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Utilization of natural stabilizer to prepare liposomal conjugate for the newly developed aptamer

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ABSTRACT

Background: Aptamers are peptide or oligonucleotides that have the capacity to bind to a specific target molecule in human body. One of the newly developed aptamers named BAS aptamer. For targeting improvement of the aptamer, liposomal conjugate was adapted utilizing phosphatidylcholine as a natural lipid stabilizer in a comparison to synthetic one.

Method*:* Two types of blank DSPE-PEG 2000-maleimide liposomes were prepared using two types of stabilizers; synthetic (Dipalmitoylphosphatidylcholine; DPPC) and natural lipids (phosphatidylcholine) then conjugated with BAS-aptamer through introducing thiol moiety. The liposomal conjugates then evaluated by H^1-NMR and urea polyacrylamide gel electrophoresis and its efficacy on binding affinity, anticancer activity against MCF-7 breast cancer as well as its stability in plasma and buffers were estimated.

Results*:* The binding affinity study revealed a significantly higher affinity of the naturally stabilized BAS aptamer conjugated liposomes towards drug receptor (SIRT1) than the synthetically stabilized one (KD= 38.5 ± 0.985 and 68± 0.967; respectively). The cytotoxic effect of the liposomal conjugates was higher than those of pure BAS aptamer and its thiol derivative in all concentrations used. The stability study in plasma showed that naturally stabilized liposomes remained stable for up to 6 days while the synthetically prepared liposomes showed partial degradation after 5 days and both gave higher stability than the pure BAS aptamer while the stability study in phosphate buffer at pH 7.4 and 5.5 revealed non-significant decline in the absorbance of the drug started after 6 days in its naturally stabilized conjugate and after 3 days (at pH5.5) its synthetically stabilized liposomal.

Conclusion*:* Using natural stabilizer for liposomal conjugates gave better physical properties as well as significant binding affinity for the aptamer conjugated liposomes to the receptor (SIRT1) and higher cytotoxic effect as well as better stability in plasma and buffers.

Keywords: BAS aptamer, urea-PAGE, DSPE-PEG 2000-maleimide liposomes, DPPC, phosphatidylcholine, BAS aptamer conjugated liposomes.

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INTRODUCTION

Designing a new drug delivery system represents attractive method and alternative investment opportunity for drug firms as well as its participation in the improvement of the understanding of the pharmacokinetic and pharmacodynamics principles of the drug [1]. Many drug delivery systems had been developed by using different alternative routes of administration such as skin, buccal or nasal mucous membranes etc. Additionally, the attempts to deliver drug with a controlled, slow or targeted delivery using different advanced technologies is pursued vigorously[2].

Liposome is defined as a lipid bubble or vesicle that containing core that form the aqueous compartment and a bilayer of lipids that form the non-aqueous compartments. The conventional liposomes had been used widely for different routes of administration; however, the smart liposomes are considered as a pathway for the designation of nanocarriers that possess a significantly enhanced efficacy. The recently conducted researches on smart liposomes comprise the usage an environment of low pH in an attempt to achieve pH-triggered approaches, the application of enzyme to induce the triggering of enzyme-sensitive liposomes and those which are ultrasound responsive. In addition to light which is applied and investigated widely as a stimulus in photosensitive liposomes [3].

Aptamers are peptide or oligonucleotides that have the capacity to bind to a specific target molecule in human body; they differ from each other by the number, type and sequence of nucleotides [4]. One of the newly developed aptamers named BAS aptamer (consist of 40 nucleotides in circular manner) which has a specific target receptor (SIRT1) which is responsible for its anticancer activity [5]. Targeting of anticancer drug is one of the desired properties; therefore, many approaches had been adapted for this purpose including liposomal conjugates where synthetic lipid usually applied as stabilizer.

The aim of this work is to prepare and evaluate liposomal conjugates using natural lipid (pure phosphatidylcholine as stabilizer) for the newly developed single strand DNA drug BAS aptamer and investigate the contribution of such conjugates on the behaviour, stability and anticancer activity against breast cancer cells through cell line study in comparison to liposomal conjugate for BAS aptamer stabilized by synthetic lipid.

MATERIALS AND METHODS

Materials

Reagents and solvents used are analytical grade and commercially available. DNA Oligonucleotide for BAS aptamer and BAS ligation splint were provided by Bioneer- Korea. In addition to materials for molecular biology process and chemicals including 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)-2ow](DSPE-PEG(2000)-maleimide) natural lipid (Phosphatidylcholine) purity 94% provided by Sigma-Aldrich Ltd. USA and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) and TEMED (tetramethylethylenediamine) that supplied by Fisher Scientific, USA

Aptamer preparation

BAS-aptamer available as solution of 2.5 µM/ml concentration, to this aptamer functional group containing disulfide bond ($(CH_2)_6$-S-S-$(CH_2)_6$) was introduced at 3/ linkage of the BAS-aptamer, the product was desalted and lyophilised, then disulfide bond was reduced to produce linear BAS-aptamer-SH, this work was done by the manufacturer (Bioneer, Korea) [6]. Since BAS aptamer is a circular DNA product, therefore, the supplied linear BAS-aptamer-SH should be circularized.

Circularization of the supplied BAS-Aptamer-SH

Circularization of the supplied BAS-Aptamer-SH includes the following steps: -

Characterization of the supplied BAS-Aptamer-SH (Identification and purification)

Denaturing urea polyacrylamide gel electrophoresis (urea PAGE) was used to denaturing the supplied secondary DNA structure by 6-8 M urea, and polyacrylamide gel matrix for the separation of fragments between 2 to 500 bases. The type of acrylamide solution decides the movement of the sample; a higher concentration helps in breaking up of the lower molecular weight fragments. The unstructured DNA molecules can also be separated by combining urea at a temperature of 45-55°C. The solution was heated for 20s in the microwave and mixed, then 33µl of 30% APS (Ammonium pre-sulfate) was added to melt the acryl amide and 4 µl TEMED (N,N,N,N,Tetra methyl ethylene diamine**)** was added to made polymerization and get acrylamide gel [7].

Determination of Nucleic Acids on Gel by Gel Red Staining

Gel Red interacts with the bases of nucleic acids and used because of its stability, sensitivity and environmentally safe fluorescent nucleic acid dye[7]. The samples obtained from gel electrophoresis column were incubated with Gel Red solution for 30 minutes by adding 15 µl of Gel Red stain 10,000X stock reagent and 5 ml of 1 M NaCl to 45 ml H_2O then visualised at 254 nm ultraviolet transilluminator. Bands of desired length were extracted from a polyacrylamide gel**,** using ladder DNA 25 as reference[8].

Extraction of Nucleic Acids bands obtained from Polyacrylamide Gel

After visualization, ssDNA was extracted from a polyacrylamide gel by cut out the wanted band (with 40 nucleotides) exactly from the gel. Then the gel was cut into small pieces and put into a 1.5 ml Eppendorf tube and shaked overnight at room temperature after adding two volumes of elution buffer (200 ml double distilled water, 8 ml of 5 M NaCl, 2 ml of 1 M Tris-Cl (pH 7.5), 0.4 ml of 0.5 M EDTA (pH 8.0)). Then centrifugation was performed, and the supernatant was retrieved carefully. The supernatant was subjected for further purification using ethanol precipitation method[9].

Ethanol Precipitation method

Firstly, the concentration of the supernatant solution obtained from the previous step was adjusted to 0.3 M by adding 1/10 volume of 3 M sodium acetate solution, followed by the addition 2 volumes of cold 100% ethanol and the mixture obtained subjected to an overnight incubation at -20°C. Next day the mixture was spun for 30 min using a micro centrifuge then the precipitated DNA was collected, and the supernatant was discarded[10] The precipitated DNA was washed by spinning (using micro centrifuge) with 1 ml of 70% ethanol for 5seconds and discarded the supernatant, the washing process was repeated twice, and the precipitate was dried and re-suspended in water. The obtained precipitate was linear BAS-Aptamer-SH which subjected for further processes (phosphorylation and ligation) for circularization[11].

Phosphorylation of the purified Linear BAS-Aptamer-SH

Reaction mixture of ATP (10 mM), Linear BAS Aptamer-SH (1660 pmol), Reaction buffer A (670Mm glycine-KOH (PH 9.5 at 25ºc, 67Mm $MgCl_2$, 10 Mm DTT), T4 Polynucleotide Kinase (332 U) and nuclease-free water enough amount to obtained volume of 664 µl of the reaction mixture was incubated for 1h at temperature of 37° C, followed by heating 75° C in dry bath for 10 min. The obtained phosphorylated BAS-Aptamer-SH (linear) was circularized using T4 DNA ligase [9].

Ligation (circularization) of linear phosphorylated BAS-Aptamer-SH

The following reaction mixture was prepared containing linker sequenced DNA (1660 pmol), Phosphorylation reaction mixture (500 nM) and T4 DNA Ligase Buffer (10X).

The phosphorylated reaction mixture prepared in the previous step was first heated at 90º C for 1 min and cooled at room temperature for 10 min. Then linker sequenced DNA (1660 pmol) and T4 DNA Ligase Buffer (10X) were added, mixed thoroughly followed by an overnight incubation at 16° C. The mixture was identified, purified by gel electrophoresis using poly acrylamide gel electrophoresis on a 10% denaturing gel (8 M urea). The required circular DNA was separated using the same ethanol precipitation method described above[12].

Confirmation of the separated circular BAS aptamer-SH

Confirmation of circularity of the BAS –Aptamer-SH obtained from the previous step was done by using EcoRI digestion method where nuclease free water (16µl) and 2µl 10x buffer ECORI was added to 1µl containing 1µg separated circular BAS-Aptamer-SH, mixed gently and spin down with minispin centrifuge for few seconds then the precipitate taken, incubated at 37ºC for 16 hours[13], then identified by gel electrophoresis using the procedure described above.

Purification of the obtained circular BAS-Aptamer -SH

The impurities including remaining linear BAS-Aptamer-SH and adenylated intermediate which might available with the obtained precipitate of circular BAS-aptamer-SH, were removed by using Exonuclease I enzyme which did not digest the circular BAS-Aptamer-SH but digests the linear one as well as the adenylated intermediate. This was done by adding Exonuclease I to the precipitate, mixed and incubated at 37° C for 45 minutes[13]. Then the pure circular BAS-Aptamer-SH was separated applying the same gel electrophoresis technique described above.

Characterization of purified circular aptamer BAS-SH

This was done using: -

H^1-NMR

The H^1-NMR spectrum performed for circular BAS aptamer –SH, using Inova H^1-NMR spectrometer (Varian company, USA) 500 MHz and DMSO solvent as internal standard [14].

UV absorption maxima (λ max) determination

BAS- Aptamer-SH (4.55 µM) was dissolved in phosphate buffer pH 7.4 and acetate buffer pH 5.5 each one separately to prepare a solution with concentration (0.0758 µM/mL). The stock solution was scanned using Perkin Elmer lambda 2 UV-visible spectrophotometer and 5 cm quartz cells, over a wavelength range of 200 to 400 nm, in comparison to pure BAS aptamer[15].

