Production of Targeted Estrone Liposomes Using a Herringbone Micromixer

Mohamed Agam, Vinod Paul, Mohamed Abdelgawad, *Member, IEEE*, and Ghaleb A. Husseini

***Abstract*— Liposomes are spherical vesicles formed from bilayer lipid membranes that are extensively used in targeted drug delivery as nanocarriers to deliver therapeutic reagents to specific tissues and organs in the body. Recently, we have reported using estrone as an endogenous ligand on doxorubicin-encapsulating liposomes to target estrogen receptor (ER)-positive breast cancer cells. Estrone liposomes were synthesized using the thin-film hydration method, which is a long, arduous, and multistep process. Here, we report using a herringbone micromixer to synthesize estrone liposomes in a simple and rapid manner. A solvent stream containing the lipids was mixed with a stream of phosphate buffer saline (PBS) inside a microchannel integrated with herringbone-shaped ridges that enhanced the mixing of the two streams. The small scale involved enabled rapid solvent exchange and initiated the self-assembly of the lipids to form the required liposomes. The effect of different parameters on liposome size, such as the ratio between the flow rate of the solvent and the buffer solutions (FRR), total flow rate, lipid concentrations, and solvent type, were investigated. Using this commercially available chip, we obtained liposomes with a radius of 66.1 ± 11.2 nm (mean ± standard deviation) and a polydispersity of 22% in less than 15 minutes compared to a total of ∼11 hours using conventional techniques. Calcein was encapsulated inside the prepared liposomes as a model drug and was released by applying ultrasound at different powers. The size of the prepared liposomes was stable over a period of one month. Overall, using microfluidics to synthesize estrone liposomes simplified the procedure considerably and improved the reproducibility of the resulting liposomes.**

***Index Terms*— Microfluidics, estrone, herringbone micromixer, liposomes, drug delivery.**

I. Introduction

CHEMOTHERAPY is a well-known and effective therapeutic treatment for different types of cancer; however, these strong chemicals also attack and adversely affect other fast-growing healthy cells in the body, such as white blood cells, skin cells, and hair follicles. Drug delivery is a novel field of biomedical research where the drug is delivered to the tumor site with minimal interactions with other healthy cells. The main component in designing new targeted drug delivery techniques is the synthesis of nanocarriers that can encapsulate drugs inside their structure and deliver the therapeutic agents to the tumor site. For this purpose, researchers have developed different nanocarriers such as liposomes, quantum dots, dendrimers, and polymeric nanoparticles [1].

Liposomes are spherical vesicles made of a bilayer of phospholipids, with dimensions varying between tens of nanometers to hundreds of micrometers, that can encapsulate drugs in their hydrophilic core and/or hydrophobic agents in their phospholipid bilayer [2], [3]. They can also be conjugated with moieties that will target specific receptors on the surfaces of the diseased cells. Liposomes were used for the first time as drug delivery carriers in 1971 [4]. Since then, they have shown promise as therapeutic reagents for different types of cancer, including breast cancer [5], [6], [7], [8], [9], [10], cervical carcinoma [11], hepatocarcinoma [12], and ovarian and lung carcinoma [13], among many others. The FDA first approved them to treat Kaposi sarcoma in 1995.

Cancer cells overexpress receptors on their surfaces, including fucose receptors [14], transferrin receptors [15], folate receptors (F.R.) [16], epidermal growth factor receptors (EGFR) [17], and estrogen receptors (E.R.) [18], [19]. Studies have shown that more than two thirds of breast cancer cells have E.R.s [20], and these receptors can be found on the cell

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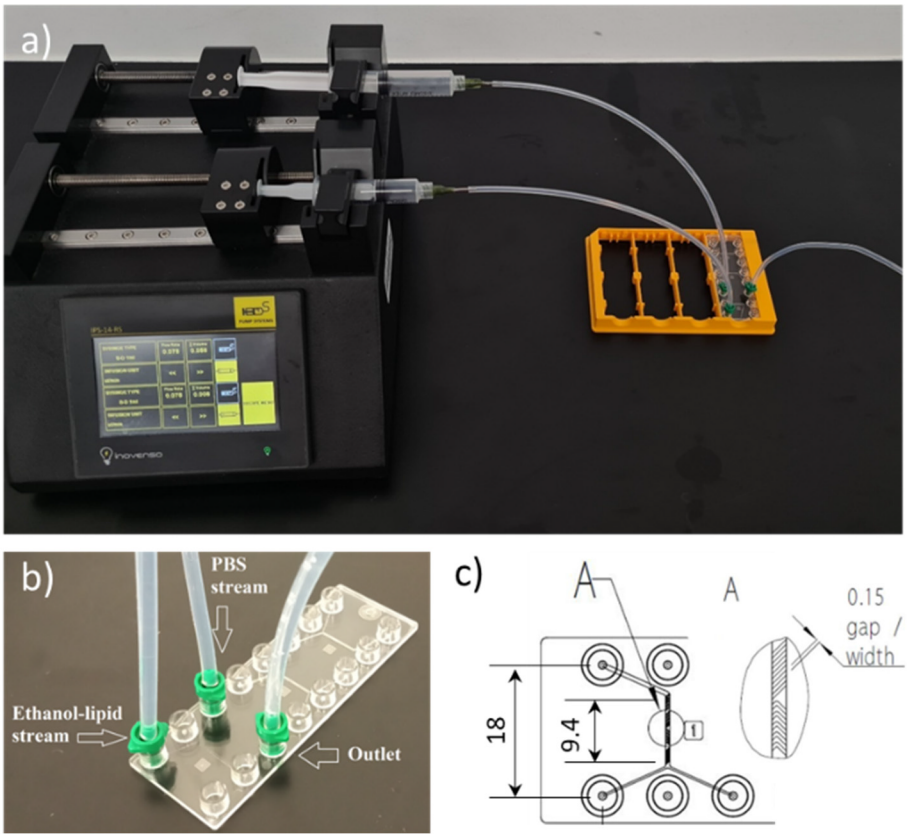

Fig. 1. a) The liposomes synthesis microfluidic setup. b) Close up on the tubing connections to the micromixer chip. c) Details of the herringbone micromixer chip (all dimensions in mm). Reprinted with permission from [42].

membrane and nucleus. Several drugs with estrogen or their analogs were synthesized to target E.R.s [21], [22]. Estrogen includes estrone, estradiol, and estriol, with the majority of estrogen being estrone in postmenopausal women. Estrone transfers its signal to E.R. proteins ERα and ERβ [23]; 60% of the signal to ERα protein and 37% to ERβ protein. Hence, estrone is a promising ligand in cancer research and treatment.

