Drug Delivery and Translational Research (2019) 9:404–413
https://doi.org/10.1007/s13346-018-0587-4

SHORT COMMUNICATION

Rapid optimization of liposome characteristics using a combined microfluidics and design-of-experiment approach

Mahsa Sedighi[1,2] · Sandro Sieber[2] · Fereshteh Rahimi[1] · Mohammad-Ali Shahbazi[3,4] · Ali Hossein Rezayan[1] · Jörg Huwyler[2] · Dominik Witzigmann[2,5]

Published online: 10 October 2018

Abstract

Liposomes have attracted much attention as the first nanoformulations entering the clinic. The optimization of physicochemical properties of liposomes during nanomedicine development however is time-consuming and challenging despite great advances in formulation development. Here, we present a systematic approach for the rapid size optimization of liposomes. The combination of microfluidics with a design-of-experiment (DoE) approach offers a strategy to rapidly screen and optimize various liposome formulations, i.e., up to 30 liposome formulations in 1 day. Five representative liposome formulations based on clinically approved lipid compositions were formulated using systematic variations in microfluidics flow rate settings, i.e., flow rate ratio (FRR) and total flow rate (TFR). Interestingly, flow rate-dependent DoE models for the prediction of liposome characteristics could be grouped according to lipid-phase transition temperature and surface characteristics. For all formulations, the FRR had a significant impact ($p < 0.001$) on hydrodynamic diameter and size distribution of liposomes, while the TFR mainly affected the production rate. Liposome characteristics remained constant for TFRs above 8 mL/min. The stability study revealed an influence of lipid:cholesterol ratio (1:1 and 2:1 ratio) and presence of PEG on liposome characteristics during storage. To validate our DoE models, we formulated liposomes incorporating hydrophobic dodecanethiol-coated gold nanoparticles. This proof-of-concept step showed that flow rate settings predicted by DoE models successfully determined the size of resulting empty liposomes (109.3 ± 15.3 nm) or nanocomposites (111 ± 17.3 nm). This study indicates that a microfluidics-based formulation approach combined with DoE is suitable for the routine development of monodisperse and size-specific liposomes in a reproducible and rapid manner.

Keywords Liposomes · Microfluidics · Design-of-experiment · Physicochemical characteristics · Nanomedicines

Introduction

Over the past 20 years, nanomedicines have attracted significant attention due to their potential for diagnosis, prevention, and treatment of various diseases [1, 2]. Contrary to bulk structures or biological and chemical molecules, these nanoscale materials present diverse advantages [3–7]. Among all varieties of multifunctional nano-based drug delivery systems,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13346-018-0587-4) contains supplementary material, which is available to authorized users.

✉ Fereshteh Rahimi
rahimi.f@ut.ac.ir

✉ Jörg Huwyler
joerg.huwyler@unibas.ch

[1] Division of Nanobiotechnology, Department of Life Sciences Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

[2] Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

[3] Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, DK-2800 Kongens Lyngby, Denmark

[4] Department of Pharmaceutical Nanotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

[5] Department of Biochemistry and Molecular Biology, University of British Columbia, Health Sciences Mall, Vancouver, British Columbia, Canada

self-assembled spherical liposomes, characterized by a bilayer phospholipid membrane surrounding an aqueous core, have received considerable attention owing to unique physicochemical and biological properties as well as the tremendous potential for drug delivery applications using both hydrophilic and hydrophobic drugs [8–10]. These liposomal formulations change the pharmacokinetic properties of the encapsulated drugs (e.g., doxorubicin, daunorubicin, or vincristine) resulting in improved safety and increased therapeutic index [1, 8]. Over the past years, many liposome formulations (e.g., doxil, DaunoXome, Myocet, Marqibo, or Onivyde), comprised of various lipids have been approved by medical agencies [1, 11–13]. However, the optimization of liposome characteristics during drug development is still challenging despite great advances in formulation strategies.

According to previous reports, liposomes can be produced using a wide range of methods, including thin-film hydration, sonication, extrusion, reverse-phase evaporation, freeze-thaw process, or high-pressure homogenization for fabrication of multi- or unilamellar vesicles [13, 14]. All these methods suffer from similar drawbacks, including poor batch-to-batch reproducibility, risk for sample contamination, non-scalability, or lack of temperature control which increases the risk of lipid degradation [14]. To overcome these limitations, microfluidics-based methodologies have been introduced as an alternative method to produce liposomes based on nanoprecipitation and self-assembly reactions during millisecond-controlled mixing at the nano-liter scale [15–17]. This reproducible and robust strategy offers the possibility to reduce both production time and the economic costs of developing advanced formulations [14]. However, several parameters influence the formulation optimization and characteristics of resulting liposomes. For example, it is necessary to optimize microfluidic flow rate settings to produce liposomes with sizes suitable for biomedical applications.

In this study, we present a systematic approach based on a design-of-experiment (DoE) model for the rapid bottom-up formulation of well-defined liposomes by microfluidic mixing using a staggered herringbone chaotic micromixer (SHM). The DoE was used to systematically optimize experiments, resulting in a minimal number of formulation runs. In addition, the DoE enabled us to assess the influence of factors important to the microfluidics process as well as their impact on the characteristics of liposomes. As a proof-of-concept, we selected the lipid compositions of five clinically approved liposomes (i.e., doxil, DaunoXome, Myocet, Marqibo, and Onivyde) and combined microfluidics with DoE to determine optimal flow rate settings for the production of liposomes with specific sizes similar to the marketed products. The developed microfluidics prediction models were finally used to incorporate hydrophobic gold nanoparticles (HGNs) into the lipid bilayer without affecting the size of resulting liposomes. Empty liposomes and HGN-loaded liposomal nanocomposites were characterized using dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FT-IR), and transmission electron microscopy (TEM).