Preparation of BAS-Aptamer conjugated liposomes

It was performed by conjugating circular BAS-Aptamer-SH with DSPE-PEG2000-maleimide liposomes (as a lipid forming liposomes). This was achieved by the following steps:

Preparation of blank maleimide liposome

Liposomes were prepared using DSPE-PEG2000-Maleimide as a lipid in the presence of DPPC (synthetic lipid) and cholesterol as stabilizers. Mixture of DPPC, cholesterol, and DSPE-PEG-Mal in a 62; 35; 3 molar ratios were mixed and dissolved in chloroform. To obtain a thin film, chloroform was evaporated by using rotary evaporator at 50 °C for 5 min. the hydration of this film was then performed by adding 5 mL of PBS (phosphate buffer saline). The mixture was sonicated using probsonicator (300 watt) for 5 minutes[16]. Same procedure was applied to prepare maleimide liposome using phosphatidylcholine 94% purity (natural lipid) and cholesterol as stabilizers.

Characterization of the prepared blank maleimide Liposomes

Characterization of the prepared maleimide liposomes was done through the following: -

A. Zeta potential, Particle size and Poly dispersity index determination: -

Measurement of the mean particle size, zeta potential and polydispersity index were done using dynamic light scattering method (Zeta Plus Particle Sizingin) that measure light scattering fluctuations of liposomal particles. The average particle size, polydispersity index and zeta potential values were determined for both types of maleimide-liposomes prepared using synthetic and natural stabilizers[17].

B. Morphological study by TEM

A drop of each type of the prepared liposomes was allowed to be deposited on the circular copper film grid of 300 mesh and then stained with formvar and left for drying, then investigated under the microscope[18].

Conjugation of BAS aptamer with the prepared maleimide liposomes

Purified circular BAS aptamer-SH was re-suspended in binding buffer and heated at 70°C in dry bath for 10 minutes then cooled rapidly on ice for 10 minutes. To this cold mixture, each type of the prepared maleimide liposomes was added at a 0.5:1 molar ratio then incubated overnight at 4°C followed by dialysis to remove the non-conjugated BAS aptamer followed by centrifugation to collect the aptamer liposomes[16].

Identification for the BAS-Aptamer conjugated liposomes

A. Urea polyacrylamide gel electrophoresis (urea PAGE)

BAS aptamer conjugated -liposomes (before and after dialysis), blank maleimide-liposomes and free circular BAS aptamer-SH were loaded into 10% denaturing (8M urea) polyacrylamide gels and stained with Gel Red staining, followed by running in 1xTBE buffer at 15 V/cm. Images for analysis were identified by UV transilluminators using MF-ChemiBIS gel imaging system.

B. H^1-NMR:

The H^1-NMR spectrum performed for BAS-Aptamer conjugated with natural maleimide liposomes using Inova H^1-NMR spectrometer (Varian company, USA) 500 MHz and using DMSO solvent as internal standard[14].

Evaluation of the binding affinity of BAS-aptamer conjugated liposomes to SIRT1 enzyme:

The binding affinity of both types of BAS-Aptamer after conjugation to maleimide liposomes (containing natural or synthetic lipids) to SIRT1 enzyme were studied in (Manchester University, department of Microbiology; UK) by surface Plasmon resonance (SPR) using ProteOn™ XPR36 protein interaction array system. The principle of this step relied on the optical phenomenon of SPR. This system was carried out at 25° C in running phosphate buffer pH 7.4 containing 100 mM NaCl and 5 mM $MgCl_2$[19].

Cell-line study for the prepared BAS-aptamer conjugated liposomes

Culturing of the cells

Human breast cancer cells, MCF-7(stored under liquid nitrogen) was obtained from American Type Culture Collection ATCC (Middlesex, UK). MCF-7 cells were placed in 15ml DMEM medium containing 10% fetal- bovine serum FBS and 1% L-Glutamine as well as 1% Penicillin-Streptomycin-Amphotericin B 100X as antiseptic. Cells were cultured in 75cm^2 flasks and incubated in 5% CO_2,95% humidified air at 37°C. After 1 day, the DMEM medium was removed and replaced with fresh DMEM (15ml) and the work repeated for 3 days. Once the cells reach 90% confluency, the DMEM medium was removed and the cells were subjected to washing with phosphate buffer saline pH 7.4 (this was repeated 3 times), then the cells were incubated with trypsin solution for 2 min at 37ºC. Centrifugation of the cells was performed and the supernatant subjected to separation step and the resulting cells were re-suspended in 1ml DMEM medium and counted under the microscope on a haemocytometer. The

cells were stored at -80 °C for 24hrs and then kept under liquid nitrogen[20, 21].

Cell viability by MTT assay at different incubation time

The cells were rapidly thawed at 37 °C, and then 10 mL fresh growth medium (DMEM medium) was added. Harvesting of the cells was done through centrifugation. Then the cells were re-suspended in fresh medium and placed in flask to grow [21] as early explained. Cells suspension (MCF-7) were subjected to MTT assay to assess the effects of aptamer on breast cancer cell viability where the cells dispensed in each of 96-well flat-bottom tissue culture plates at concentrations of 5 x 10^3 cells in DMEM medium per well for 24h incubation under standard conditions (5% CO_2,95% humified air at 37ºc), 4 x 10^3 cells/well for 48h incubation, 3 x 10^3 cells/well for 72h incubation and 750 cells/well for 7 days incubation. The cells (incubated for 24 hours) were treated with 2.5µM pure BAS-Aptamer, both types of BAS-Aptamer conjugated liposomes (natural and synthetic), and circular BAS-Aptamer –SH then incubated for 24 hours. The cell culture medium was removed and re-incubated with 30µL MTT stain solution (equivalent 3mg/ml MTT in PBS) for 4 hours at 37ºC.After that the MTT solution was removed by inversion the wells and tapping on tissue paper. A 100µL of DMSO was added to all wells, the plates were then kept at room temperature in the dark for about 10-15min. Then the absorbance of stained cells was measured by multi-scan reader at 540nm and compared to control well containing DMEM medium with cells only.

The cells incubated for 48 hours and cells incubated for 72 hours as well as those incubated for 7 days, where in each case the cells treated with 2.5µM pure BAS-Aptamer, both type of BAS-Aptamer conjugated liposomes and circular BAS-Aptamer-SH and incubated for 48 hours, 72 hours or 7 days respectively then MTT stain was added and the procedures carried on similar to that for 24hours incubation. For circular BAS-Aptamer-SH; it was added for cells incubated for 24,48 and 72 hours only [20, 21].

The half maximal inhibitory concentration (IC_{50}) value study

IC_{50} is concentration of the drug needed for 50% inhibition of the cell viability. Different concentrations 0.02, 0.04, 0.078, 0.3125, 0.625, 1.25 and 2.5 µM of each of pure BAS-Aptamer, both type BAS-Aptamer liposomes were applied on MCF-7 cells applying the same method in section 2.7.2, incubated for 72 hours and assayed using the MTT assay [22]

Stability study of BAS-Aptamer conjugated liposomes in plasma by two different methods

Samples: BAS aptamer synthetic and natural liposomes were mixed with l human plasma, then added to equal volume of water and incubated at standard incubation condition for 0, 24 hr, 48 hr, 72 hr, 5, 6 and 7days. At the end of each incubation time, the sample was quickly frozen in a deep freezer and prior to analysis the samples were thawed at 37ºC [23].

Analysis by Urea PAGE: Sample at each incubation time was mixed with equal volume of stop solution (8M urea, 50mM EDTA) and frozen at -20°C. The samples were examined using 10% denaturing (8M urea) polyacrylamide gels and stained by gel red, then visualised by UV (ultraviolet transilluminator) spectroscopy at 254 nm[24].

Analysis by HPLC-UV method: Samples were analysed using a HiChrom ACE-Excel 5 super C18 (150 x 4.6mm) column and detected by UV at 256nm. The mobile phase used was 20 % aqueous acetonitrile and acetonitrile in gradient profile. The rate of flow was 1.4 ml/min and the temperature of the column was maintained at 37 ºC. Data was acquired and stored by peak pro data acquisition system "aptamer"[25].

Stability study of BAS-aptamer conjugated liposomes in different pH (5.5 and 7.4) by HPLC

BAS-aptamer conjugated synthetic and natural liposomes were incubated with phosphate buffer pH 7.4. and pH 5.5, then the samples were incubated at 37ºC for different incubation time (0, 1, 2, 3, 4, 5, 6 and 7 days). After that, the samples were analysed by HPLC

Statistical Analysis

For results' statistical analysis, One-way ANOVA with Tukey (9.1 software) were performed considering values of $p<0.05$ as statistically significant.

Results and Discussion

Preparation of Aptamer

To facilitate the conjugation it is necessary to introduce SH group to DNA drug for coupling process by making the bio conjugation more readily available for the reaction[26]. Introducing SH group for activation of BAS-aptamer-SH makes it linear that has to be circulized. For purification and to ensure the nucleotide sequence of BAS-aptamer-SH, denaturing PAGE was used and gel red staining for visualization of the separated bands was applied then the linear BAS-aptamer-SH was extracted and purified, then activated and the two ends were ligated to get circular BAS-aptamer-SH. The circularization was confirmed using gel urea PAGE and the results is shown in figure 1 were the circulized BAS products migrated faster than linear one due to topological reasons[27].

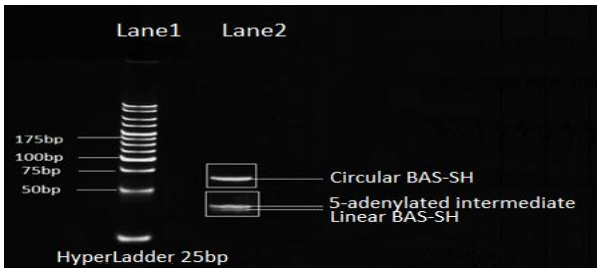


Figure 1. Circularization of BAS aptamer.

Lane 1, DNA markers 25bp; lane 2, circularization proceeds through an adenylated intermediate containing circular BAS-SH, 5-adenylated intermediate together with remaining linear BAS-SH.

Adenylated-oligo intermediate can be seen as a band just above the remaining linear BAS aptamer-SH. This 5`-adenylated intermediate is produced in the initial part of ligation. Both the remaining linear BAS-aptamer-SH and linear single-stranded adenylated intermediate were removed by treatment with Exonuclease I which digests linear ssDNA and the linear adenylated intermediate while circular BAS-aptamer-SH is resistant to these exonucleases. The confirmation of the purity of the isolated product is shown in figure 2.

Circular aptamer is resistant to exonuclease degradation and endonuclease in blood serum that confer their stability and hence their pharmacological activity[28]. Polyacrylamide gel electrophoresis (PAGE) represents a gentle, high resolution and versatile method for molecule fractionation and characterization of physico-chemical properties depending on the size, conformation, and net charge of such molecules. The reaction of polymerization can be precisely directed to generate highly ordered gels with measurable and reproducible size of pores over a wide range. This improve the possibility to obtain reproducible relative mobility (*Rf*) values as physico-chemical constants[29].

Characterization of circular BAS-Aptamer-SH

The pure circular BAS aptamer-SH was characterized by nuclear magnetic resonance spectroscopy (NMR) which depends on the energy absorption after the excitation of the nucleus of an atom from a state of low spin energy to the next higher one[30]. A proton nuclear magnetic resonance (H^1-NMR) spectrum provides information about the environments of different hydrogens in a molecule which will allow for determining the molecular structure of substance.

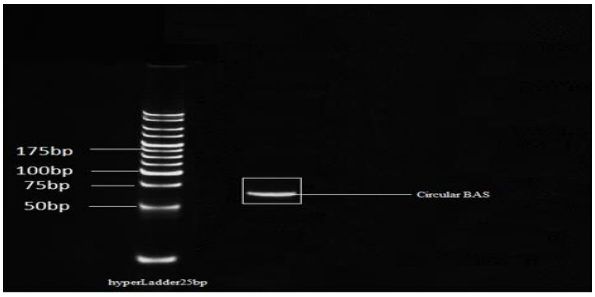


Figure 2. Confirmation of the purity of circular BAS-aptamer-SH.