The synthesis of liposomes with estrone as a targeting moiety was reported in many studies to selectively target breast cancer cells [24], [25], [26]. For example, estrone-targeted liposomes were used for *in vivo* drug delivery to the tumor site with increased circulation time and reduced drug side effects [27]. Estradiol-Associated Stealth-Liposomes were also used to deliver an anticancer gene to breast cancer cells [28]. In another study [24], estrone was used to synthesize doxorubicin-encapsulating liposomes targeted toward the (E.R.)-positive (ER+) breast cancer cells.

Liposomes can be synthesized using different techniques, such as thin-film hydration [29] [30], ethanol injection [31], [32], and detergent dialysis [33], [34]. However, these techniques are arduous, complicated, involve long processes, and have low encapsulation efficiencies. To overcome these limitations, microfluidics, which pertains to the manipulation of minute liquid volumes inside microchannels, emerged as a more efficient and lower-cost platform for liposome synthesis [35], [36], [37], [38]. With mixing being entirely diffusion-based due to the laminar flow nature inside microchannels, microfluidics offer better control over the formation of liposomes. By controlling the flow rate of the lipids stream and buffer stream injected inside the microchannel and the microchannel dimensions, liposome size, polydispersity, and throughput can be easily controlled [39]. The enormous advantages microfluidics offer lead to the common use of microreactors for liposome synthesis and the development of many off-the-shelf microreactor chips ready for use by any research group working in this field [40], [41].

The main objective of this work is to use commercially available microfluidics micromixer chips to synthesize estrone liposomes in an easy and facile manner. Lipids dissolved in different solvents were mixed with a stream of PBS using a herringbone micromixer to produce estrone liposomes with

TABLE I
FLOW RATE RATIO (FRR) AND TOTAL FLOW RATE (TFR) CONDITIONS INVESTIGATED

Flow Rate Ratio (FRR)	Total Flow Rate (TFR)
10:1	220 µL/min
30:1	220 µL/min
50:1	220 µL/min
50:1	51 µL/min
50:1	102 µL/min
50:1	204 µL/min
50:1	408 µL/min
50:1	612 µL/min
50:1	816 µL/min
50:1	1020 µL/min
50:1	1224 µL/min
50:1	1428 µL/min
50:1	1632 µL/min

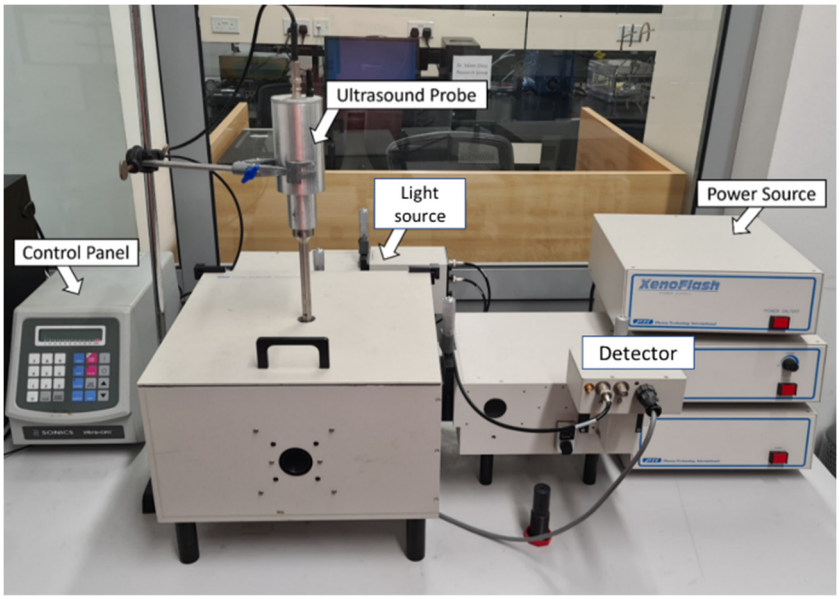

Fig. 2. The ultrasound setup used to trigger the release of encapsulated calcein from the prepared liposomes.

the desired size. We studied the effect of the flow rate ratio between the lipids solution and PBS on liposome size and polydispersity. We also studied the effect of the solvent type and total flow rate of both solutions. Additionally, we studied the effect of estrone concentration and solvent type on the size and polydispersity of the synthesized liposomes.

II. Methodology

A. Materials

The DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000-NH_2 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000]) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Sephadex G-100, Sephadex G-25, estrone (E.S.), calcein disodium salt, potassium bromide (KBr), and 2,4,6 trichloro-1,3,5 triazine (cyanuric chloride (CC)) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chloroform was obtained from Panreac Quimica S.A. (Castellar del Vall_s, Barcelona, Spain). Cholesterol was obtained from AlfaAesar (Ward Hill, MA, USA). Triethylamine (TEA) was obtained from Reidelde Ha_n (Seelze, Lower Saxony, Germany). All the above-mentioned chemicals were obtained from the indicated vendors via LABCO, Inc. (Dubai, United Arab Emirates).