Materials and methods

Materials

The lipid hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-sn glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), and 1,2-distearoyl-sn-glycero-3- phosphorylcholine (DSPC) were obtained from Avanti Polar Lipids (Alabaster, USA). Egg yolk phosphatidylcholine (EPC) and sphingomyelin (SM) were purchased from Lipoid AG (Steinhausen, Switzerland). Cholesterol, Dulbecco's phosphate-buffered saline (D-PBS), absolute ethanol (EtOH), tetraoctylammonium bromide, dodecanethiol (DDT, 98%), uranyl acetate solution, gold (III) chloride trihydrate ($HAuCl_4$-$3H_2O$), sodium borohydride (98%), tetraoctylammonium bromide (98%), methanol, chloroform, and toluene were obtained from Sigma-Aldrich (Buchs, Switzerland).

Preparation of empty clinical liposomes using microfluidics

To prepare and optimize clinical liposome formulations, a microfluidics approach based on the NanoAssemblr™ benchtop instrument (Precision NanoSystems Inc., Vancouver, Canada) was used. The microfluidic cartridge had two inlets, which were merged to a micro-channel. One inlet was used for the lipid mixtures dissolved in EtOH. Initial lipid concentrations introduced to the micromixer were adjusted to increase dilution effect by increasing FRRs to result in final lipid concentrations of 2 mg/mL. Using this strategy, post-processing steps (i.e., sample concentrating) to match lipid concentrations of different runs could be avoided, making the process more time-efficient [16, 18, 19]. The other inlet contained an aqueous buffer (D-PBS, pH 7.4). Lipids and buffer were mixed under laminar flow in the SHM structure of the microfluidic cartridge in order to form unilamellar liposomes. Disposable syringes (1 mL for EtOH and 3 mL for aqueous solutions) suitable for the fluid inlet connectors were used. The NanoAssemblr™ allowed the control of TFR (4–12 mL/min) and FRR (1.5:1 to 5.5:1 D-PBS:EtOH ratio). Optimization of clinical liposome formulations (Table 1) using microfluidics was based on a full factorial DoE (Stavex 5.2, Aicos Technologies, Basel, Switzerland). The experimental design shown in Table 1 was used for the formulation of each clinical liposome composition listed in Table 2. In a last step, liposome formulations were purified using dialysis (12–14 kDa; Medicell membranes Ltd.,

Table 1 Clinical liposome formulations approved by medical agencies [13]. Liposome formulations are listed according to year of clinical approval. The major structural lipid of each liposome composition is italicized and the corresponding phase transition temperature (T_m) is given

Liposome product	Year	Composition	T_m	Molar ratio	Size	Active agent	References
Doxil	1995	*HSPC*:Chol:DSPE-PEG2000	53 °C	55:40:5	< 100 nm	Doxorubicin	[11, 20]
DaunoXome	1996	*DSPC*:Chol	55 °C	66.7:33.3	45 nm	Doxorubicin	[21]
Myocet	2000	*EPC*:Chol	− 5 to − 15 °C	55:45	180 nm	Doxorubicin	[22]
Marqibo	2012	*SM*:Chol	~ 40 °C	58:42	115 nm	Vincristine	[23, 24]
Onivyde	2015	*DSPC*:Chol:DSPE-MPEG 2000	55 °C	59.8:39.9:0.3	110 nm	Irinotecan	[25]

HSPC, hydrogenated soy phosphatidylcholine; *DSPC*, 1,2-distearoyl-sn-glycero-3-phosphorylcholine; *EPC*, egg yolk phosphatidylcholine; *SM*, sphingomyelin; *DSPE-PEG2000*, 1,2-distearoyl-sn glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; *Chol*, cholesterol

London, UK) against Poncini water to remove residual solvent (room temperature and overnight stirring at 150 rpm).

Physicochemical characterization of liposomes

Size and polydispersity index (PDI) of liposomes were determined by DLS using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Particle size and zeta potential were measured in aqueous solution at 20 °C.

Stability of liposomes

The stability tests were performed for 1 month. The samples were stored at 4 °C under a static condition (without stirring), and at specific time points, size and PDI were analyzed by DLS. All experiments were performed in triplicate.

DoE approach

To analyze the influence of TFR and FRR on the physicochemical characteristics of liposomes formulated using microfluidics, a DoE was used (MODDE software, Umetrics). Results from three independent experiments were analyzed using both multiple linear regressions (MLR) and partial least square (PLS) regression to optimize the prediction models. The collected data was used to determine the statistical significance between model coefficients. The prediction error was minimized using the least squares regression analysis. The analysis of variance (ANOVA) was carried out to validate models based on R^2 and Q^2 as the goodness of fit and prediction, respectively. In addition, the model validity and reproducibility were determined. The model and replicate errors were investigated by lack of fit (LOF) test.

