. 1 – ^{1}H-NMR of 1',2-tris-nor-squalene aldehyde **2** in $CDCl_3$ and PEGylated Squalene **3** in D_2O at 1h, 3 days, 5 days, 10 days.

In silico molecular dynamics simulation

In order to visualize the auto assembly process into micelles of the PEGylated Squalene **3** molecules, molecular dynamics (MD) simulation study of the system, using the Yasara software, was performed. In this context, 10 molecules of SQ-PEG were randomly distributed and solvated with water molecules in a simulation box (Fig. 2A).

Interestingly, after 8 ns of MD simulation the formation of two micelles was observed (Fig. 2B); one of them containing three PEGylated Squalene molecules and the other seven molecules. In both cases the micelles have the same structure, with the squalene part aggregating inside of micelle and the PEG part coating Squalene assemblage.

Transmission Electron Microscopy investigations

Morphological and dimensional characteristics of PEGylated Squalene **3** in water were investigated by transmission electron microscopy (TEM). The analysis of TEM images revealed the formation of spherically-shaped micelles (Fig. 3A and 3B), similar to the *in silico* predicted models.

Remarkably, the dispersion of micellar aggregates in aqueous media is uniform+ and micelles are not agglomerated in small or large clusters. The diameters of PEGylated Squalene micelles are 20 to 55 nm as proved by calculations obtained from micellar size distribution of PEGylated Squalene (Fig. 3C).

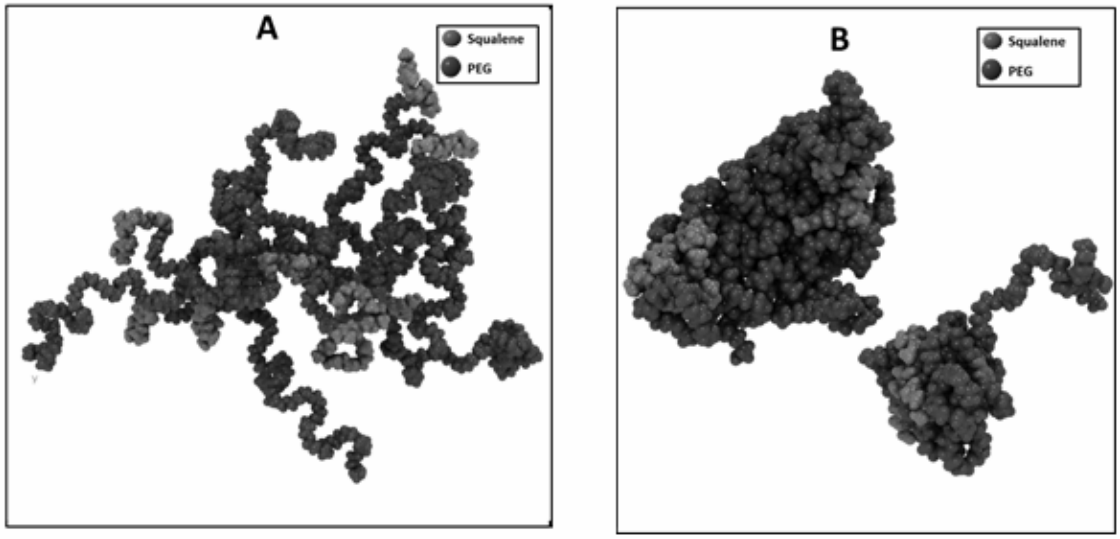


Fig. 2 – *In silico* auto-assembly into micelles of the SQ-PEG molecule. A) Is the starting distribution of the simulation and B) is the final distribution after 8 ns. The compounds that make the molecule were color-coded for a better visualization of their orientation, and the water was omitted.