B. Preparation of DSPE-PEG2000-CC-ES Modified Lipids

The lipids DSPE-PEG_{2000}-NH_2 were modified by conjugating the estrone molecule via a cyanuric chloride linker. Estrone and cyanuric chloride were reacted in a molar ratio of 1:1 in the presence of triethylamine (TEA) at 0°C. To initiate the reaction, both estrone and cyanuric chloride were dissolved in dry chloroform maintained at 0°C. The estrone solution containing 2 molar equivalents of TEA was added to the cyanuric chloride solution dropwise, and the reaction was continued for 3 hours. The estrone-cyanuric chloride conjugate was then reacted with DSPE-PEG-NH_2 lipids dissolved in dry chloroform. The reaction was continued at 0°C for three hours and then continued overnight at room temperature. The formed DSPE-PEG_{2000}-CC-ES conjugates were dried and stored at -20°C until further use. To confirm the conjugation, the CC-ES conjugate was analyzed using Fourier transform infrared spectroscopy (FTIR).

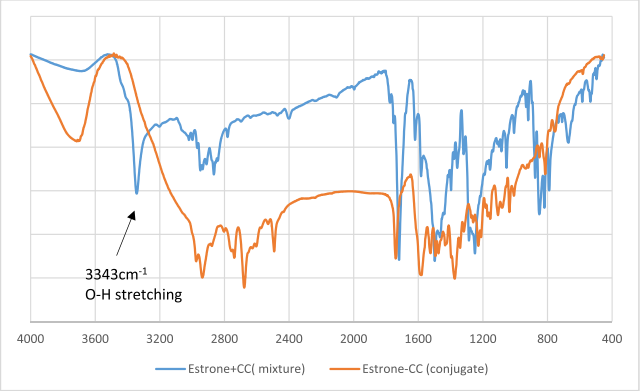

Fig. 3. Infrared (IR) spectra of estrone and cyanuric chloride. The hydroxyl group stretching at 3343 cm^{-1} was not detected for the conjugate, confirming the conjugate formation.

C. Preparation of Estrone Liposomes

A herringbone micromixer chip was purchased from the Microfluidic ChipShop GmbH (Jena, Germany). The chip is made of P.C. (polycarbonate), which is a transparent thermoplastic that is less hydrophobic than PDMS (Polydimethylsiloxane) and has a better filling behavior [42]. There are 3 devices per chip; the main mixing channel in each device was 600 μm x 200 μm (WxH), and 9.4 mm long and contained slanted ridges to enhance mixing as reported earlier [43], Figure 1 (a). The modified lipids and buffer streams were injected into the micromixer using a dual syringe

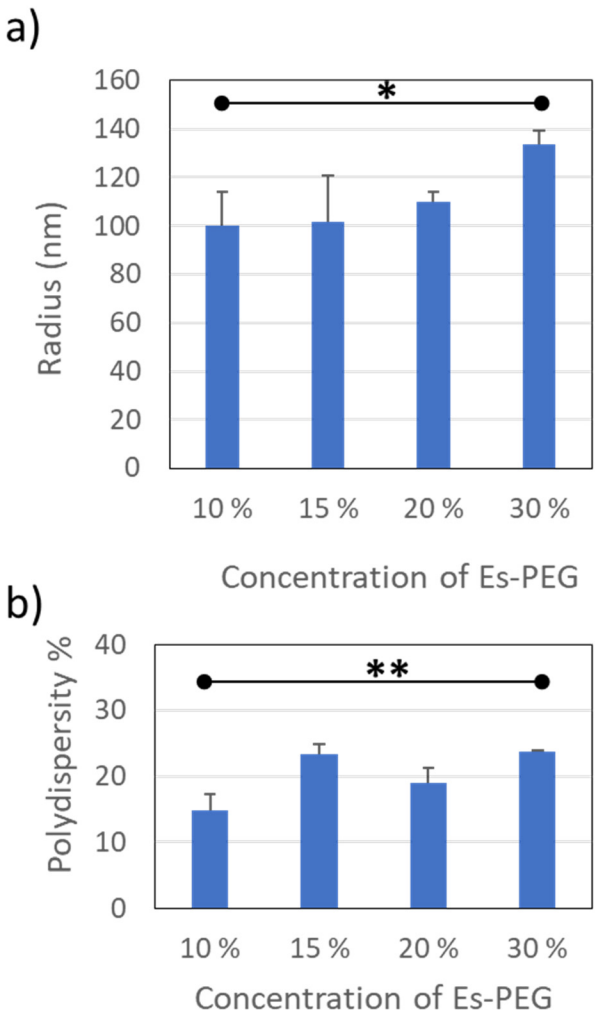

Fig. 4. a) The size of the prepared liposomes increases significantly (p=0.038) when the estrone concentration in PEG increases from 10% to 30%. (b) The polydispersity index also increased significantly (p=0.0031) for the same estrone concentration change. Data was produced using ethanol as a solvent at FRR = 30:1 and a total flow rate of (220 μL/min). Error bars present one standard deviation.

pump (Inovenso, Istanbul, Turkey). The synthesized liposomes were collected from the device outlet. All experiments were run in triplicates, with each run performed in a different device.

We studied various flow rate ratios (FRRs) between PBS and the lipids streams to investigate their effect on the size of synthesized liposomes. We varied the FRR between 1:1 and 50:1 while keeping the total flow rate constant at 220 μL/min. We also studied the effect of changing the total flow rate while keeping the FRR constant at 50:1. The total flow rate varied between 51 and 1632 μL/min. Table I summarizes the conditions investigated in this study.

In addition to flow parameters, we also changed chemical parameters. The lipids solution comprised 2 mg/ml of DPPC, 1 mg/ml of cholesterol, 0.5 mg/ml of DSPE-PEG (2000), and 0.05 mg/ml estrone-PEG at 10% concentration. The amount of DSPE-PEG (2000) and estrone-PEG were varied to achieve different concentrations of 10%, 15%, 20%, and 30%. Also, we tested the effect of changing the lipids carrying solvent. Both methanol and ethanol were tested.