Table 2 Design-of-experiment (DoE)-based microfluidic flow settings. Different flow rate ratios (FRR) and total flow rates (TFR) used for the formulation development of each clinical liposome formulation are represented

Run no.	FRR	TFR
1	5.5	8
2	4.5	6
3	4.5	10
4	3.5	4
5	3.5	8
6	3.5	12
7	2.5	6
8	2.5	10
9	1.5	8

HGN synthesis

HGNs were synthesized using the protocol previously reported by Rasch et al. [26]. Briefly, 20 mL of a 41.65-mM $HAuCl_4$ solution in H_2O was mixed with 80 mL of a 150-mM tetraoctylammonium bromide solution in toluene as an organic phase by stirring at 600 rpm for 60 min. The purple organic phase was collected by extraction, and the colorless aqueous phase was discarded. A 0.6-mL DDT was then injected into the toluene phase to achieve a 3:1 ratio of thiol groups to Au atoms. After 15 min, the toluene phase turned colorless and then 20 mL of a 10-mM sodium borohydride solution was added rapidly, resulting in a dark brown microemulsion. After 12 h of stirring, the brown toluene phase was extracted and centrifuged at 8000 rpm for 5 min along with methanol as anti-solvent (1:4 toluene to methanol ratio) to precipitate the gold nanocrystals. Gold nanocrystals were dispersed in chloroform and size-selective precipitation was performed by centrifugation at 8500 rpm for 10 min to collect any poorly dispersed or aggregated nanocrystals. The supernatant was gently mixed with 250 μl of methanol and then centrifuged at 8500 rpm for 5 min. The process of adding methanol, centrifuging, and separating the supernatant from precipitates was repeated several times, and the precipitates collected in steps 5–7 were used for nanocomposite formulations.

HGN characterization

The size and shape of HGNs were determined by TEM imaging (CM-100; Philips, Eindhoven, Netherlands) [27]. Since toluene is able to degrade carbon-coated copper grids, toluene was completely removed by evaporation under N_2 and HGNs were dispersed in EtOH. After that, 5 μL of samples in EtOH

was deposited on a 400-mesh grid after plasma treatment for 30 s. The diameter of at least 100 HGNs was measured for size. The qualitative analysis of synthesized HGNs was performed using a FTIR spectrometer to assess functional groups on the surface. The FTIR spectra in the range of 550–4000 cm^{-1} were obtained using a FTIR ATR-IR (Bruker Tensor 27).

HGN encapsulation into liposomes

Lipid stock solutions and HGNs were dissolved using 40% THF in EtOH at a lipid to HGN ratio of 0.107 (*w*/*w*) [28]. This lipid gold nanocrystal solution was rapidly mixed with an aqueous solution (D-PBS; pH 7.4) as described for preparation of empty clinical liposomes using the Nanoassemblr™ benchtop instrument. FRR and TFR were selected based on DoE prediction models. Nanocomposites were purified using dialysis (12–14 kDa) to remove residual solvent.

HGN-liposome nanocomposite characterization

TEM analysis was used to detect HGNs in liposomes as described above [27]. In brief, after sample deposition, grids were washed with water and then incubated for 10 s with a 2% uranyl acetate solution. The excess of uranyl acetate was removed and the samples were dried overnight at room temperature before imaging.

Statistical analysis

All experiments were performed in triplicate. Mean and standard deviation were then calculated. To access statistical significance for paired comparisons, a one-way ANOVA combined with Bonferroni's multiple comparison was used. Significantly different results are indicated for p values lower than 0.001(*). All calculations were performed using Origin Pro 8.6 software (Origin Lab Corp., MA, USA).

Results and discussion

In recent years, several groups have demonstrated that microfluidics is a reproducible, scalable, cost-effective, and simple method for the production of various nanoparticles. Rotational flow and wrapping of fluid streams in microfluidic channels results in rapid mixing of aqueous and organic fluids [15]. Small unilamellar liposomes can be formed in milliseconds due to the polarity increase in the SHM channels that facilitates a nano-precipitation reaction followed by supersaturation and self-assembly of lipid molecules [16]. Importantly, key physicochemical properties of liposomes, such as size and