Table 1 shows the interpretation of the main characteristic peaks in the ^{1}H-NMR spectrum of the circular BAS aptamer-SH (figure 3) where it shows a peak with a chemical shift of δ 2.42 for the hydrogen atoms attached to sulphur atoms, indicating the successful introducing of -SH group to the pure BAS aptamer.

λmax determination

Circular BAS aptamer-SH scanning by UV spectrophotometer in pH 7.4 showed maximum absorbance at 258 nm and in pH 5.5 was 255 nm in comparison to BAS aptamer which showed absorbance at pH 7.4 equal to 257 nm and at PH 5.5 equal to 259 nm. Figure 4 showed the UV spectra of both in PH 7.4 where no effect of pH on λ_{max} of the compounds.

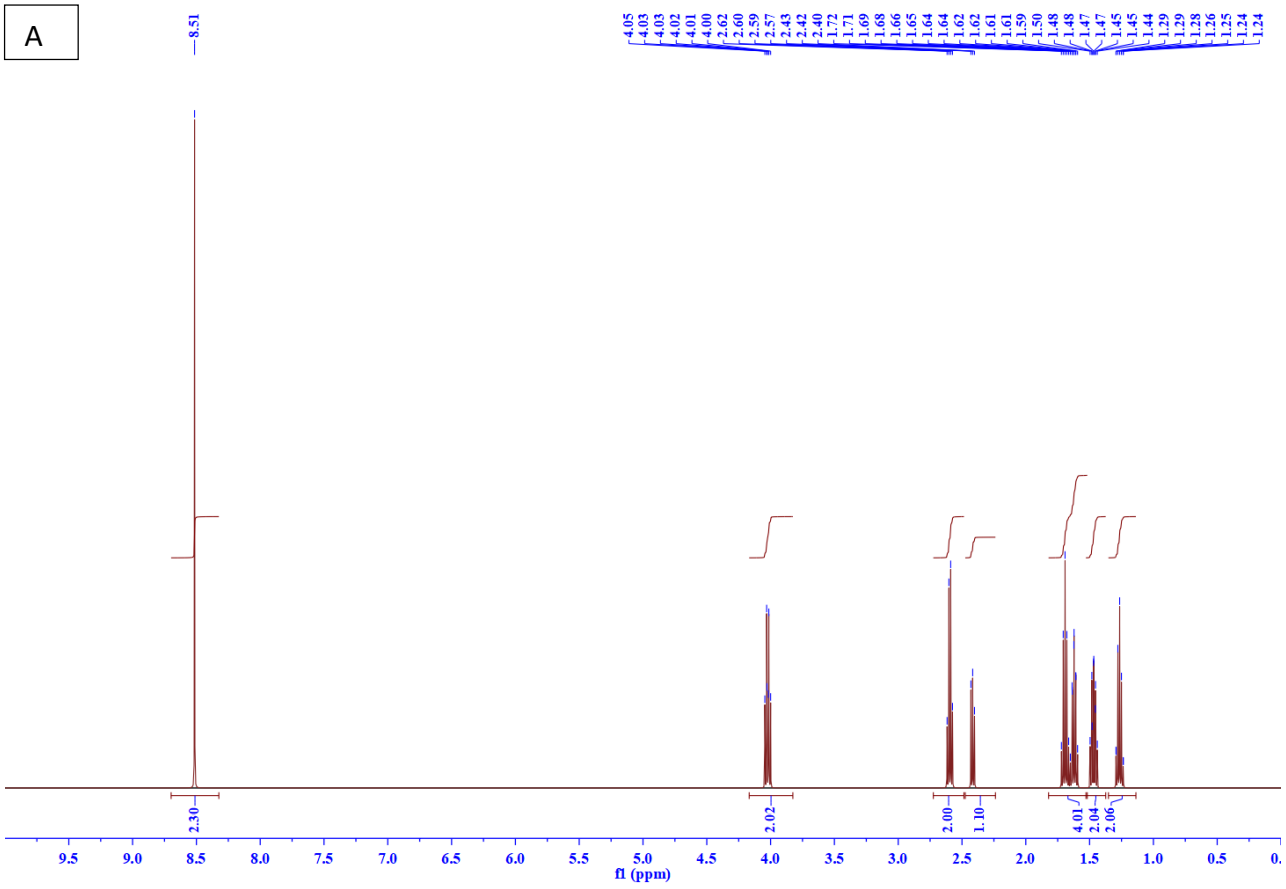
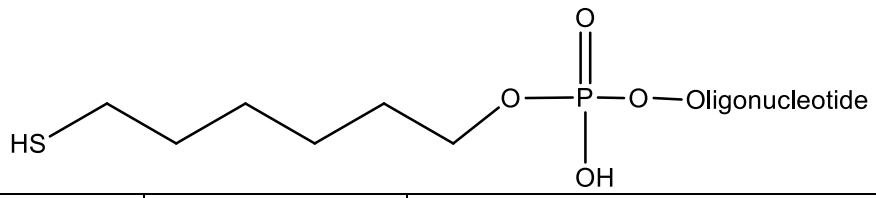


Figure 3. ^{1}H-NMR spectrum of circular BAS-aptamer-SH.

Table 1. Interpretation of ^{1}H-NMR spectrum of BAS-aptamer-SH.



Chemical shift ppm	NO. of H	Interpretation
1.25-1.72	8	Multiplet, for CH_2 protons of the CH_2-CH_2-CH_2-CH_2
2.42	1	Triplet, for SH
2.6	2	Triplet of Singlet for CH_2 that attached to the SH
4.02	2	Triplet, for CH_2 protons that attached to O-P
8.51	2	Singlet, for 2 OH groups

Preparation of BAS aptamer conjugated liposomes:

This involved two steps:

1. Preparation of blank maleimide liposomes.
2. Conjugation of the circular BAS aptamer-SH with the blank maleimide liposome.

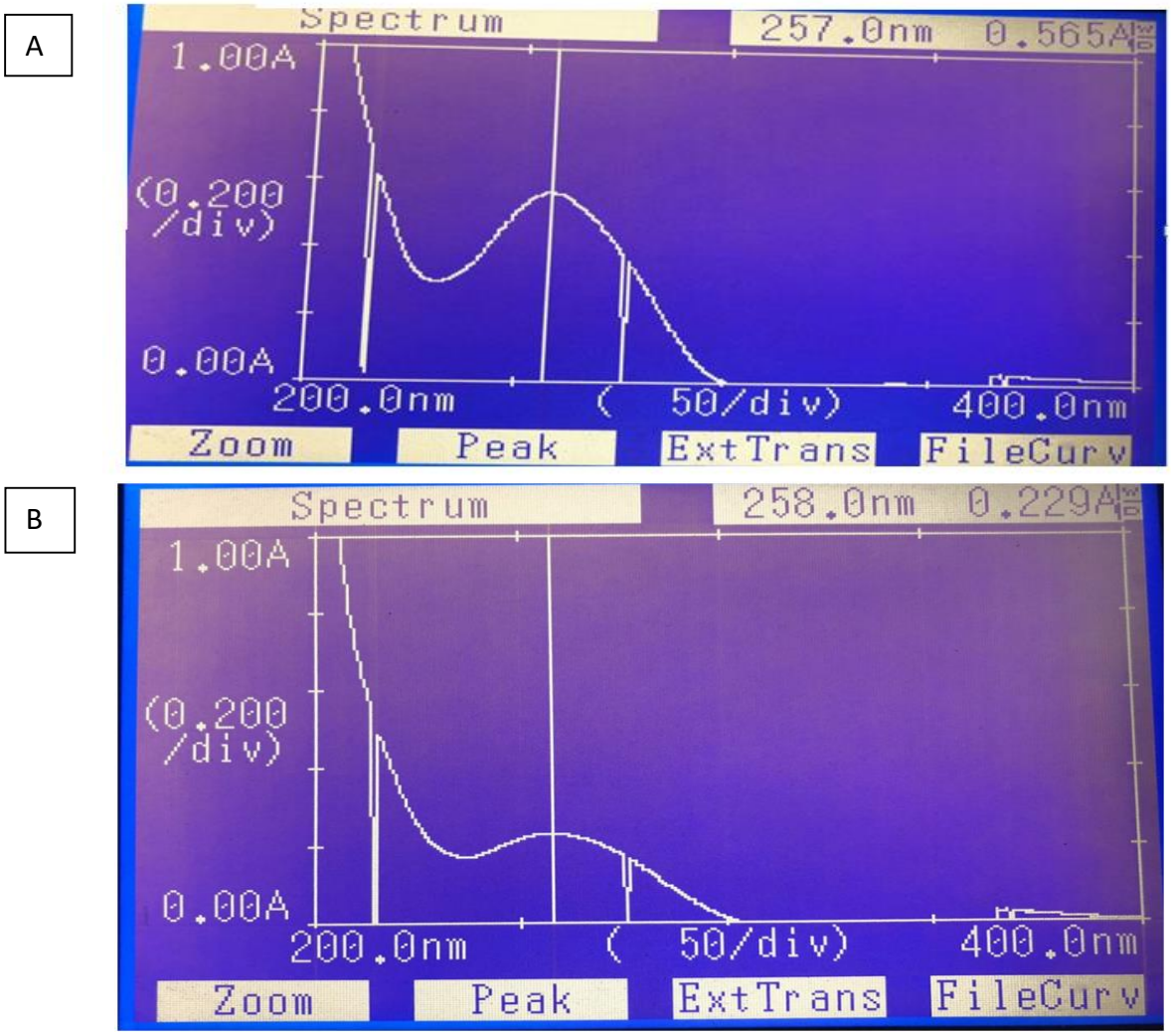


Figure 4. UV Spectra of (A)BAS-aptamer-SH (B) Pure BAS-aptamer in pH 7.4.

Preparation of the blank maleimide liposomes

DSPE-PEG (2000) maleimide phospholipid was used to prepare functionalized surface liposome (its structure shown in figure 5). For optimization of the prepared liposome, two types of maleimide liposomes were prepared:

First: DSPE-PEG-Maleimide liposome was prepared in presence of cholesterol and DPPC (synthetic lipid) as stabilizers.

Second: DSPE-PEG-Maleimide liposomes were prepared in presence of cholesterol and phosphatidyl choline (94% purity natural lipid) as stabilizers.
Maleimide was utilized with a thiol-reactive moiety in lipoplex preparations of *N*- (4- (*p* -maleimidophenyl) butyryl) dipalmitoylphosphatidyl ethanolamine (MPB-DPPE) / lipospermine/ deoxyribonucleic acid / lipospermine/ deoxyribonucleic acid (DNA) for the improvement of the delivery of gene. The modification of Maleimide could be utilized for the targeting of the surface of cell and the utilization of it for the cellular particles internalization was greatly increased[31] .