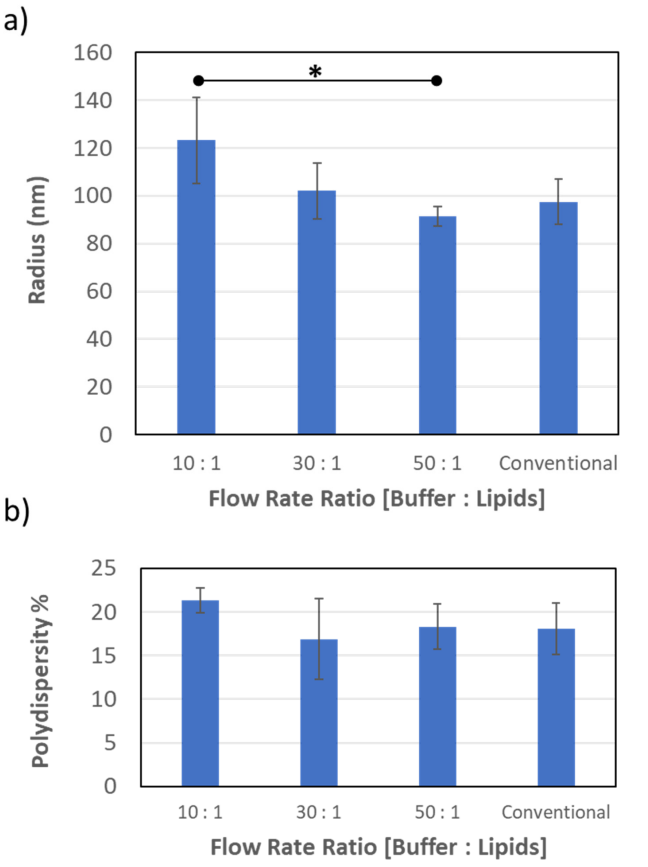

Fig. 5. Effect of FRRs on (a) liposomes radius (p =.014) and (b) polydispersity index. Data were obtained using ethanol as a solvent with an estrone concentration of 10% and a total flow rate of 220 μL/min. Data of liposomes prepared using the conventional thin-film hydration technique are added for comparison. Error bars present one standard deviation.

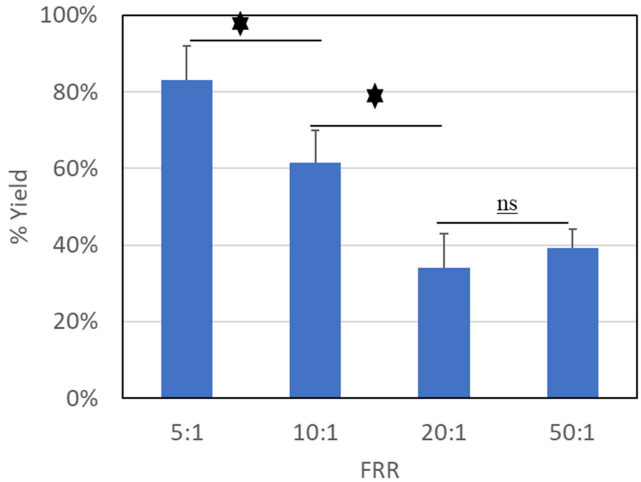

Fig. 6. The percentage yield of liposomes synthesized at different flow rates. An asterisk (*) denotes a p-value less than 0.05, indicating statistical significance. Notably, no statistically significant difference was observed in the percentage yield between the flow rates of 20:1 and 50:1 (p-value = 0.6 for the comparison between the percentage yields of FRR 20:1 and 50:1).

D. Determination of the Liposome Size

The liposome size was determined by dynamic light scattering (DLS) using the DynaPro® NanoStar™ sizer (Wyatt