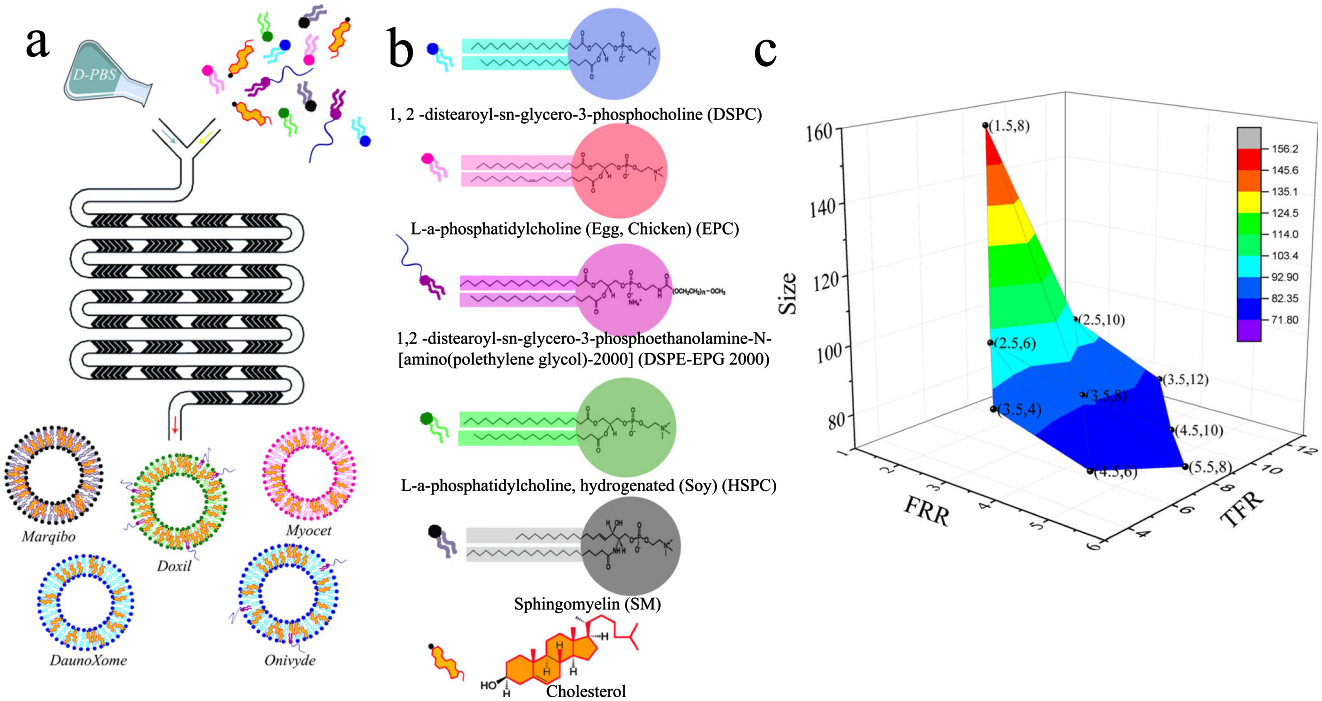

Fig. 1 Schematic representation of rapid microfluidic-based liposome formulation development. **a** Liposomes based on clinically approved lipid compositions are formulated using a staggered herringbone micromixer (Staggered herringbone micromixer adapted from Belliveau et al. [15]). **b** Chemical structures of different lipids used in clinical liposome formulations. **c** Schematic representation of design space investigated during microfluidic-based full factorial DoE approach. Influence of fFRR and TFR on the physicochemical characteristics of liposome was determined

PDI, are highly influenced by flow rate settings during microfluidic-based formulation. Thus, optimization of the entire process is difficult and time-consuming when a one-factor-at-a-time method is used.

Here, we propose a systematic approach based on DoE for the rapid optimization of liposome formulations. The complete optimization procedure includes five steps: (i) design of microfluidic settings based on a full-factorial DoE, (ii) formulation of liposomes using specific flow rate settings for each run, (iii) physicochemical characterization of resulting liposomes, (iv) determination of DoE prediction models, (v) formulation of liposomes with specific sizes using predicted flow settings. To demonstrate the validity of our strategy, five different liposome compositions based on clinically approved liposome formulations, i.e., doxil, DaunoXome, Myocet, Marqibo, and Onivyde [21–25], were selected (Table 1).

The DoE design space presented here is based on nine different runs with systematic variations in FRR and TFR (Table 2). This strategy offers the possibility to assess the effect of both FRR (from 1.5:1 to 5.5:1) and TFR (4 to 12 mL/min) on physicochemical characteristics of liposomes (size, PDI, and zeta potential) using a minimal number of runs. Notably, this design can be expanded to any other parameter of interest. After DoE-based specification of microfluidic settings (Table 2), different lipid mixtures based on clinically approved compositions were prepared in EtOH (Table 1,