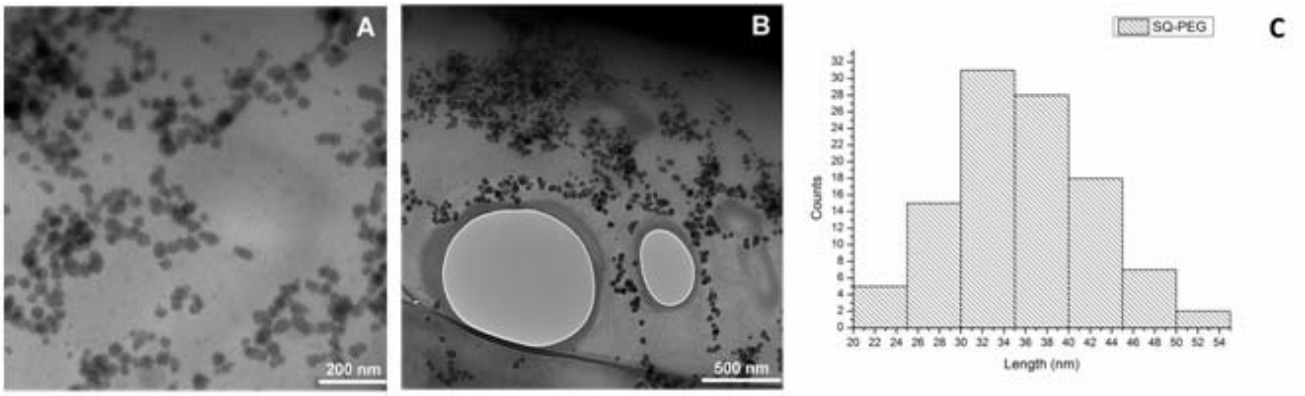


Fig. 3 – TEM micrographs for PEGylated Squalene. (A) with 200 nm scale; (B) with 500 nm scale; (C) Size distribution.

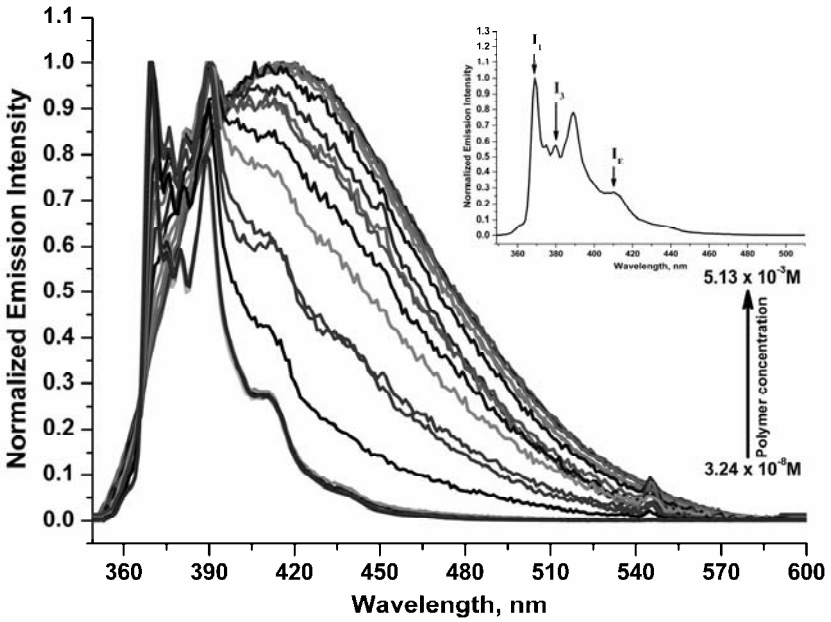


Fig. 4 – Normalized Emission spectra of Pyrene aqueous solution (5 x 10^{-7} M) in presence of different concentration of PEGylated squalene (3.24 x 10^{-8} M to 5.13 x 10^{-3} M), λ_{ex} = 334 nm (I_1, I_3, I_E – Normalized intensity of peak 1, 3, and excimer respectively).

Determining Critical Micellar Concentration (CMC) of PEGylated Squalene using Fluorescence

Polymeric micelles are characterized by the critical micelle concentration (CMC), therefore, upon dilution below the CMC, micelles disassemble into free molecular unimers.[13] Herein a method based on the emission of fluorescent probe (pyrene) was applied, that provided information about self-assembling of compound and formation of hydrophobic domain in aqueous solutions.[14,15] The fluorescence spectra of pyrene give information about local polarity of the probe surroundings based on the intensity ratio of the first and the third emission maxima (I_1/I_3). Higher or lower values of this ratio are related to polar or non-polar environments of the probe.[16] Another important parameter that gives information about the polarity of the pyrene surroundings is the intensity ratio of excimer (excited state of dimer of pyrene) and the third emission maxima (I_E/I_3)[17], as depicted in insert of Fig. 4. The precise CMC value of PEGylated squalene aqueous solution was determined by calculating I_1/I_3 and I_E/I_3 from fluorescence emission spectra registered as concentration *vs*. fluorescence intensity at excitation wavelengths λ_{ex} of 334 nm.