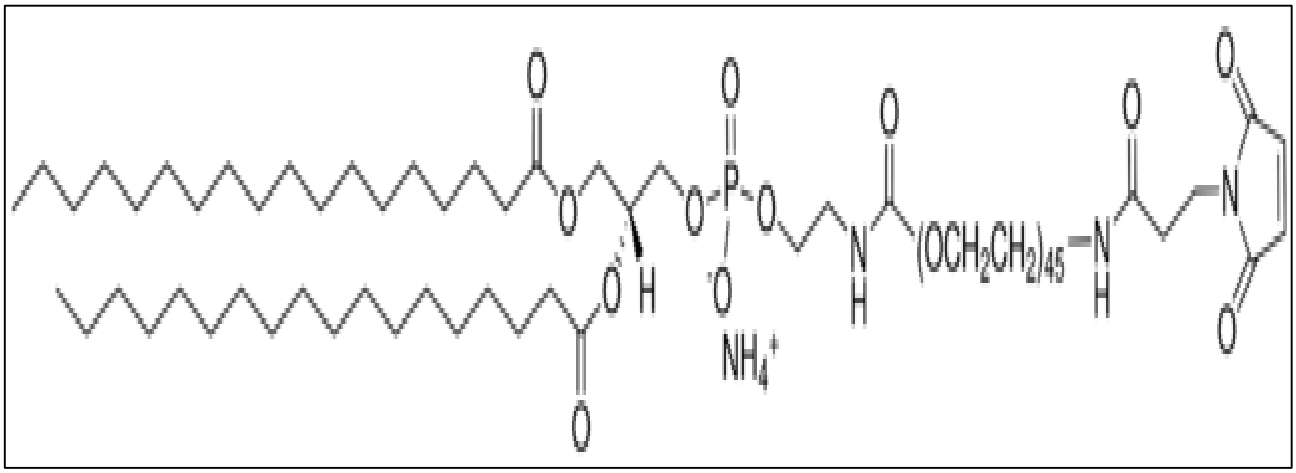


Figure 5. Structure of DSPE-PEG-Maleimide[32]

Characterization of the prepared blank maleimide liposome

The two types of the prepared blank maleimide liposomes (using the synthetic or the natural stabilizers) were characterized as follow:

A. Zeta Potential, Particle size and Poly dispersity index (PDI) determination: -

The method of Zeta potential measurement used to determine the electrostatic potential at the surrounding electrical double layer of particle in solution. Particles that showed values of zeta potential ranged from −10 and +10 mV regarded as nearly neutral particles, whereas those with zeta potentials values of higher than +30 mV or lower than −30 mV regarded as strongly cationic or strongly anionic, respectively. Given that the most cellular membranes carried a negative charge, the values of zeta potential have an influence on the tendency of the nanoparticle to cross the membranes, with cationic particles generally give rise to more toxicity in association with the disruption of cell wall.
The Zeta potential of the blank maleimide liposome (using natural or synthetic stabilizers) were -28.93 mV and -25.09 mV respectively, indicating good stability of the prepared blank liposomes.
Table 2 shows that the particle size of the prepared maleimide liposomes using PC (as natural stabilizer) were significantly smaller ($p<0.05$) than the particle size of liposomes prepared using DPPC (as synthetic stabilizer) that could be due to the difference in molecular organization indicating the efficiency of using natural stabilizer. The Polydispersity index (PDI) value of (0.05-0.7) is considered to be desirable for uniform distribution and homogeneity of particles, while PDI value > 0.7 to less than 1 is considered to have a broad distribution of particle size[33]. Both types of the prepared liposomes showed a uniform distribution for particle size.

B. Morphological study by TEM

Both types of blank maleimide liposomes (with synthetic or natural stabilizers) were examined by TEM and the photographs are shown in figures 6. The TEM images revealed spherical structures surrounding an inner core indicating liposome formation, similar shape observed with pilocarpine nitrate liposomal formulation[34].

Table 2. Particle size, PDI and zeta potential of the prepared blank maleimide liposomes.

Sample 1	Diameter (nm) ± SD	PDI ± SD	Zeta Potential (mV) ± SD
maleimide liposome (with natural stabilizer (PC))	209.64 ± 2.81	0.356 ± 0.049	-28.93 ± 0.106
maleimide liposome (with synthetic stabilizer)	400.81 ± 1.93	0.484 ± 0.0046	-25.09 ± 0.156

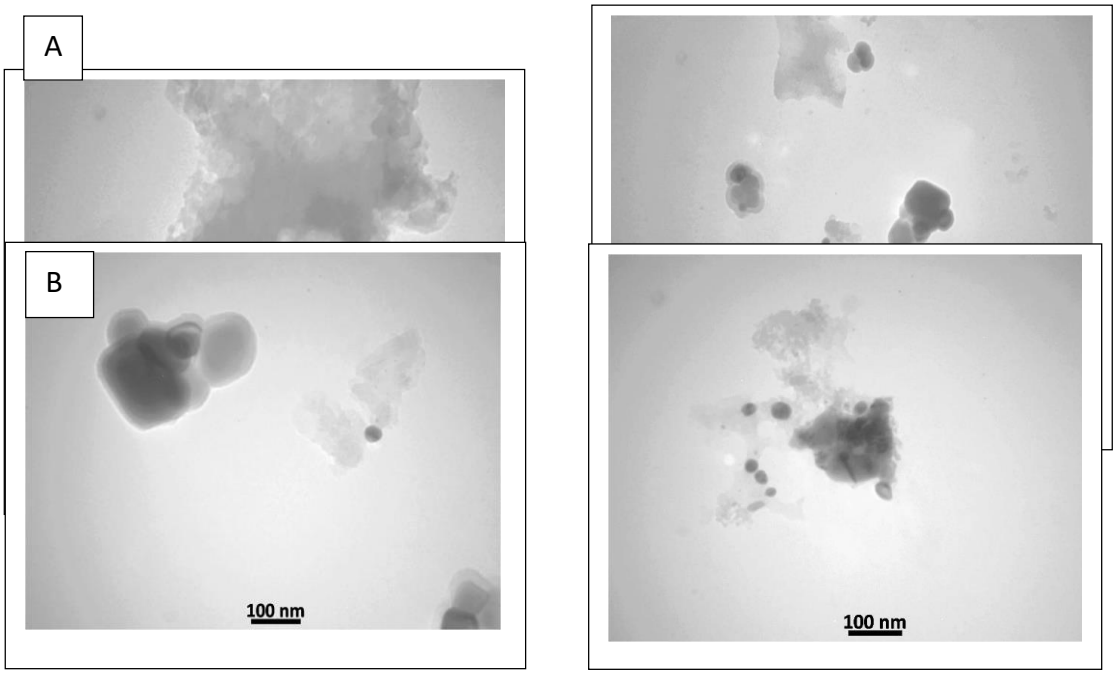


Figure 6. TEM photographs of (A): blank maleimide liposomes (with natural stabilizer) (B): blank maleimide liposomes (with synthetic stabilizers)

Conjugation of BAS-aptamer-SH with the blank maleimide liposomes

The two types of the prepared blank maleimide liposomes (those prepared with synthetic and natural stabilizer), were conjugated with the circular BAS-aptamer-SH and the conjugation was carried out using thiol-maleimide crosslinking[35], which is one of strategies to develop a drug delivery system able to selectively bind to its target site[36]. The BAS aptamer conjugated liposome containing natural stabilizers will be referred to as BAS aptamer natural liposomes and those containing synthetic stabilizer will be referred to as BAS aptamer synthetic liposomes during this study.

Identification for the BAS-Aptamer conjugated liposomes

A-Using urea polyacrylamide gel electrophoresis (urea PAGE)

In electrophoresis, each compound would have a characteristic mobility feature. In figure 7 the liposomes (blank Maleimide-liposome before conjugation (Lane 4) and after conjugation with the drug and dialysis (lane 3)) shows no spot or band (black background) since they have no mobility in gel electrophoresis chromatogram[37] while a white band for pure BAS aptamer-SH (lane 2) was observed. A faint white band (lane 1) for BAS aptamer conjugated liposome before dialysis since it contains small amount of pure BAS aptamer-SH in addition to the conjugated liposomes, therefore dialysis was efficient to obtain highly purified conjugated liposomes. The results also showed the difference in the mobility of pure circular BAS aptamer-SH (lane 1) and the pure conjugated liposomes (lane 3) which confirmed the conjugation process. Similar results observed with other aptamers[38].

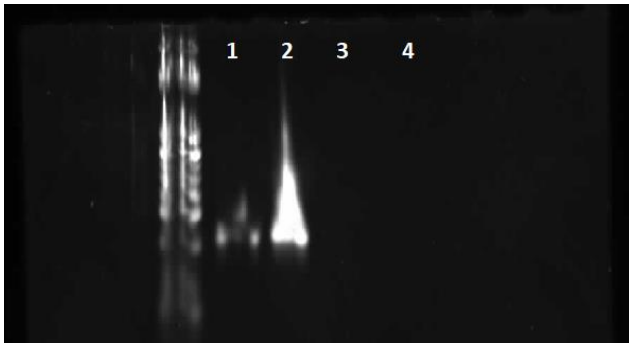


Figure 7. Characterization of liposomes before and after BAS aptamer conjugation using Urea PAGE gel electrophoresis.

Lanes: 1, BAS aptamer-conjugated Liposome before dialysis; 2, free BAS aptamer; 3, BAS aptamer-conjugated Liposome after dialysis; 4, Mal-functionalized Liposome

B- H^1-NMR measurement

H^1-NMR measurement for BAS-Aptamer conjugated natural liposomes is shown in (figure 8, table 3). The disappearance of peak with chemical shift of 6 2.42 that was appeared in 1H-NMR of BAS aptamer-SH (figure 3) for -SH which further indicated that the conjugation occurred at the position of SH group.

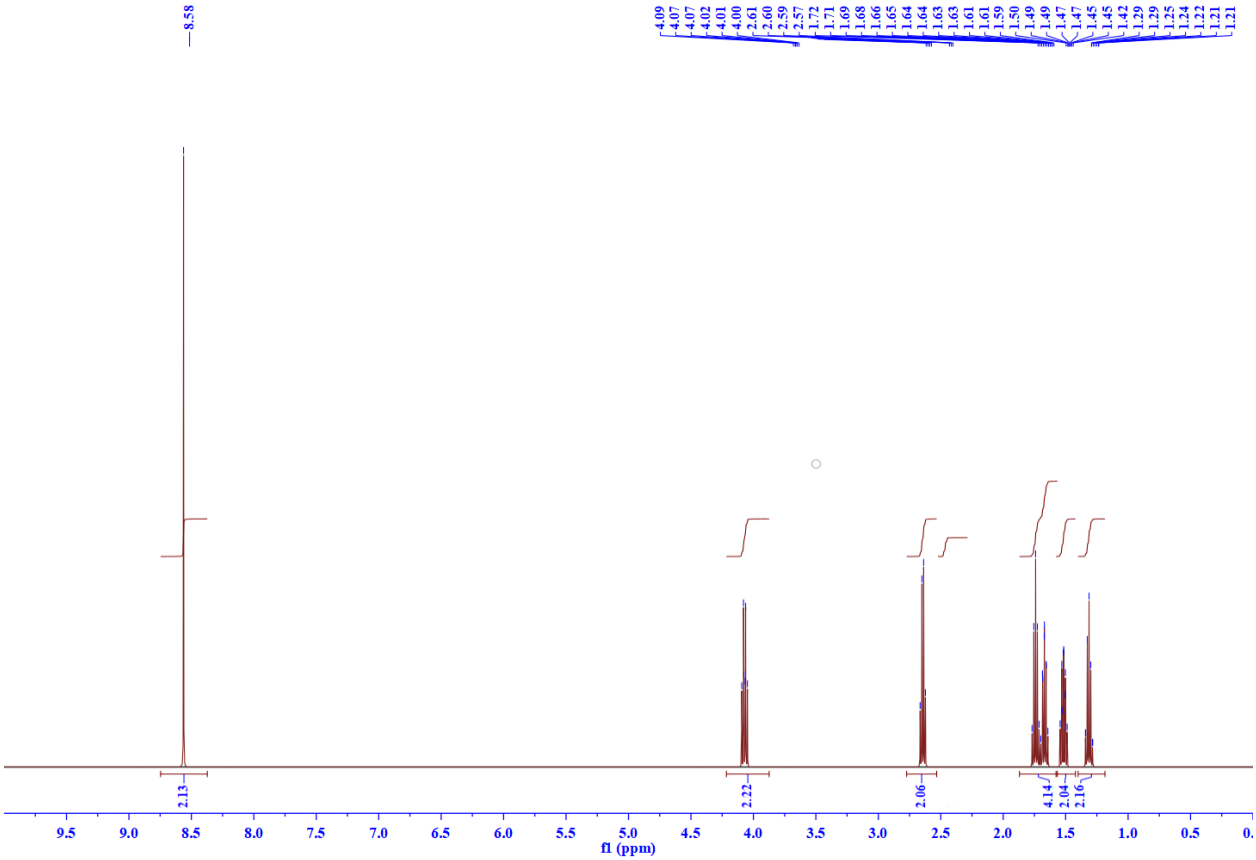
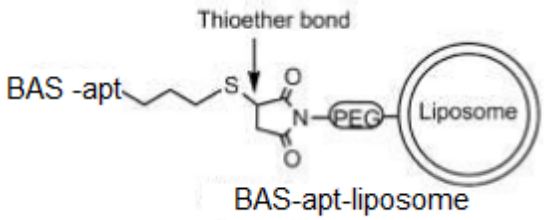


Figure 8. [1]HNMR of BAS-aptamer conjugated liposomes.