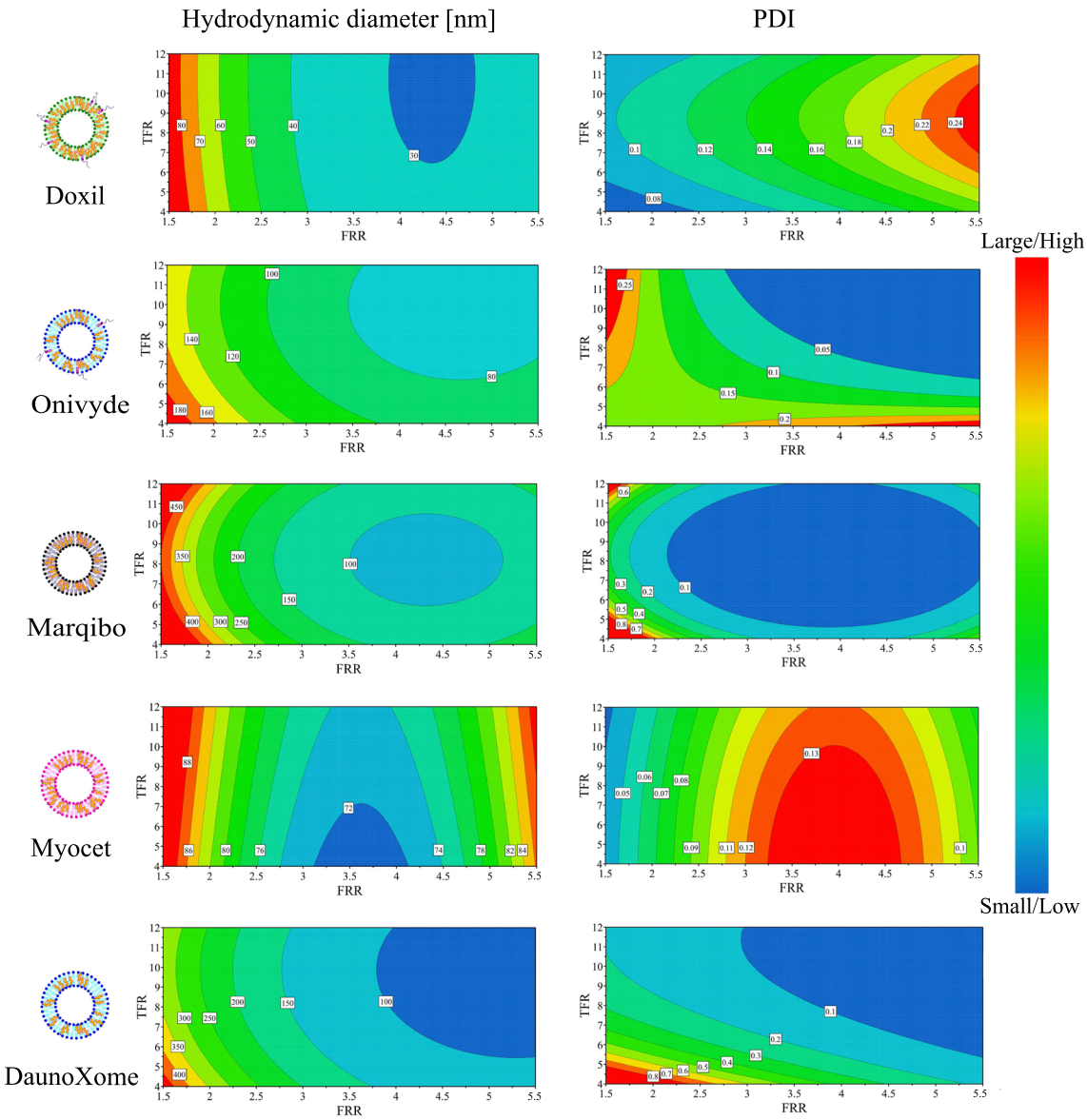

Fig. 2 DoE-based optimization of clinical liposome formulations using microfluidics. TFR and FRR were altered to investigate their effect on hydrodynamic size and size distribution (polydispersity index, PDI) of resulting liposomes. DoE-based prediction models are represented as two-dimensional contour plots with color codes indicating liposome sizes from small (blue) to large (red) and PDI values from low (blue) to high (red). Specific sizes and PDI values are indicated in boxes in each design space

Figs. 1a and 2b). Microfluidic flow settings were adjusted for each run and liposomes were prepared by mixing of ethanolic lipid solution with D-PBS. Figure 1c schematically illustrates the design space to investigate the influence of FRR and TFR on liposome size based on a combined microfluidic-DoE approach.

Previous studies have shown that microfluidics is a robust method to precisely control TFR and FRR between aqueous and solvent inlet streams [16]. Interestingly, all procedures for the formation of the presented liposomes were performed in less than 1 h, demonstrating that microfluidics is highly time-efficient in contrast to other common liposome preparation methods, such as film rehydration-extrusion (Table 3).

In all experiments, zeta potential of resulting liposomes did not change significantly with varying TFR and FRR. This indicates that surface properties of different liposome compositions are maintained despite varying microfluidic settings. The zeta potential for each formulation is presented in Table S1. The predicted DoE models for flow setting-dependent size or PDI of liposomes with different lipid compositions are shown in Fig. 2. For all formulations, the smallest hydrodynamic diameter was achieved at medium FRR. Smaller FRRs caused an increase in liposomal size. Notably, design spaces could be grouped according to lipid-phase transition temperature and surface characteristics. Lipid compositions containing surface-modifying lipids (i.e., doxil, Onivyde) or lipids with low-transition temperature (i.e., Myocet) resulted in smaller liposomes at defined FRR as compared to other lipid compositions (i.e., Marqibo, DaunoXome). This result was also confirmed by resulting PDI values. Size distributions of doxil, Onivyde, or Myocet were always lower than 0.25, whereas PDI values of Marqibo and DaunoXome increased significantly at low FRRs. This might be explained by the reduced rate of diffusion within the micromixer channels at low FRRs [14]. Taken together, these results confirmed that FRR is a key parameter to control size and PDI of liposomes, as previously reported for other nanoparticles [14, 29, 31, 32].

In sharp contrast, TFR did not significantly influence the size of resulting liposomes (Fig. 2 and Fig. S1a) [16, 33]. However, there was a significant change in PDI with increasing flow rate for liposomes containing DSPC, i.e., Onivyde and DaunoXome (Fig. 2 and Fig. S1b). At medium FRR, increasing TFR resulted in smaller size distributions. The different PDI levels for DaunoXome and Onivyde can be explained by the presence of DSPE-PEG, which results in homogenous liposome formation. The constant PDI at varying TFR for doxil in comparison to Onivyde might be due to the usage of HSPC, which consists of a lipid mixture with varying fatty acid tail lengths, and, thus, results in homogenous particle formation even at low flow rates (i.e., 4 mL/min). These results confirmed that lipid compositions highly influence the features of liposome [34]. Importantly, the size and PDI for all liposomes remained constant for TFRs above 8 mL/min. This behavior of vesicle formation at increased TFR demonstrates the ability of microfluidics as high-throughput formulation method, which is one of the key aspects for large-scale manufacturing of liposomes.