Fig. 4 presents fluorescence spectra of aqueous solution with constant pyrene concentration (5 × 10^{-7} M) and different concentrations of PEGylated Squalene. It was observed that by increasing the concentration of PEGylated Squalene from 3.24 × 10^{-8} M to 5.13 x 10^{-3} M, the fluorescence intensity of pyrene unimers ($\lambda_{emission}$ = 369 ÷ 389 nm interval) and excimers ($\lambda_{emission}$ = 410 nm) was increasing correspondingly. From these spectra, the Boltzmann Sigmoidal fitting[18,19] was drawn for I_1/I_3 ratio as a function of log C (mg/mL), and revealed a non-linear decreasing of the I_1/I_3 values with the increasing of PEGylated Squalene concentration (Fig. 5). At low concentrations of polymer, up to 3.24 × 10^{-5} M, I_1/I_3 ratio presented a constant value of approximately 1.69, which was associated with a polar environment.[20] While the concentration of the PEGylated squalene was increased, I_1/I_3 ratio rapidly decreased to a value of approximately 0.57, which represents a non-polar environment. Simultaneously, I_1/I_3 ratio was decreasing while the I_E/I_3 ratio was increasing (Fig. 5 insert) due to the amplification of excimer intensity, suggesting that the pyrene was surrounded by hydrophobic part of PEGylated Squalene when micellar aggregates were formed.

CMC value of 0.1620 mg/mL (8.75 × 10^{-2} mM) for PEGylated Squalene was determined by the intersection of the two branches from the Boltzmann Sigmoidal Fitting of I_E/I_3 as a function of logC, where I_E is the fluorescence intensity of excimer and I_3 is the third vibronic peak from the emission spectra of 5 × 10^{-7} M pyrene aqueous solutions with different PEGylated squalene concentrations (Fig. 5).

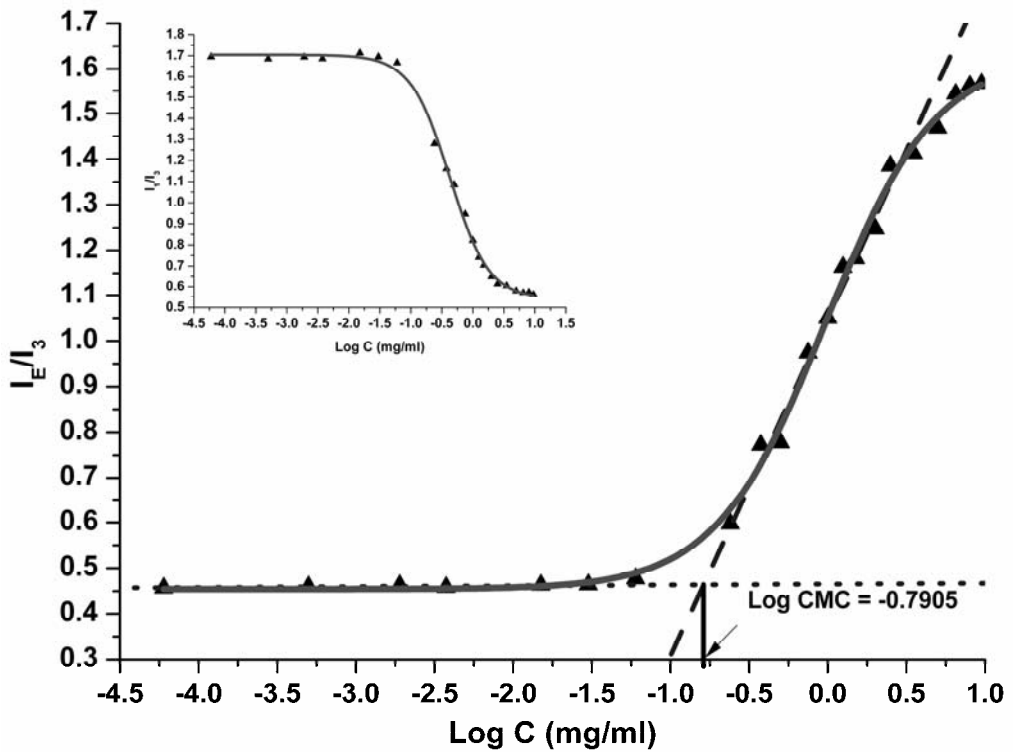


Fig. 5 – Determination of CMC from Boltzmann Sigmoidal Fitting of I_E/I_3 as a function of logC (mg/mL, concentration of PEGylated Squalene). Insert is variation and Boltzmann Sigmoidal Fitting of I_1/I_3 as a function of logC; pyrene concentration = 5 x 10^{-7} M and λ_{ex}=334 nm.