Table 3. Interpretation of HNMR chart of BAS-aptamer conjugated liposome



Chemical shift ppm	NO. of H	Interpretation
1.21-1.72	8	Multiplet, for CH_2 protons of the CH_2-CH_2-CH_2-CH_2
2.57	2	CH_2 protons that attached to the CH_2-S and overlap with the CH of maleimide ring
4.0	2	Triplet, for CH_2 protons that attached to O-P
8.58	2	Singlet, for 2 OH groups

Binding affinities of BAS aptamer conjugated liposomes to its target (SIRT1 enzyme).

Binding affinity is the strength of the binding interaction between a single biomolecule (e.g. protein or DNA) to its ligand/binding partner. This affinity is typically determined and reported by the equilibrium constant of dissociation (KD) that is utilized to assess and rank the strengths order of interactions among biomolecules. If the value of KD was small, it indicates a great binding affinity between the drug and its target. Binding affinity is also affected by non-covalent intermolecular interactions such as electrostatic interactions, hydrogen bonding, Van der Waals forces and hydrophobic bonds between the two molecules. In addition, binding affinity between a ligand and its target molecule may be affected by the presence of other molecules [39].

Figure 9 and 10 show the curve used to determine the affinity of both types of the prepared BAS aptamer

conjugated liposomes (natural and synthetic) and those prepared using synthetic stabilizer (referred to as BAS aptamer synthetic liposome) for its target site (SIRT1) using surface Plasmon resonance. From these data the association constant (Ka), dissociation constant (Kd) and the equilibrium rate constant (KD) were calculated. Assuming that the binding of the drug to its SIRT1 is second order reversible rate reaction in 1:1 ratio by

Drug + receptor ⇌ Drug-receptor

The KD constant can be calculated by Kd/Ka ratio[40].

Table 4 demonstrates the Ka, Kd, and KD constants for BAS aptamer natural and synthetic liposomes as well as for pure BAS aptamer. The KD value for the natural liposomes is significantly lower ($p<0.05$) than the synthetic liposomes mean that the binding affinity of the natural liposome is much higher than the binding affinity of the synthetic liposome which can be attributed to the stability of the prepared liposome and indicating the efficacy of using natural stabilizer (natural phosphatidylcholine) on the stability and binding affinity of the drug conjugated liposome.

There is no significant difference between the binding affinity of BAS aptamer natural liposome and pure BAS aptamer, while the binding affinity of BAS aptamer synthetic liposome is significantly lower (higher KD value) than both natural liposome and pure BAS aptamer. The administration of the drug in the liposomal form may minimize the engulfment of the drug by the macrophage and immune body system than the pure drug. As well as the presence of liposomes may facilitate the entry of the drug into the cells (in vivo) than the pure hydrophilic drug (BAS aptamer)[12]. The high binding affinity indicates better pharmacological.

Cytotoxic activity of the prepared BAS aptamer-natural liposome and BAS aptamer-synthetic liposome.

The in vitro cytotoxic activity of the prepared BAS aptamer natural and synthetic liposomes against MCF7 (breast cancer cells) using cell-line study in comparison to the pure BAS aptamer and circular BAS aptamer-SH after different incubation time was tested using MTT colorimetric assay method which is simple, well-characterized method used to evaluate detrimental intracellular effect on metabolic activity[41].

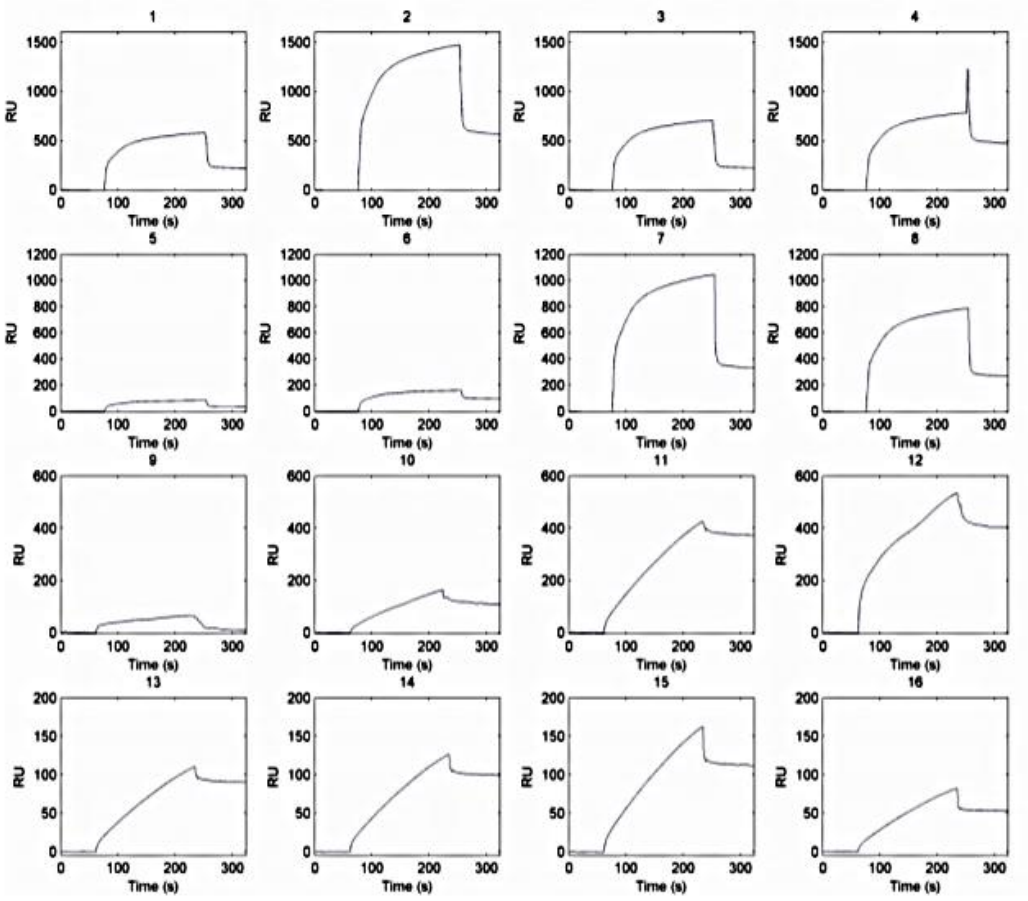


Figure 9. Raw (RU) data for affinities.

All the data injection starting RU are normalized to zero. Plot 1-4 are BAS aptamer-natural liposome binding sensorgrams for 6.2, 12.5,25, 50, 100 nM SIRT1; Plot 5-8 are BAS aptamer-natural liposome binding sensorgrams for 200, 400, 800 nM SIRT1; Plot 9-12 are BAS aptamer-synthetic liposome binding sensorgrams for 6.2, 12.5,25, 50, 100 nM SIRT1; Plot 13-16 are BAS aptamer-synthetic liposome binding sensorgrams for 200, 400, 800 nM SIRT1.

Table 4: binding parameters for the interaction between BAS aptamer natural and synthetic liposomes with SIRT1 enzyme using surface Plasmon resonance

	K_a (M^{-1} s^{-1}) ± SD	K_d (s^{-1}) ± SD	KD (nM) ± SD
BAS aptamer natural liposome	1.4(±0.6) ×10^{-5}	5.4(±0.3) ×10^{-4}	38.5± 0.985
BAS aptamer synthetic liposome	8.9(±1.4) ×10^{-6}	6.1(±0.2) ×10^{-4}	68 ± 0.967
Pure BAS aptamer [95]	1.480 (±0.24) ×10^{-4}	0.00401 ± 0.59	27.09 ± 0.959

Ka: association constant, Kd: dissociation constant, KD: equilibrium rate constant.

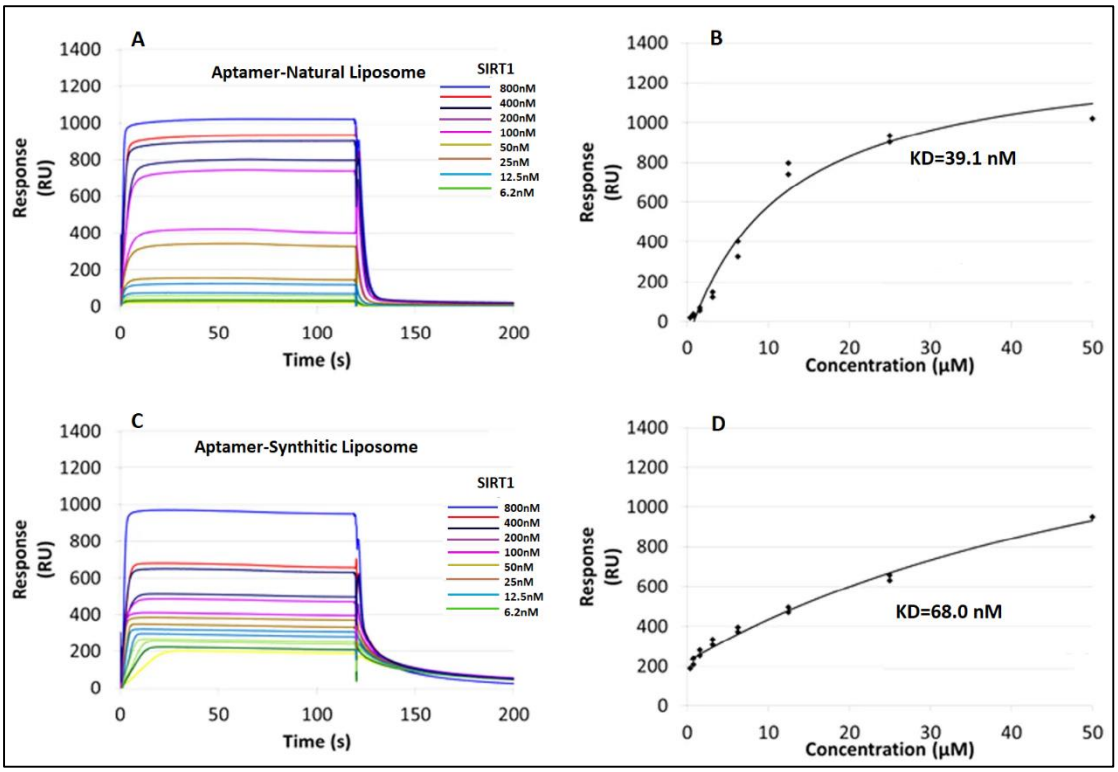


Figure 10. Determination of the affinities of BAS aptamers natural and synthetic liposomes for SIRT1 using surface Plasmon resonance.