The statistical analysis of the DoE prediction models indicated that the MLR model has a higher fit for all the tested compositions as compared to the PLS model. The optimized model was determined according to R^2 and Q^2 values presented in Table S2. A smaller difference than 0.3 between R^2 and Q^2 represents an index for a good model fit. The significant coefficients determined in the model are shown in Fig. S2. As an example, the FRR × FRR term in the Myocet size model confirmed the significant impact of FRR on the size of Myocet-based liposomes formed in the microfluidics channel. In

Table 3 Expert opinion on the most important advantages and disadvantages of top-down versus bottom-up formulation strategies. Preparation of liposomes using conventional film rehydration-extrusion method as compared to rapid microfluidics mixing technique [18, 29, 30]

Formulation strategy	Advantages	Disadvantages
Top-down formulation strategy (i.e., film rehydration-extrusion)	• High lipid concentrations possible	• Several process steps are needed • Often temperature • Problem in the sterilization of liposomal preparations • Limited or challenging scalability • Lack of temperature control which increases the risk of lipid degradation
Bottom-up microfluidics formulation	• Time efficient due to decreased number of formulation steps • Possibility of in situ monitoring of the liposome formation process • Continuous production and scaling up by microfluidics parallelization • Precise control of liposome size via adjustment of flow settings	• Formulations mostly need to be concentrated due to low final lipid concentration • Limited studies on effect of drug encapsulation on liposome size

addition, Table S2 shows the ANOVA analysis, demonstrating the statistical significance and validation of each model generated. All models confirmed the significant impact of FRR on liposome size and PDI, while varying TFR mainly resulted in a difference of production rate.

After demonstrating that a combined microfluidics DoE approach is a rapid and controllable strategy for the production and optimization of liposomes, we also assessed the long-term stability of liposomes formulated by microfluidic manufacturing at a storage temperature of 4 °C. Clinical liposomes were prepared by microfluidics at different FRR and TFR as described in Table 2 and stored at 4 °C for 1 month. All liposome formulations except doxil-based liposomes showed good size stability over the course of storage and liposome sizes remained mainly unaffected (Fig. 3). In the case of doxil, limit size liposomes were produced [35], which resulted in a minor size increment during storage and also alteration of size distribution. Notably, changes in PDI during storage were remarkable for samples prepared at lowest TFR (Fig. S3). According to the stability tests, homogenous liposomes (i.e., without significant differences in vesicle size) showed a higher long-term stability after preparation at higher FRR. This is in agreement with a report by Correia et al. [33] who showed improved stability of liposome formulations with increased FRR. In addition, it has previously been shown that liposomes with 1:1 lipid:cholesterol ratio have an ideal stability [36, 37] confirming our observations in the case of the Myocet formulation with a 1.1:0.9 EPC:Chol ratio. Other groups reported that a 2:1 ratio between lipids and cholesterol might increase liposome stability (e.g., in DaunoXome and Onivyde) [38]. Figure 3 illustrates the change in size for diverse lipid:cholesterol ratios and presence of PEG in lipid compositions over time.

Based on our results, both claims are valid and the optimal lipid-to-cholesterol ratio for storage stability of liposomes manufactured by microfluidics technology is between 1:1 and 2:1.