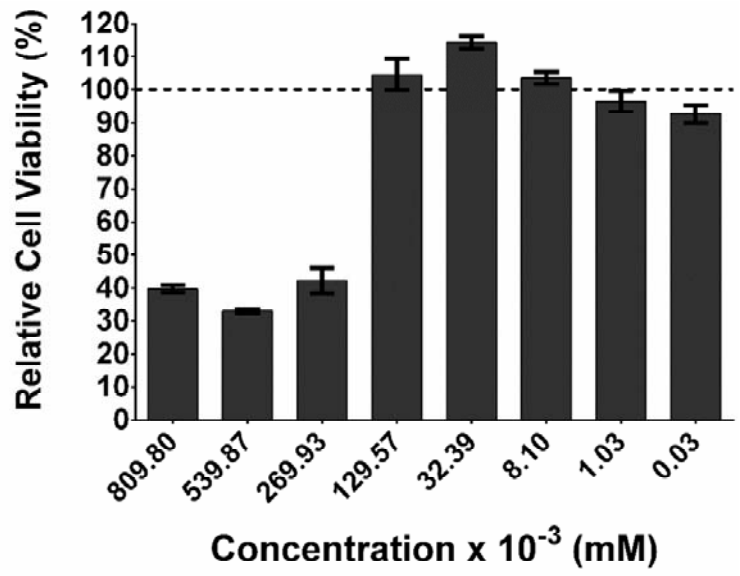


Fig. 6 – MTS Cytotoxicity Assay of PEGylates Squalene on NHDF cells.

MTS Cytotoxicity Assay

Cytotoxicity of PEGylated Squalene was tested by determining the mitochondrial reductase activity (MTS).[21] Eight concentrations of PEGylated Squalene in ultrapure water were used to determine the concentration value, including at CMC, where the polymer becomes cytotoxic for Normal Human Dermal Fibroblasts (NHDF) cells (see Experimental Section). Cell viability was calculated and expressed as percentage relative to the viability of untreated cells (100% viability). The aqueous solutions of PEGylated Squalene at low concentrations (0.03÷129.57 × 10^{-3} mM) have superior cell viability, meanwhile at higher concentrations (269.93÷809.80 × 10^{-3} mM), cell viability dropped to values of ~40 %

(Fig. 6). At CMC (8.75×10^{-2} mM) the cell viability is ~ 100%.

EXPERIMENTAL

Materials

All the chemicals were purchased from Sigma-Aldrich Chemie GmbH (Germany) and utilized without any further purification. Normal Human Dermal Fibroblasts (NHDF) cells (PromoCell); CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega), 96-well culture plate (Corning).

Methods

Nuclear Magnetic Resonance spectra were recorded on Bruker Avance III 400 instrument operated at 400 and 101 MHz for ^{1}H and ^{13}C nuclei, respectively, at room temperature. Chemical shifts were reported in ppm, and referred to tetramethylsilane (TMS) as internal standard.

Transmission Electron Microscopy images were obtained on a HT7700 Hitachi Transmission Electron Microscope. The necessary calculated amount of PEGylated Squalene was concentrated to dryness using reduced pressure to remove all the acetonitrile and the remained residue was collected with ultrapure water to obtain a stock solution with the desired concentration, in our case 2.6 (μg/μL). After complete solubilization of PEGylated Squalene in aqueous media by magnetic stirring, a volume of 6 μL was deposited on carbon coated copper grid and air dried for 24 hours at ambient temperature (23 ºC). After drying, the samples were examined in high resolution mode, under an operating potential of 100 kV.