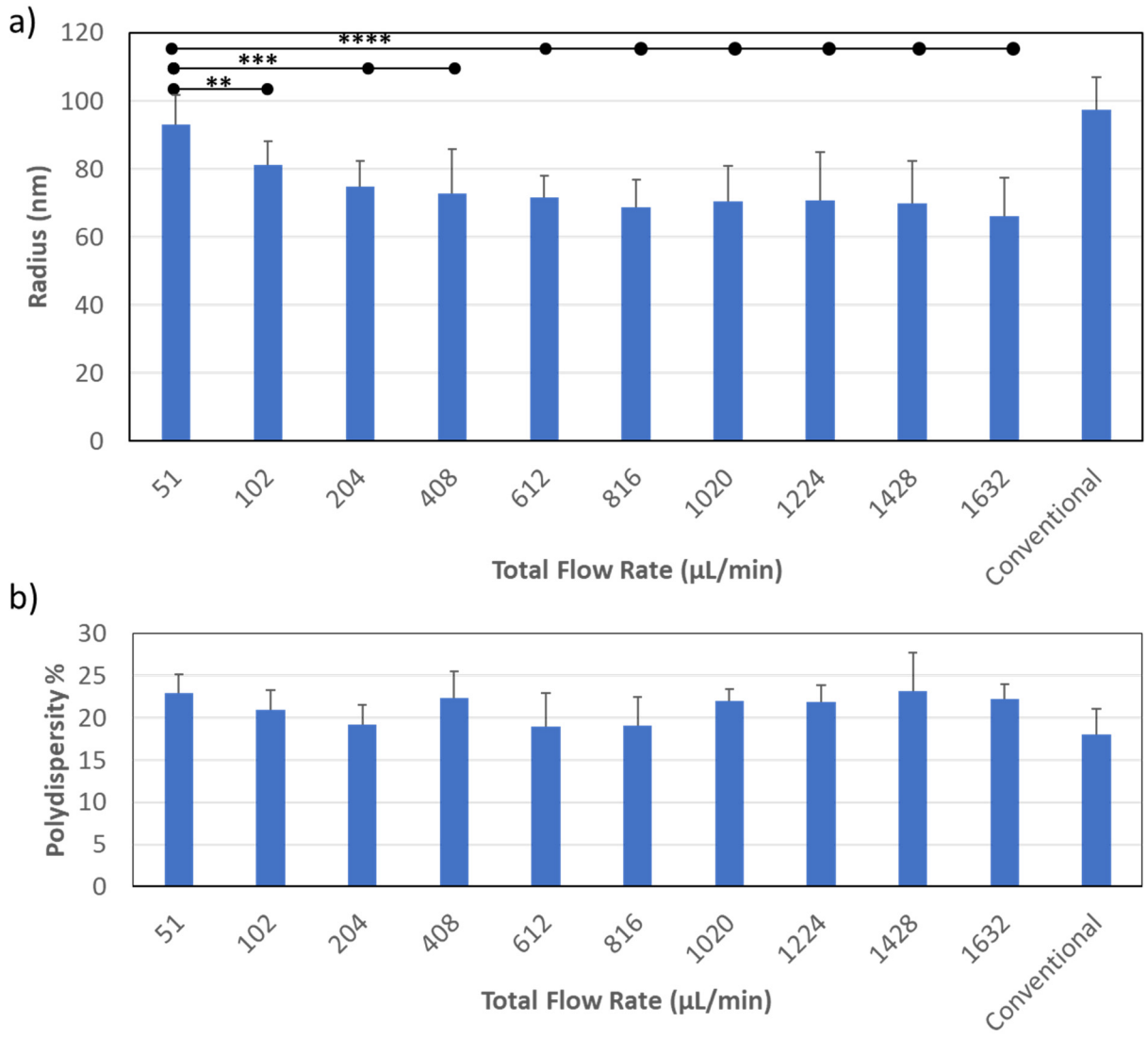

Fig. 7. The radius of the prepared liposomes (a) decreased significantly with the increase in the total flow rate of both the lipids solution and PBS at constant FRR, whereas the polydispersity (b) did not change significantly. Flow rates higher than 204 μL/min did not produce a further significant reduction in liposomal radius. Data produced at FRR = 50:1 using ethanol as a solvent. The concentration of estrone in PEG was 10%. Data of liposomes prepared using the conventional thin-film hydration technique are added for comparison. Error bars represent one standard deviation.

Technology Corp., Santa Barbara, CA, USA) [36]. A liposome sample of 10 μL was diluted 1:10 in PBS and loaded into a cuvette to determine the hydrodynamic radius of the prepared liposomes.

E. Quantification of Phospholipids

The Stewart assay measured the quantity of phospholipids in the resulting liposomes [44]. The method relies on calorimetrically determining a red-colored complex created by the interaction between ammonium ferrothiocyanate and phospholipid head groups. Liposomes, synthesized under various flow rates, underwent centrifugation at 32,000 RCF for 60 minutes to form liposome pellets, from which the supernatant was removed to eliminate free lipids. Subsequently, the liposome pellets were reconstituted in PBS before lipid content analysis. Three sets of liposomes were generated at each flow rate ratio, and an average yield percentage was documented.

F. Calcein Release

Calcein disodium salt was used to test the release mechanism from the synthesized liposomes, as a model hydrophilic drug [45]. Calcein was added to the PBS stream before being injected into the microfluidic chip, and the resulting liposomes were collected at the outlet as usual. In order to remove the unencapsulated calcein from the liposome solution, the solution was dialyzed using a Slide-A-Lyzer ® dialysis cassette with a molecular weight cutoff of 7000 Da. The dialysis was carried out at 4°C for 72 hours in PBS buffer, and the buffer was exchanged with fresh buffer in between.

To release the calcein from the prepared liposomes, ultrasound was applied to the sample at three different intensities, 6.2, 9, and 10 mW/cm^2 at 20 kHz using an ultrasonic probe (Sonics and materials VCX 750, Newtown, Connecticut, United States), Figure 2. The release of calcein from the liposomes was measured using a phosphorescence spectrofluorometer (QuantaMaster QM 30 Photon Technology

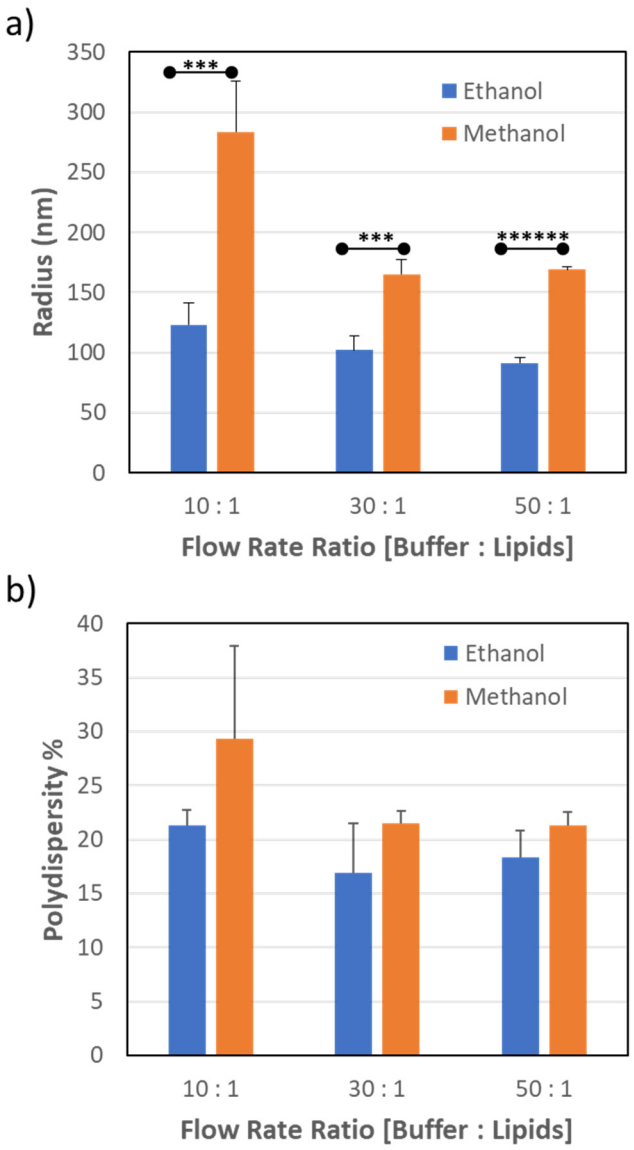

Fig. 8. Effect of solvent type on (a) liposomal radius and (b) polydispersity index. The total flow rate was maintained at 220 μL/min, and the concentration of estrone in PEG was 10%. Changes in polydispersity due to solvent type were not significant. Error bars represent one standard deviation.