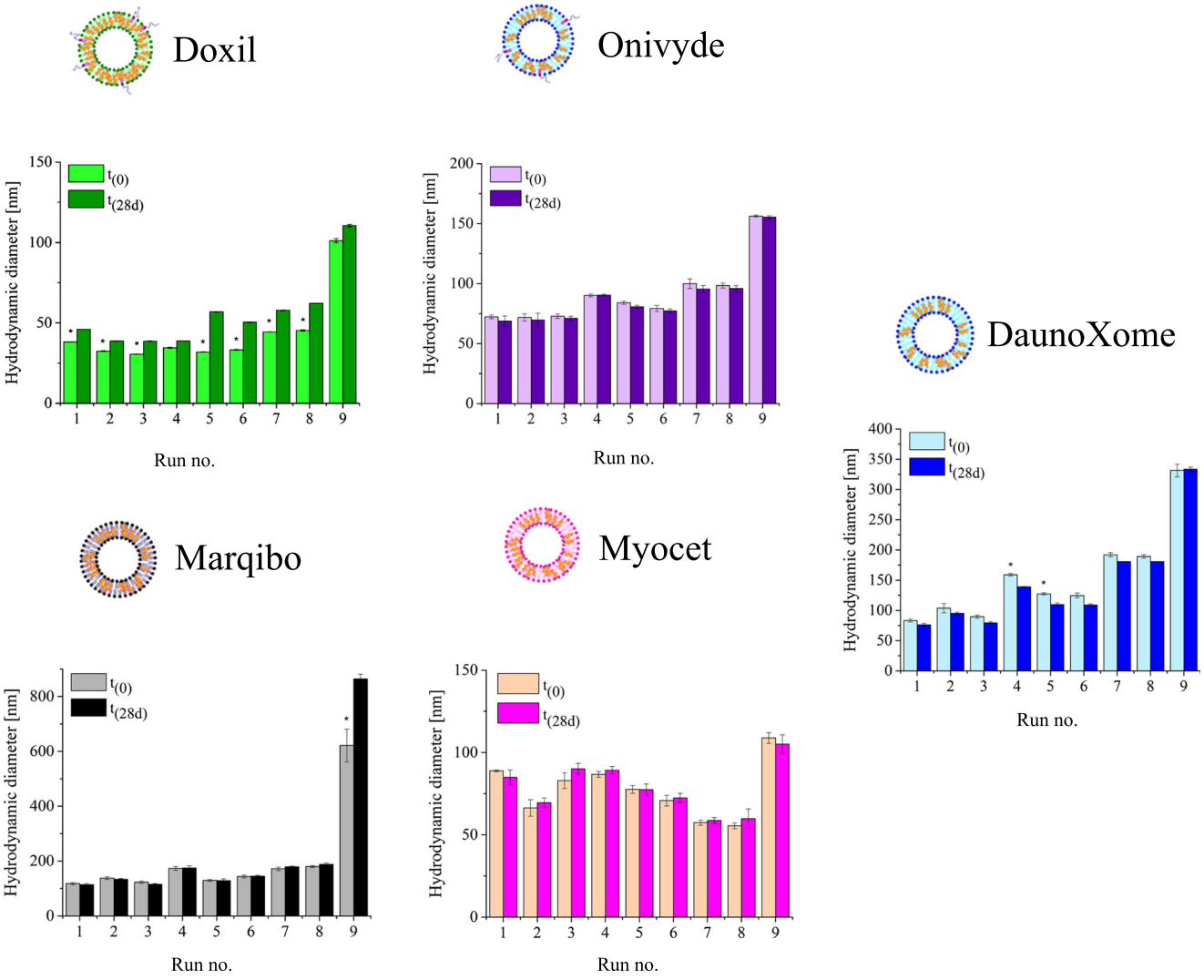

Fig. 3 Storage stability of clinical liposomes formulated by microfluidics. Liposomes were stored at 4 °C for 28 days. The bright and dark diagrams represent the sizes of liposomes after preparation ($t_{(0)}$) and after storage at 4 °C for 28 days ($t_{(28)}$). Run numbers indicate the microfluidics settings (i.e., FRR/TFR) based on Table 2

To validate our DoE-based formulation strategy, we used the flow rate settings identified during the DoE to incorporate HGNs into liposomes with a defined size (Fig. 4). Gold nanoparticles have attracted great attention in the field of diagnosis and therapy due to their unique physicochemical properties, facile synthesis pathway, and various functionalization capabilities [27, 39–42].

Therefore, we synthesized HGNs with diameters between 1.5 and 3 nm for the incorporation into liposomes (Fig. 4a, b) [26]. The FTIR analysis of HGNs (Fig. 4c) showed peaks at 720 cm^{-1} and 2850–2900 cm^{-1}, corresponding to C-S and CH_2–CH_2 symmetrical and unsymmetrical stretching, respectively. The S-H vibration peak at 2572 cm^{-1} was not observed, suggesting the formation of Au-sulfide bonds and replacement of H with Au in S-H groups of DDT. These results confirmed the successful synthesis of ideal HGNs for the incorporation into the liposomes.

HGN-liposome composites were formulated by rapid mixing of an ethanolic solution containing HGNs/lipids and an aqueous phase (D-PBS) at optimized FRR and TFR. Notably, HGNs were not dispersible in pure EtOH and addition of at least 40% THF was needed for adequate dispersion [28]. Resulting HGN-liposome nanocomposites had a similar hydrodynamic diameter as compared to empty clinical liposomes with sizes of 111 ± 17.3 nm and 109.3 ± 15.3 nm, respectively (Fig. 4d, e). Electron microscopy analysis confirmed the incorporation of HGNs (dark spot) into liposomes without formation of HGN clusters (Fig. 4e). Recently, it has been shown that hydrophobic alkyl chains of phospholipids and the presence of cholesterol can improve hydrophobic association of nanoparticles in the bilayer space and the entrapment efficiency [27, 43, 44]. Therefore, it is tempting to speculate that HGNs associate with hydrophobic lipid tails in the lipid bilayer due to a strong thermodynamic driving force [26]. The HGN-lipid ratio was adjusted to result in encapsulation of a single HGN per liposome. Thus, highly efficient mixing in the microfluidic channels allowed efficient loading of HGNs without affecting physicochemical characteristics of liposomes. Importantly, flow rate settings selected based on DoE models resulted in predicted diameters demonstrating the potential of our appraoch to formulate liposomes with specific physicochemical characteristics. This facilitates the process development resulting in reduced time and costs. In addition, this bottom-up formulation strategy is generally considered as less harsh in comparison to conventional top-down methods for the preparation of unilamellar liposomes based on mechanical disruption of multilamellar vesicles [16]. In our opinion, the key advantage of microfluidics is the continuous process which allows for a much easier scale up and parallelization when compared to the conventional methods (Table 3).