(A) Sensorgrams of the binding response to aptamer natural liposomes measured for concentrations of 6.2, 12.5, 25, 50, 100, 200, 400 and 800 nM SIRT1. The KD = 39.1 nM as determined from a global fit of the kinetic simultaneous Ka/Kd model, assuming Langmuir (1:1) binding, and x^2= 0.985, (B) Plot of the steady-state affinity for `A' using the Req values derived from sensorgrams in (A) fitted locally. (C) Sensorgrams of the binding response to aptamer synthetic liposome measured for concentrations of 6.2, 12.5, 25, 50, 100, 200, 400 and 800 nM SIRT1. The KD = 68 nM as determined from a global fit of the kinetic simultaneous Ka/Kd model, assuming Langmuir (1:1) binding, and x2= 0.967, (D) Plot of the steady-state affinity for `C' using the Req values derived from sensorgrams in (C) fitted locally.

Figure (11) shows the time response curve for the prepared drug conjugated liposome (both synthetic and natural) in comparison to BAS- aptamer and BAS- aptamer SH, the results showed that in all cases the cytotoxic effect of the drug is increased as the incubation time increased in all concentrations used (0.63, 1.25 and 2.5µM). the cytotoxic effect of the prepared liposomes was higher than those for BAS aptamer and BAS aptamer-SH which may be attributed to the stability of the liposome in the cells and the improved targeting effect of the drug upon conjugation with liposome towards the enzyme (SIRT1) available in the cells [42]. In addition, the results showed the highly significant effect on time-response curve for the BAS aptamer natural liposome in all the concentration used which further confirmed the contribution of the natural lipids used as stabilizers in the prepared liposome on the cytotoxic effect of the drug where it reached 100 % killing effect on the breast cancer cells MCF-7 using 2.5 µM concentration.

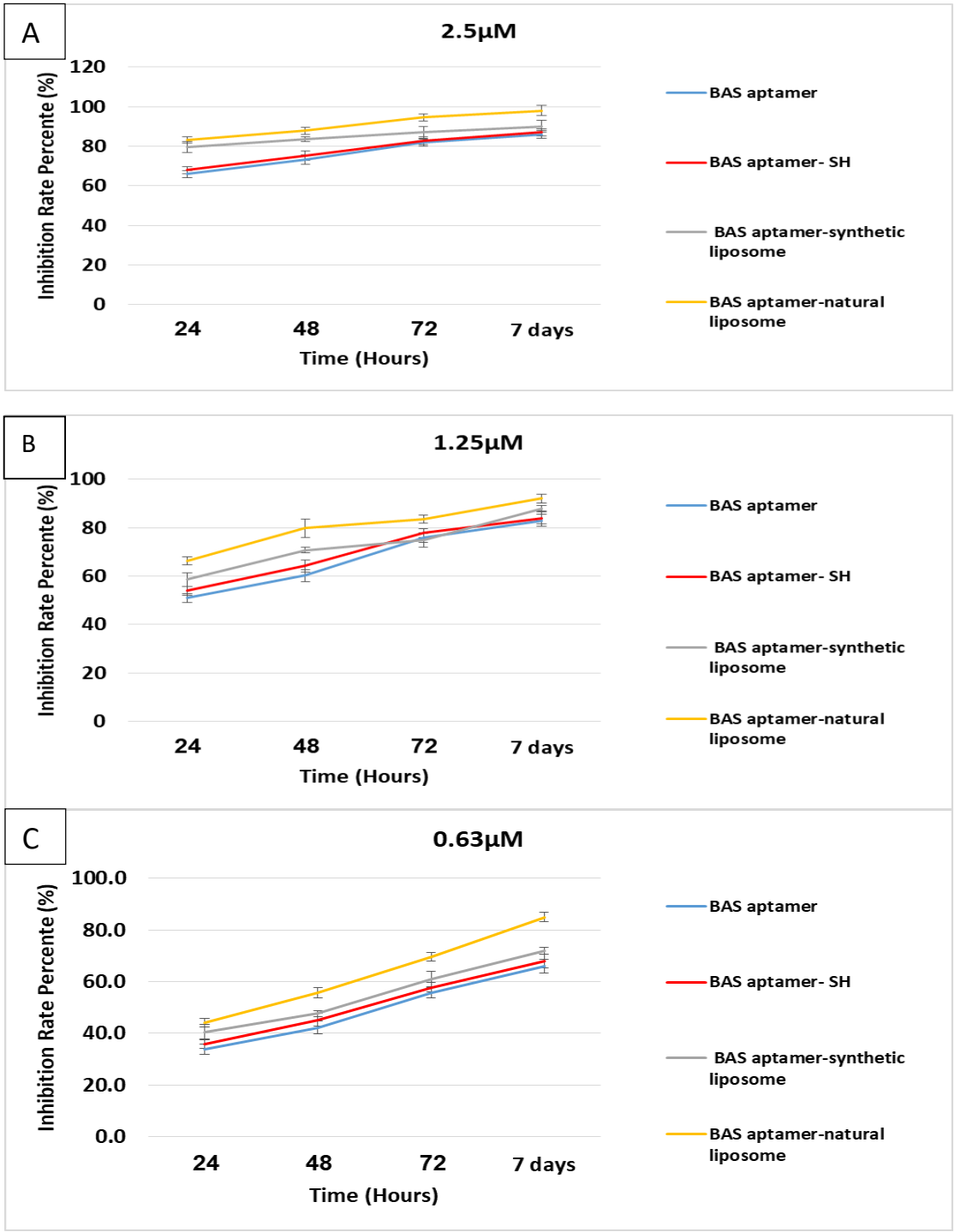


Figure 11. Time-response curves for comparative *in vitro* cytotoxicity of BAS aptamer-natural and synthetic liposome, BAS aptamer and BAS aptamer-SH on MCF-7 cancer cell line.

Using (A): Concentration 2.5 µM, (B): Concentration 1.25 µM, (C): Concentration 0.63 µM. The result represents the mean of 3 independent experiment $P<0.05$ vs control.

Figure 12 shows the concentration-response curves for the cytotoxic effect of the BAS aptamer liposomes (natural and synthetic) in comparison to BAS aptamer (after 24, 48, 72 hours and 7 days) and BAS aptamer -SH after (24, 48 and 72 hours) on MCF-7 breast cancer cell-line study. The results agreed with that observed with (figure 11) where BAS aptamer liposome showed higher inhibition rate (cytotoxic effect) than pure BAS aptamer and BAS aptamer-SH and continued even for 7 days and the natural liposome showed much higher cytotoxic effect which further confirmed the efficacy of natural liposome.

Determination of the Half Maximal Inhibitory Concentration (IC_{50}) Value

IC50 considered as the most commonly used and informative way of determination of a drug's efficacy. IC50 also indicate the amount of drug required to inhibit a half of biological process and can be considered as representative measure for the drug potency in pharmacological study[43].

In this work, the dose-response curve was constructed by plotting the percentage of cell death versus log concentration for free BAS aptamer, BAS aptamer-synthetic liposome and BAS aptamer-natural liposome in MCF-7 cells as shown in figure (13 A, B, C). The IC_{50} values for free BAS aptamer, BAS aptamer-synthetic liposome and BAS aptamer-natural liposome were (0.61, 0.42, 0.49μM) in MCF-7 cells respectively.

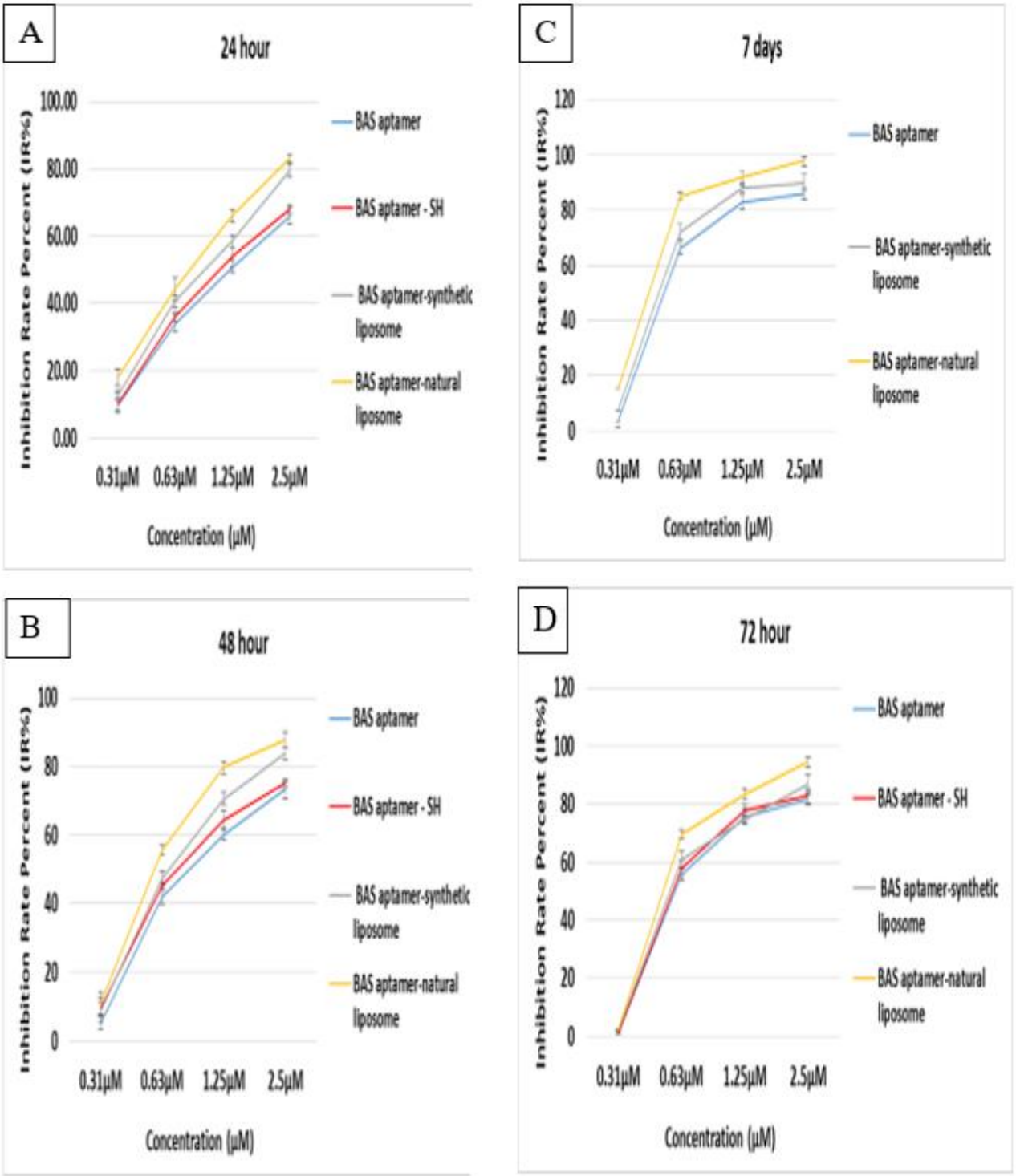


Figure 12. Comparative concentration-response curve for in vitro cytotoxicity of BAS aptamer-natural and synthetic liposomes, BAS aptamer and BAS aptamer-SH on MCF-7 cell line.
(**A)**: Exposure time is 24 hours, (**B)**: Exposure time is 48 hours, (**C)**: Exposure time is 72 hours**, (D)**: Exposure time is 7 Days.