Molecular dynamic simulation was performed by means of a YASARA-Structure software package version 14.12.2. The "AutoSMILES" algorithm was used to generate the force field parameters for the MD simulation. The SQ-PEG molecule built from 426 atoms and the molar mass ratio of SQ/PEG of 1:4. 10 SQ-PEI-PEG molecules were introduced randomly in a rectangular simulation box with the dimensions 116 Å X 102 Å x 104 Å and solvated with 119487 TIP3P water molecules. The system was subjected to energy minimization by means of the steepest descent algorithm and annealing optimization. The resulted conformations were used as the starting point for the MD simulation production run. The water density was set to 0.997 $g \cdot cm^{-3}$ in order to simulate a constant pressure of P = 1 bar at the temperature equal to T = 298 K. Electrostatic interactions were modelled using the particle mesh Ewald (PME) method.

Fluorescence measurements. For the fluorescence spectra acquisition were used pyrene aqueous solutions at constant concentration (5×10^{-7} M) as a function of different concentrations of PEGylated Squalene. Stock solutions of 5.13×10^{-3} M to 3.24×10^{-8} M were kept at 2-4 °C for 24 h (Table 1.S). Spectra of obtained samples were recorded at room temperature (23 ºC) with Fluoromax-4® Spectrofluorometer from HORIBA SCIENTIFIC®. The optical path was 10 mm. For emission spectra the following parameters were used for all the samples: excitation wavelength, λ_{ex} = 334 nm, front entrance slit – 2 nm Bandpass, front exit slit – 2 nm Bandpass, emission wavelength interval, λ_{em} = 349 – 600 nm, increment – 1 nm, integration time – 0.1 s, detector accumulations – 3 averaged scans.

MTS cytotoxicity assay. For cytotoxicity experiment, 8 different concentrations were prepared with the calculated amounts of PEGylated Squalene which were solubilized in ultrapure water and kept at 2 – 4 ºC until the day of experiment. The preparation of the samples for biological assay were conducted in sterile, clean rooms to avoid contamination of samples. Cytotoxicity was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). Cytotoxicity was measured using the standard MTS assay as reported previously.[22] Normal Human Dermal Fibroblasts (NHDF) cells were seeded into a 96-well culture plate at a density of $5x10^3$ cells per well in 100 μL culture medium (alpha-MEM medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/25 μg). After 24 hours the medium in each well was replaced with 100 μL mixture containing fresh medium and PEGylated Squalene samples. 7 biological replicates were performed for each concentration of polymer. After 44 hours, 20 μL of CellTiter 96® Aqueous One Solution reagent were added to each well, and the plates were incubated for another 4 hours before reading the result. Absorbance at 490 nm was recorded with a plate reader (EnSight, PerkinElmer).

Synthesis

Synthesis of 2-hydroxy-3-bromosqualene: 3-bromo-2,6,10,15,19,23-hexamethyl-6,10,14,18,22-tetracosapentaen-2-ol (Scheme 1.a). Into a 500 mL round bottom flask, squalene (***I***) (20.54 g; 50 mmol; 1 equivalent) was dissolved in THF (125 mL), upon cooling at 0°C, and then bidistilled water was added dropwise until the solution became opalescent. Then a small amount of THF was added to clear the solution. N-bromosuccinimide (NBS) (10.68 g: 60 mmol; 1.2 equivalents) was added over a period of 30 min at 0°C, and then left for stirring 90 minutes at room temperature (23 ºC). The product was worked as follows: the crude reaction mixture was diluted with bidistilled water (120 mL) and extracted with petroleum ether (2 × 75 mL), the organic layer was washed with saturated brine (2 × 75 mL), dried over anhydrous sodium sulfate, filtered and evaporated to dryness *in vacuo*. The product was verified for purity using Thin-Layer chromatography (TLC), as eluent was used petroleum ether:diethyl ether, 95:5, the plates were stained in $KMnO_4$ solution. The resulted oil was purified by 'flash chromatography' – a column of appropriate diameter is selected and packed 1/3 with appropriate silica gel (230 – 400 mesh), the sample was diluted and slowly introduced on the silica bed using column wall as support. The sample was eluted using petroleum ether to remove all the unreacted squalene, then petroleum ether:diethyl ether, 95:5 and 90:10 to give 5.49 g (27%) of 2-hydroxy-3-bromosqualene as a yellow oil. **^{1}H-NMR** (400 MHz, $CDCl_3$, TMS) δ (ppm) = 5.24 – 5.07 (m, 5H), 3.98 (dd, *J* = 11.3, 1.7 Hz, 1H), 2.15 – 1.91 (m, 20H), 1.68 (s, 3H), 1.60 (s, 15H), 1.34 (s, 3H), 1.33 (s, 3H) (Fig. 1.S); **^{13}C-NMR** ($CDCl_3$, 101 MHz): δ (ppm) = 135.16 (CH_3-C=C-); 134.91 (CH_3-C=C-); 132 ($(CH_3)_2$C=); 124.3 (-CH=); 123.5 (-CH=); 72.47 ($(CH_3)_2$(OH)C-); 71.05 (Br-CH-); 39.7 (-CH_2-); 38.2 (-CH_2-); 32.19 (-CH_2-); 28.3 (-CH_2-); 26.7 (-CH_2-); 25.72 (-CH_3); 17.71 (-CH_3); 16 (-CH_3) (Fig. 2.S).