International, Edison, NJ, USA). A sample of 75 μl of the liposome suspension was diluted in 3 ml of PBS, and the initial fluorescence intensity was taken for 60 seconds, which was set as the baseline. After 60 seconds, ultrasound was applied to the cuvette in pulses (20 seconds ON and 10 seconds OFF) to prevent temperature increase due to the ultrasound energy. Calcein release from the liposome was observed as an increase in fluorescence intensity. The samples were irradiated with ultrasound until there was no significant increase in fluorescence intensity. The fractional release of calcein was calculated using the equation:

$$\text{Fractional release of calcein} = \frac{F(t) - F(Baseline)}{F(Final) - F(Baseline)}$$

$F(t)$ is the fluorescence intensity at time t and $F(Final)$ is the fluorescence intensity measured when all the calcein encapsulated within the liposome was released by adding 2% (w/v) TritonX -100 to the liposome solution, which lyses the liposome and releases all the calcein encapsulated.

G. Cellular Uptake

MCF-7 and MDA-MB-231 cells, cultured in RPMI media, were initially seeded into 6-well plates. Following seeding, the plates were incubated overnight in a humidified incubator at 37°C with 5% CO_2. Subsequently, the cells were treated with calcein-encapsulated non-targeted liposomes and estrone-conjugated liposomes synthesized at a Flow Rate Ratio (FRR) of 10:1. After treatment, the cells were further incubated for 3 hours, then detached and subjected to analysis using flow cytometry (Beckman Coulter FC500, Brea, California, United States) to assess the cellular uptake of liposomes. The analysis involved measuring the fluorescence intensity of calcein taken up by the cells, with the excitation and emission wavelengths set at 488 nm and 520 nm, respectively.

H. Statistical Analysis

All the results reported here are the average ± standard deviation (S.D.). Two-tailed t-tests were used to determine the statistical significance of the results; a p-value $<.05$ was considered statistically significant.

III. Results and Analysis

A. Confirmation of the Estrone-Cyanuric Chloride Conjugation

In order to conjugate the estrone to DSPE-PEG-NH_2, cyanuric chloride was used as the linker due to its thermally controlled reactivity. The first chloride ion of the cyanuric chloride was released at 0° C and substituted with the hydroxyl group of estrone. IR spectra were obtained for a mixture of estrone and cyanuric chloride, for which the peak of O-H stretching was observed at 3343 cm^{-1}. When the IR spectra of the estrone-cyanuric chloride conjugate were taken, this peak was not detected, confirming the successful reaction between the two compounds forming a conjugate. The FTIR spectrum obtained for the mixture and the conjugate is shown in Figure 3.

IV. Effect of the Concentration of Estrone in PEG

The radius of the resulting liposomes increased significantly ($p=0.038$) by about 33% when the estrone concentration in PEG was increased from 10% to 30%, Figure 4 (a). This size increase could be due to the increase in estrone molecules conjugated to the surface of the phospholipid's bilayer. Similar results were reported before, where the size of estrone-coupled liposomes was larger than plain liposomes [25]. There was also a significant change ($p=0.0031$) in the polydispersity index when the concentration increased from 10% to 30 %, Figure 4 (b). However, contrary to the particle size, there was no clear trend for the change in the polydispersity.

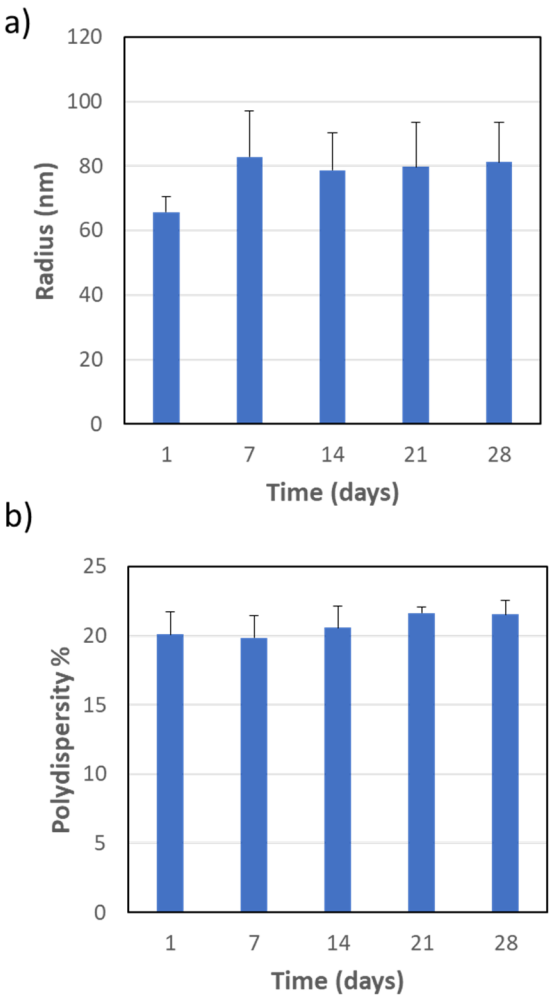

Fig. 9. Stability was assessed over a one-month period to monitor (a) the radius of estrone liposomes and (b) the polydispersity index. The concentration of estrone in PEG was 10%, with a total flow rate (TFR) of 220 μL/min and a flow rate ratio (FRR) of 50:1.