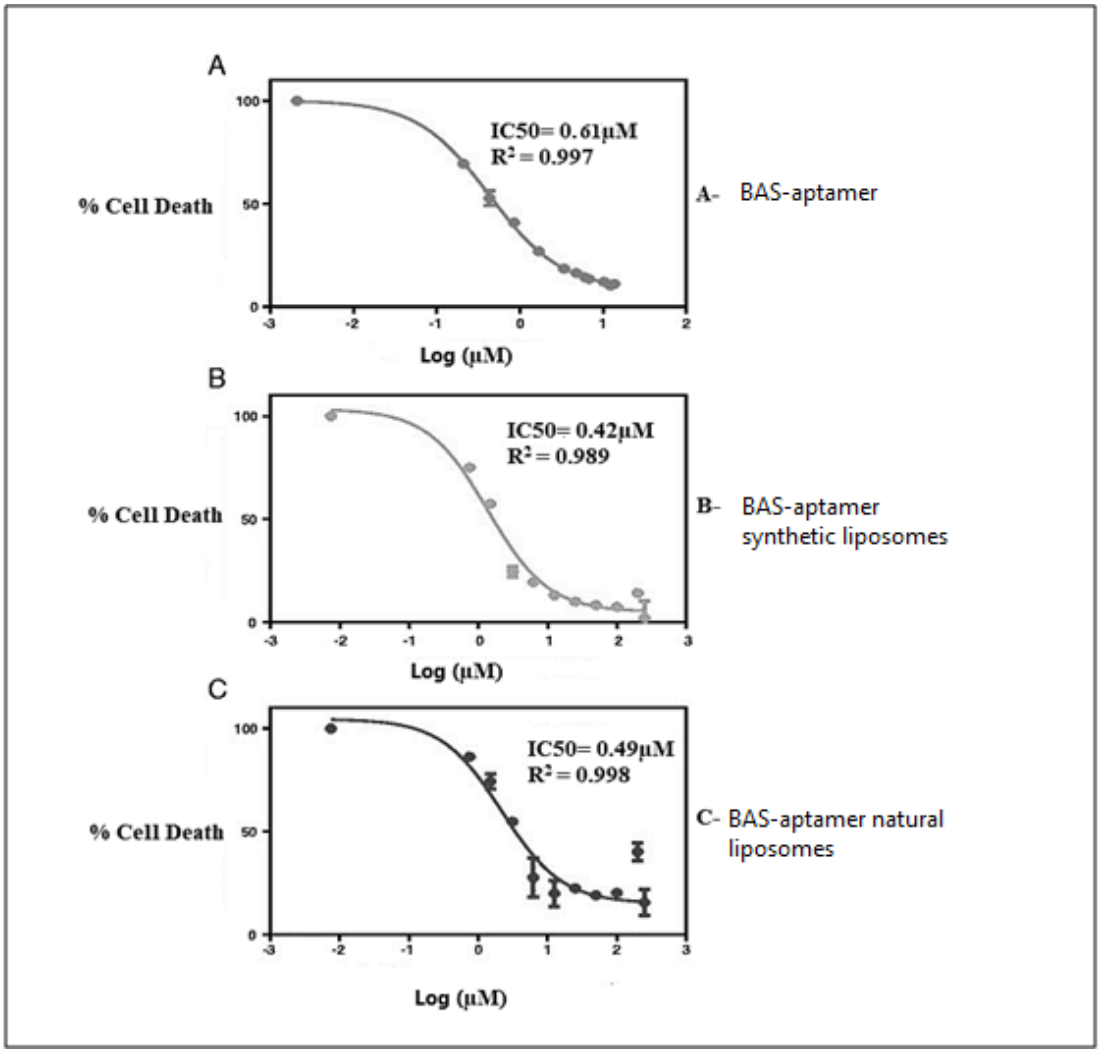


Figure 13. Dose-response curves for IC_{50} determination for free BAS aptamer, BAS aptamer-synthetic liposome and BAS aptamer-natural liposome in MCF-7 cells.

The Results revealed that the potency of pure BAS aptamer and BAS aptamer liposomes are high since a very low concentration of each can inhibit half of the total number of total viable cancer cells. Both BAS aptamer liposomes gave higher potency than the pure drug, which agreed with the time and dose response curve observed earlier in figure 11 and 12.

Stability of BAS-aptamer conjugated liposomes in plasma by two different methods

The stability of the prepared BAS aptamer natural and synthetic liposomes was studied by incubating them in plasma and assayed using urea PAGE and HPLC-UV in parallel.

The results in figure 14 shows that both liposomes were highly stable. The drug-natural liposomes remained stable for up to 6 days and only partial degradation appeared on the 7th day while synthetic liposomes showed partial degradation after 5 days, indicating that the natural liposomes were more stable than the synthetic one that could be attributed to the better effect of natural pure phosphatidyle choline (used in natural liposomes) in stabilizing the liposomal morphology. These results were further proved by using HPLC-UV assay method as shown in figure 15.

Both liposomes showed impressive stability in comparison to pure BAS aptamer which showed reported degradation within 4 hours, which proved that liposomes can improve the stability of drug in which it is encapsulated or conjugated, and agreed with reported data[44, 45].

Stability of BAS- aptamer conjugated liposome at different pH (7.4 and 5.5)

To test the pH effect for stability on both BAS-aptamer natural and synthetic liposomes, HPLC-UV stability assay was carried after incubating BAS-aptamer natural liposome and synthetic liposome in phosphate buffer at pH 5.5 and 7.4. The UV absorbance of the peaks was determined after each incubation time as shown in table 5. Figure 16 and 17 revealed the stability of the drug (BAS aptamer) in both types of liposomes in different pH using HPLC-UV assay. There was no significant difference in the stability of natural liposomes in both pH 5.5 and 7.4 at each incubation time, same results observed with synthetic liposomes. This indicated that pH had no effect on the stability of the drug in both liposomes. However, the results revealed that natural liposomes showed only slight non-significant decline in the absorbance of the drug started after 6 days that continued non-significantly till

the 7th day in both pH. While synthetic liposomes showed decline after 3 days (at pH 5.5) and continued significantly till the 7th day, also this 7th day decline was significantly lower than the decline appeared in the 7th day with natural liposomes. While at pH 7.4; the synthetic liposomes showed decline after 5 days which continued significantly till 7th day where the decline at the 7th day was significantly lower than that for natural liposomes.

The results indicated that the natural liposomes were more stable than the synthetic liposomes in both pH 7.4 and 5.5 and the possibility of using any of these pHs in the formulation of the drug-liposomal conjugates.

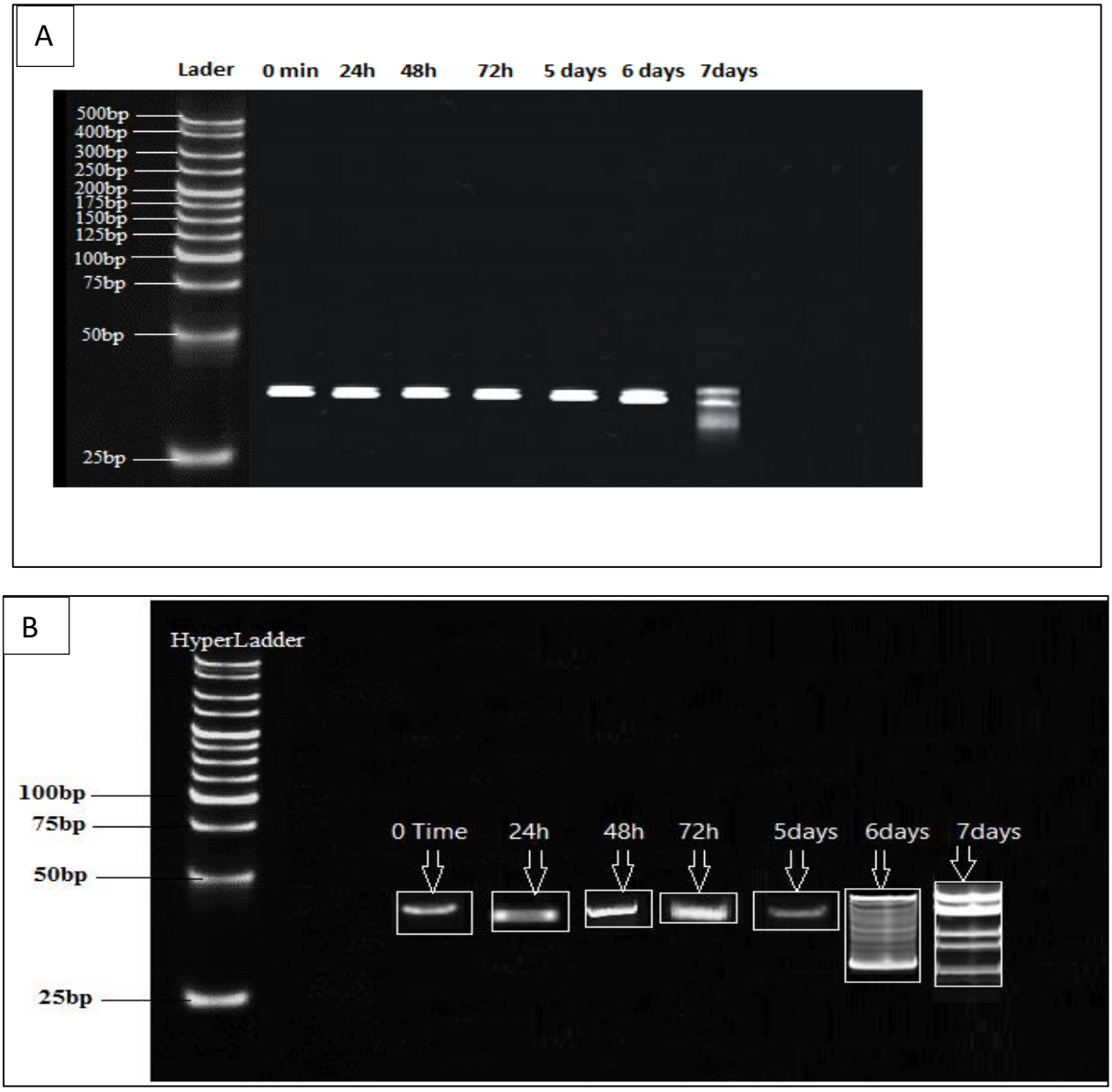


Figure 14. Stability of **(A)** BAS aptamer- natural liposome. **(B):** BAS aptamer synthetic liposome in human plasma by 10% urea PAGE method.

Lane1(Hyper Ladder 25bp) Lane2(the liposomes at 0 time) Lane3(liposomes at 24h) Lane4(liposomes at 48h) Lane5(liposomes at 72h) Lane6(liposomes at 5 days) Lane7(liposomes at 6 days) Lane8(liposomes band at 7 days).