Synthesis of 2,3-oxidosqualene, 22,23-epoxy-2,6,10,15,19,23-hexamethyl-2,6,10,14,18-tetracosapentane (Scheme 1.b). To a solution of 2-hydroxy-3-bromosqualene, previously obtained, (2.379 g, 4.69 mmol, 1 equivalent) in methanol (60 mL), K_2CO_3 (1.296 g, 9.37 mmol, 2 equivalents) is added and the reaction mixture is stirred at room

temperature (23 ºC) for 2 h under nitrogen atmosphere, then concentrated under reduced pressure. Bidistilled water (120 mL) is added and extracted with ethyl acetate (4 × 30 mL). The organic layer was washed with saturated brine (2 x 60 mL), dried over anhydrous sodium sulfate, filtered and evaporated to dryness *in vacuo* to obtain 2,3-oxidosqualene as a yellow oil in a very pure form (1.836 g, Yield: 77%) was used directly in the next step with no further purification. **^{1}H-NMR** (400 MHz, $CDCl_3$, TMS) δ (ppm) = 5.18 – 5.07 (m, 5H), 2.70 (t, J = 6.3 Hz, 1H), 2.10 – 1.95 (m, 18H), 1.67 (s, 3H), 1.61 (s, 3H), 1.59 (s, 12H), 1.29 (s, 3H), 1.25 (s, 3H) (Fig. 3.S). **^{13}C-NMR** (101 MHz, $CDCl_3$, TMS) δ (ppm) = 135.12 (CH_3-C=C-), 134.97 (CH_3-C=C-), 134.90 (CH_3-C=C-), 131.24 ((CH_3)$_2$C=), 124.93 (-CH=), 124.39 (-CH=), 124.26 (-CH=), 64.20 (-CH-O), 58.31 (-C-O), 39.72 (-CH_2-), 39.67 (-CH_2-), 36.3 (-CH_2-), 28.25 (-CH_2-), 27.45 (-CH_2-), 26.76 (-CH_2-), 26.65 (-CH_2-), 25.69 (-CH_3), 24.89 (-CH_3), 18.74 (-CH_3), 17.67 (-CH_3), 17.63 (-CH_3), 16.02 (-CH_3), 15.99 (-CH_3) (Fig. 4.S).

Synthesis of 1,1′,2-Tris-norsqualene aldehyde (2) (Scheme 1.c). To a solution of periodic acid (1.731 g, 7.59 mmol, 1,765 equivalents) in bidistilled water (10 mL), a solution of 2,3-oxidosqualene (1.836 g, 4.3 mmol, 1 equivalents) in dioxane (24 mL) is added and the reaction mixture is stirred at room temperature (23 ºC) for 2 h. Bidistilled water (120 mL) is added and extracted with ethyl acetate (3 x 40 mL). The organic layers are washed with saturated brine (100 mL) and bidistlled water (100 mL), dried over anhydrous sodium sulphate, filtered and evaporated to dryness in vacuo. The product was verified for purity using Thin-Layer chromatography (TLC), as eluent petroleum ether: diethyl ether, 90 : 10 was used, the plates were developed in $KMnO_4$. The resulted oil was purified by 'flash chromatography' – a column of appropriate diameter is selected and packed 1/3 with appropriate silica gel (230 – 400 mesh), the sample was diluted and slowly introduced on the silica bed using column wall as support. The sample was eluted using petroleum ether:diethyl ether = 90:10 to give 1.36 g (73%) of 1,1′,2-Tris-norsqualene aldehyde (***2***) as a very pale yellow to colorless oil as interest compound. **^{1}H-NMR** (400 MHz, $CDCl_3$, TMS) δ (ppm) = 9.75 (t, J = 1.8 Hz, 1H), 5.16 – 5.09 (m, 5H), 2.50 (t, J = 7.5 Hz, 2H), 2.31 (t, J = 7.5 Hz, 2H), 2.12 – 1.98 (m, 16H), 1.68 (s, 3H), 1.60 (s, 16H) (Fig. 5.S). **^{13}C-NMR** (101 MHz, $CDCl_3$, TMS) δ (ppm) 202.69 (-CH=O), 135.14 (CH_3-C=C-), 134.90(CH_3-C=C-), 134.78(CH_3-C=C-), 132.84 (CH_3-C=C-), 131.24 ((CH_3)$_2$C=), 125.43 (-CH=), 124.53 (-CH=), 124.40 (-CH=), 124.25 (-CH=), 42.15 (-CH_2-), 39.72 (-CH_2-), 39.52 (-CH_2-), 31.85 (-CH_2-), 28.24 (-CH_2-), 26.76 (-CH_2-), 26.65 (-CH_2-), 26.54 (-CH_2-), 25.69 (-CH_3), 17.67 (-CH_3), 16.08 (-CH_3), 16.04 (-CH_3), 15.99 (-CH_3) (Fig. 6.S).