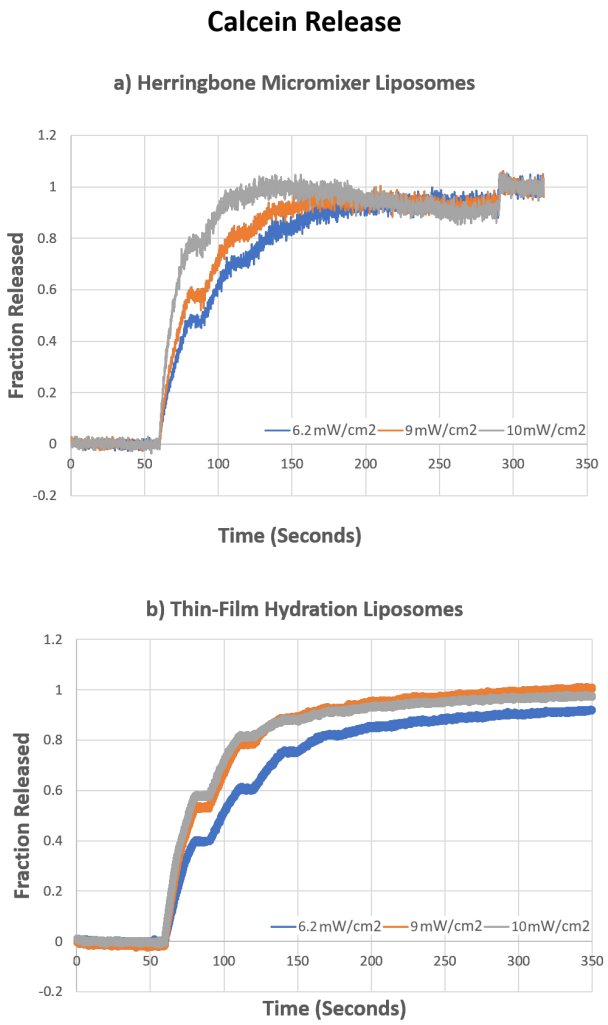

Fig. 10. a) Calcein release from the microfluidics-prepared estrone liposomes at 3 ultrasound power densities at 20 kHz. The liposomes used in this experiment were prepared at FRR = 30:1 and a TFR of 220 μL/min with an approximate radius of 100 nm. b) Calcein release from the conventionally prepared liposomes (thin-film hydration liposomes) at 3 ultrasound power densities.

A. Effect of the Flow Rate Ratio (FRR)

Three FRRs (10:1, 30:1, and 50:1) were tested at a fixed total flow rate of 220 μL/min at a concentration of 10% of Estrone in PEG, which is the concentration that gave the smallest particle size. Increasing the FRR from 10:1 to 50:1 significantly reduced the liposomes' average radius from 123.3 nm to 91.5 nm (p=0.014), Figure 5. This is a similar trend to the results reported in the literatures [46], [35], and [36]. At higher FRRs, the lipids flow rate is low, leading to a thin lipids stream in the middle of a large buffer continuum and, consequently, faster mass transfer of the ethanol into the buffer. This quick reduction of the solvent concentration does not allow the bilayered phospholipid fragments to grow to a larger size before forming the spherical liposomes to reduce their surface energy [47]. However, the higher the FRR, the lower the liposomes throughput from the device due to the relatively larger quantities of the buffer injected against the lipid solution. This may require additional concentration techniques, such as centrifugation or filtration [48] downstream, to increase the concentration of liposomes in the final suspension.

The polydispersity index also decreased when the FRR increased from 10:1 to 50:1; however, this reduction was statistically insignificant ($p = 0.085$), Figure 5 (b). This can be explained in light of the reduction in the width of the lipids stream at higher FRR. This improves mass transfer by diffusion and solvent exchange between the two streams. Therefore, the liposomes are formed rapidly under a constant solvent concentration, which improves size uniformity.

The yield of the liposomes, as quantified using the Stewart assay, changed significantly with the FRR, as shown in Figure 6. The percentage yield decreased significantly from 83% at an FRR of 5:1 to only 34% at an FRR of 20:1. This reduction in yield can be attributed to the loss of lipid molecules as they diffuse away into the relatively vast buffer content surrounding the ethanol stream.

B. Effect of the Total Flow Rate (TFR)

Increasing the total flow rate of the lipids solution and PBS at a constant FRR resulted in decreasing the size of

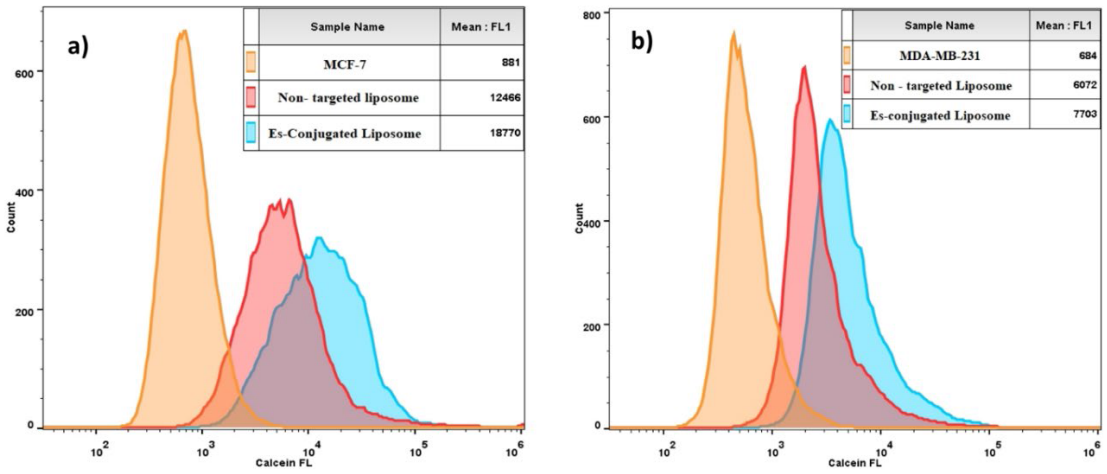

Fig. 11. The uptake of liposomes by cells was examined after incubation with both non-targeted and targeted liposomes, focusing on (a) MCF-7 cells and (b) MDA-MB-231 cells. The inclusion of the estrone moiety resulted in an enhanced cellular uptake for both cell types, albeit with a more pronounced effect in estrone-positive MCF-7 cells. The liposomes were formulated at a Flow Rate Ratio (FRR) of 10:1, with an estrone concentration of 10%.