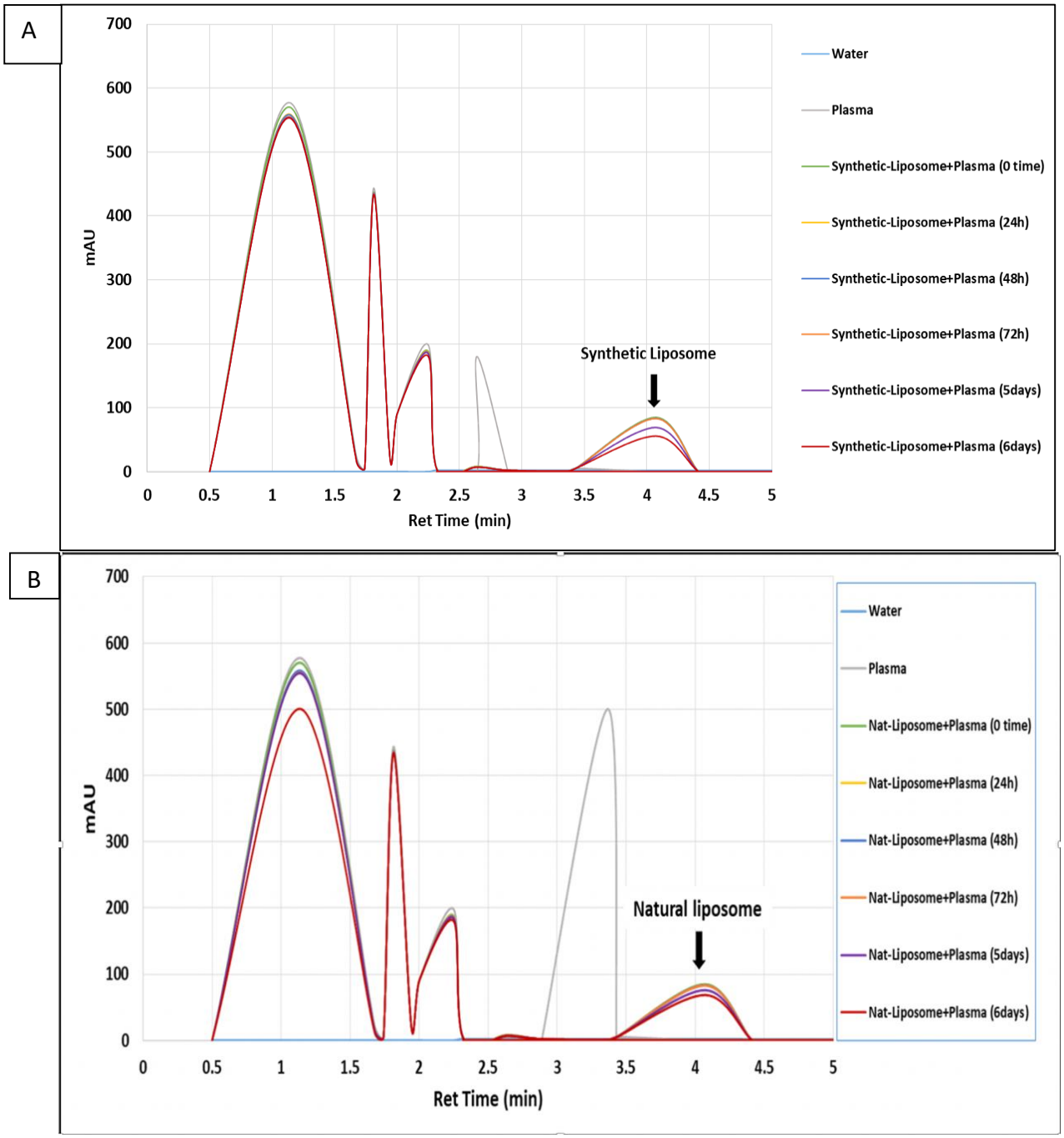


Figure 15 (**A)**: Stability of the BAS aptamer-synthetic liposome in human plasma by HPLC-UV method.

The retention time from 0.5- 2.5 for plasma and the retention time from 3.5-4.5 for BAS aptamer-synthetic liposome. (**B)**: Stability of the BAS aptamer- natural liposome in human plasma by HPLC-UV method. The retention time from 0.5- 3.0 for plasma and the retention time from 3.5-4.5 RT for BAS aptamer-natural liposome. Note: The retention time of pure BAS aptamer was reported to be 4.3 min[22].

Table 5. HPLC absorbance values of aptamer- natural liposome and aptamer- synthetic liposome after incubation with phosphate buffer pH 7.4 and 5.5 for different time.

Formula	Absorbance (mAU) at each incubation day							
	0 day	1 day	2 day	3 day	4 day	5 day	6 day	7 day
pH 5.5 BAS-aptamer Natural liposome	84.92 mAU	83.967 mAU	83.6 mAU	82 mAU	80.1 mAU	80 mAU	75.5 mAU	71.6 mAU
pH 7.4 BAS-aptamer Natural liposome	85.02 mAU	84.1 mAU	84.09 mAU	83 mAU	82.99 mAU	82.9 mAU	80.6 mAU	70.54 mAU
pH 5.5 BAS-aptamer Synthetic liposome	79.92 mAU	78.96 mAU	78.6 mAU	78.1 mAU	71.01 mAU	71 mAU	66.69 mAU	54.54 mAU
pH 7.4 BAS-aptamer Synthetic liposome	84.7 mAU	83.9 mAU	83.6 mAU	82.98 mAU	81.99 mAU	81.8 mAU	60.69 mAU	50.54 mAU

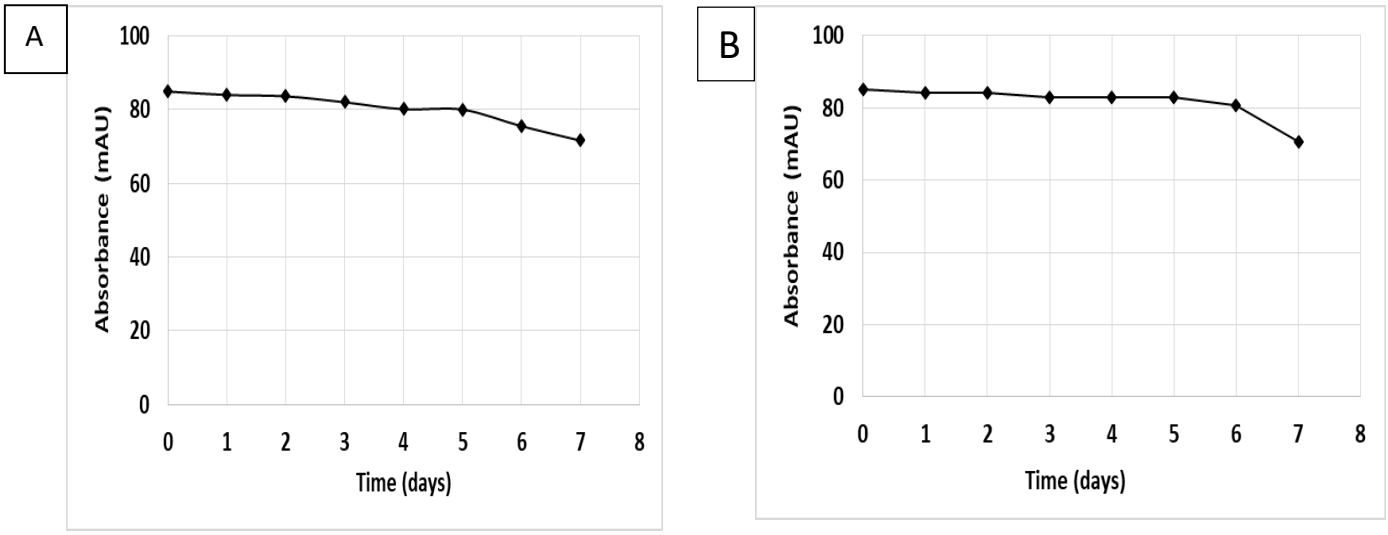


Figure 16. Stability of BAS-aptamer natural liposome: A: At pH 5.5 B: at pH 7.4

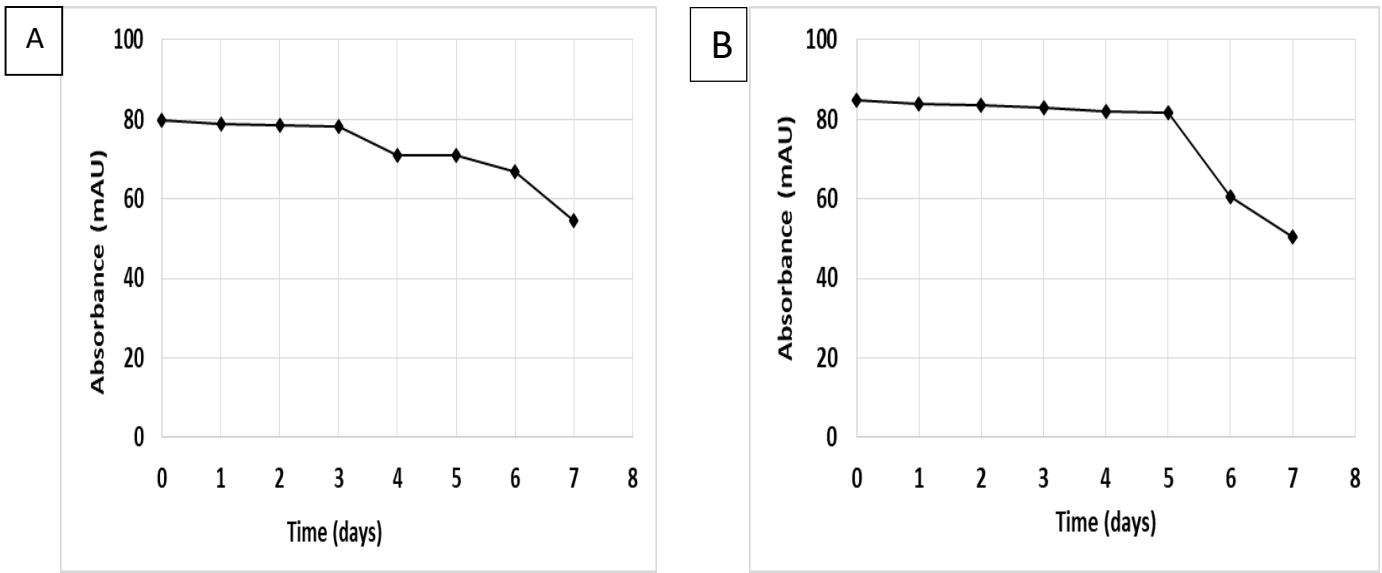


Figure 17. Stability of BAS-aptamer synthetic liposome A: At pH 5.5, B: at pH 7.4

CONCLUSION

This study provides that the introducing thiol group to the BAS aptamer facilitate the conjugation with maleimide liposomes. The applied gel electrophoresis method was efficient for identification of the purity of BAS aptamer-SH and the conjugation of aptamer with blank maleimide liposomes. Using natural phosphatidylcholine as stabilizer for the drug conjugated liposomes gave better physical properties for the blank prepared liposomes as well as significant binding affinity for the aptamer conjugated liposomes to the receptor (SIRT1) and higher cytotoxic effect that continued even for 7 days against breast cancer (MCF7) cells by cell-line study in comparison to synthetic stabilizers and the drug before conjugation. The prepared conjugated liposomes showed impressive stability in plasma indicating the efficiency of the liposomes in improving the stability. The results also revealed the significant stability of the conjugated liposomes with natural stabilizer in pH 7.4 and 5.5 in comparison to synthetic stabilizers which revealed that the stability of the drug in its liposomes against any change in pH that may happen in the cancer cells as well as it may help in formulation of a suitable injectable dosage form.

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