Synthesis of PEGylated Squalene (SQ-PEG) (3) (Scheme 1.d). Synthesis of amphiphilic polymer PEGylated Squalene was achieved using the protocols described in literature.[11,12] Into a 100 mL pear shaped flask, 1,1',2-Tris-norsqualene aldehyde (***2***) (104 mg, 0.27 mmol, 1 equivalent) previously obtained was dissolved in acetonitrile (10 mL) under magnetic stirring at room temperature for 15 minutes. Poly-(ethyleneglycol)-bis(3-aminopropyl)-terminated (H_2N-PEG-NH_2) (MW ~ 1500 Da) (445 mg, 0.297 mmol, 1.1 equivalents) was solubilized in acetonitrile (15 mL) and added over squalene-aldehyde solubilized in acetonitrile. The solution was allowed to magnetic stirring for 24 hours at room temperature (23 ºC) under nitrogen atmosphere. The product PEGylated Squalene (***3***) was formed in quantitative yield (Scheme 1) and used with no further purification. The solution with concentration 11.8 mM (21.9 mg/mL) was kept in acetonitrile at 2 – 4 ºC for further experiments. **^{1}H-NMR** (400 MHz, $CDCl_3$, TMS) δ (ppm) = 7.64 (1H, t, J=4.8, CH=N), 5.15 - 5.08 (5H, m, CH=C), 3.70 - 3.64 (140 H, m, CH_2-CH_2-O), 3.19 (2H, t, J=6.4, CH_2-NH_2), 2.53 - 2.49 (2H, m, CH_2), 2.33 - 2.30 (2H, m, CH_2), 2.09 - 1.97 (16H, m, CH_2-CH_2), 1.88 - 1.83 (3H, m, CH_3), 1.68 (3H, m, =C(CH_3)-CH_3), 1.61 (12H, m, =C(CH_3)) (Fig 7.S). **^{13}C-NMR** (101 MHz, $CDCl_3$, TMS) δ (ppm) = 161.67 (C=N), 134.91 (CH_3-C=C), 131.25 (C(CH_3)$_2$), 124.40 (CH_2-C=CH), 124,26 (CH_2-C=C), 72.57 (OCH_2-CH_2), 70.58 (O-CH_2-CH_2-O), 70.35 (O-CH_2), 70.12 (O-CH_2), 69.97 (O-CH_2), 61.71(NCH_2), 39.73 (CH_2-CH_2), 39.58 (CH_2-NH_2), 31.85 (CH_2), 28.25 (CH_2), 26.77 (CH_2), 26.66(CH_2), 26.55 (CH_2), 25.71 (CH_2), 17.69 (CH_3),16.06 (CH_3), 16.01 (CH_3) (Fig. 8.S).

CONCLUSIONS

We described a simple method to obtain PEGylated Squalene and showed its ability to form micelles in aqueous environment at CMC of 8.75 × 10^{-2} mM (determined by fluorescence), having their sizes of ~40 Å, analyzed by TEM. Also, obtained micelles presented promising behavior as drug carrier, demonstrating its ability to encapsulate pyrene at its CMC. Moreover, it proved to be nontoxic for NHDF (Normal Human Dermal Fibroblasts) cells.

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