the resulting liposomes, Figure 7. For example, increasing the total flow rate from 51 μL/min to 1632 μL/min at a FRR of 50:1 resulted in reducing the liposomes' average radius from 93.1 $\pm$ 8.7 nm to 66.1 $\pm$ 11.2 nm, respectively ($p = 0.00003$). Generally, the effect of the total flow rate on the size of the produced liposomes is not well understood, with some studies showing size reduction [35], [41], [49], while others reported a size increase [50]. Yet, others showed no effect on size as the total flow rate increases [36]. Higher flow rates should translate to larger liposome size due to reduced lipids' residence time inside the channel at high velocities [39]. However, as reported in previous studies, the mixing efficiency of the herringbone micromixer is not affected by the increases in the Peclet number ($Pe = UL/D$) [43], where U is the average flow velocity, L is the channel characteristic length, and D is the diffusion coefficient. The presence of the herringbone ridges at the bottom of the channel results in enhanced mixing at higher flow rates, which leads to a smaller liposomal size. As for the polydispersity, it was not generally affected by the increase in the flow rate and remained almost constant at around 20%.

C. Effect of the Solvent Type

Liposomes were prepared using two solvents, methanol and ethanol, as the lipid-carrying stream. Using ethanol resulted in significantly smaller liposomes than methanol, whereas the polydispersity did not decrease significantly, Figure 8. This could be due to the difference in the diffusion coefficient of both solvents in water, which is reported to affect the size of nanoparticles produced by nanoprecipitation [51]. The diffusion coefficients of methanol and ethanol in water are (1.387 - 1.28) x 10^{-9} [52] and 1.23 $\times$ 10^{-9} m ^{2}/s, respectively. This prompted the use of ethanol as a solvent over methanol, highlighting its additional benefit of being non-toxic.

D. Size Stability of the Prepared Liposomes

A stability test was conducted to determine whether the size of the liposomes would remain stable or change over time. Following the synthesis of the liposomes, their size was immediately measured. Subsequently, they were stored in the refrigerator and their size was measured again after 1, 2, 3, and 4 weeks. The size of the liposomes exhibited a slight and insignificant increase after the first week but then stabilized for the remaining three weeks, as shown in Figure 9. This slight initial increase in size could be attributed to the aggregation of smaller liposomes. Regarding polydispersity, no significant changes were observed over time, maintaining an acceptable value of around 20% throughout the four-week period, as depicted in Figure 9(b).

E. Calcein Release Under the Effect of Ultrasound Waves

Ultrasound is a well-known mechanism for triggering drug release from nanocarriers and enhancing drug transport through tissues and other membranes [24]. Many researchers advocate for the use of acoustic wave triggering to accelerate drug release and nanoparticle accumulation in tumors. In this section, we present the results of using ultrasound as a triggering mechanism to release calcein, a model drug, from liposomes prepared via microfluidics.

The test results indicate that the prepared liposomes were responsive to ultrasound as a trigger mechanism, as shown in Figure 10(a). As expected, the release rate increased with higher ultrasound intensities, with the first 20-second pulse releasing approximately 48%, 60%, and 78% of the encapsulated calcein at the three tested intensities of 6.2, 9, and 10 mW/cm^2, respectively. Within less than 3 minutes, a maximum calcein release of about 94% was achieved, regardless of the intensity of the ultrasound waves applied.

Furthermore, our research demonstrated that liposomes prepared with the herringbone microfluidic technique showed an

enhanced release of calcein when activated by ultrasound, in comparison to those produced by the conventional thin-film hydration method. The comparison of liposomes synthesized using the herringbone microfluidic approach versus the traditional method is depicted in Figure 10.

F. Cellular Uptake Studies

Cellular uptake analyses were conducted by comparing the fluorescence intensity of calcein within the cells. Both MCF-7 and MDA-MB-231 cells were subjected to treatment with targeted and non-targeted liposomes. Notably, the fluorescence intensity of cells treated with targeted liposomes exceeded that of those treated with non-targeted liposomes, as depicted in Figure 11. Specifically, the mean fluorescent intensity (MFI) of MCF-7 cells treated with estrone-conjugated liposomes was 1.5 times higher than that observed with non-targeted liposomes. Similarly, for MDA-MB-231 cells, the same treatment resulted in a 1.25-fold increase in fluorescent intensity. The enhanced uptake of estrone-conjugated liposomes by MCF-7 cells can be attributed to the higher concentration of estrogen receptors on their cell surfaces compared to MDA-MB-231 cells. The incorporation of the estrogen moiety into the liposomes imparted site-specificity, facilitating receptor-mediated endocytosis and enabling a more substantial delivery of the payload to the cancer cells.

V. Conclusion

We demonstrated the feasibility of synthesizing estrone liposomes using commercially available microfluidic chips, positioning microfluidics as a viable synthesis platform for researchers in the field of nanomedicine. This method offers significant advantages such as ease of use, reproducibility, and precisely controlling particle size. The commercial chips are compatible with a variety of solvents; in our experiments, we utilized both ethanol and methanol as solvents for the lipid solution, with ethanol yielding significantly smaller liposome sizes. Moreover, we showed that adjusting chemical and flow parameters, such as estrone concentration, total flow rate, and flow rate ratio within the chip, allows for the control of the liposomes' size. While higher flow rate ratios tend to produce smaller particles, excessively high ratios should be avoided as they decrease the prepared liposome concentration in the output solution.

The size of the liposomes remained nearly constant (with statistically insignificant changes) over a month, indicating their stability. To confirm that the prepared liposomes were responsive to ultrasound triggers, we successfully released calcein encapsulated within the liposomes by applying ultrasound waves at various intensities. These results suggest that the microfluidics technique is an effective method for synthesizing estrone liposomes, providing a solution to overcome the limitations of traditional methods. The work presented here introduces a simple and user-friendly platform to the nano drug delivery toolkit.

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