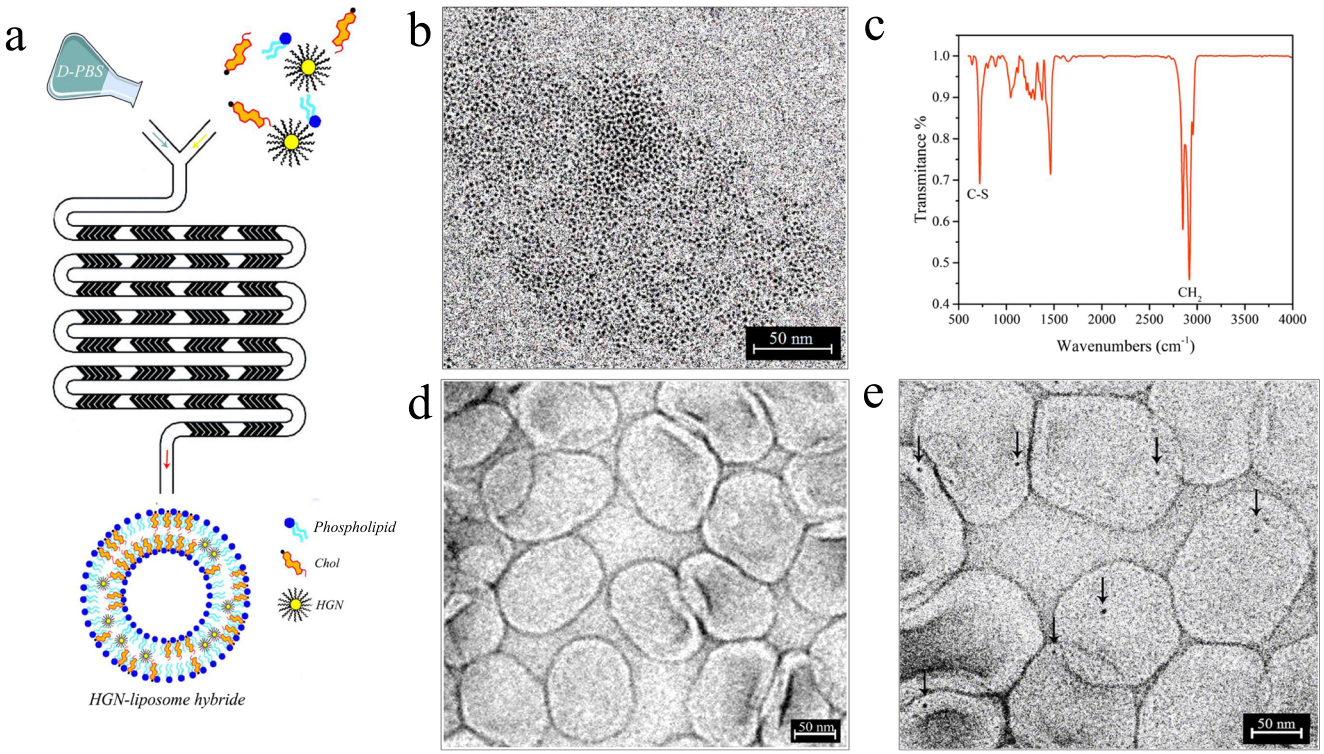

Fig. 4 Validation of DoE prediction model. **a** Schematic representation of microfluidics-based encapsulation of hydrophobic gold nanoparticles (HGNs) into a clinical liposome formulation (Staggered herringbone micromixer adapted from Belliveau et al. [15]). **b** Representative transmission electron micrograph of HGNs. **c** Fourier-transform infrared (FTIR) spectrum of HGNs functionalized with dodecanethiol (DDT). Transmission electron microscopy (TEM) analysis of **d** empty liposomes and **e** HGN nanocomposites. Single HGNs are clearly visualized within liposomes (black arrows)

Conclusion

In this study, we demonstrate that combining microfluidics with DoE is a robust and rapid strategy to formulate liposomes with well-defined and reproducible size. DoE analysis confirmed the statistical significance of flow rate settings on the resulting size and PDI of liposomes. The determined models could efficiently be used to predict liposome characteristics as a function of microfluidic parameters (i.e., FRR and TFR). Thus, the size of liposomes formulated by microfluidics could be easily adjusted via simple changes of flow rate settings based on design spaces. This strategy presents an interesting alternative to time-consuming traditional methods for the formulation of liposomes. Advantages including simple screening and capability of facile scale-up make the presented approach suitable for many applications. In the future, this might facilitate the screening of various nanocarriers during preclinical development and may open up a path towards broad applicability of advanced technologies in the preparation of conventional drug delivery systems.

Acknowledgments We thank the bioimaging center of the Biozentrum Basel for their support with electron microscopy techniques and Tomas Skrinskas for proofreading the manuscript.

Funding information This study received financial support from the "Stiftung zur Förderung des pharmazeutischen Nachwuchses in Basel," "Freiwillige Akademische Gesellschaft Basel," and the Swiss National Science Foundation (SNF grant No. 174975 and 173057).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Wicki A, Witzigmann D, Balasubramanian V, Huwyler J. Nanomedicine in cancer therapy: challenges, opportunities, and clinical applications. J Control Release Off J Control Release Soc. 2015;200:138–57.
2. Liu Y, Miyoshi H, Nakamura M. Nanomedicine for drug delivery and imaging: a promising avenue for cancer therapy and diagnosis using targeted functional nanoparticles. Int J Cancer. 2007;120:2527–37.
3. Lu Y, Chen W. Sub-nanometre sized metal clusters: from synthetic challenges to the unique property discoveries. Chem Soc Rev. 2012;41:3594–623.
4. Mieszawska AJ, Mulder WJM, Fayad ZA, Cormode DP. Multifunctional gold nanoparticles for diagnosis and therapy of disease. Mol Pharm. 2013;10:831–47.
5. Shahbazi M-A, Almeida PV, Correia A, Herranz-Blanco B, Shrestha N, Mäkilä E, et al. Intracellular responsive dual delivery by endosomolytic polyplexes carrying DNA anchored porous silicon nanoparticles. J Control Release Off J Control Release Soc. 2017;249:111–22.
6. Shahbazi M-A, Shrestha N, Mäkilä E, Araújo F, Correia A, Ramos T, et al. A prospective cancer chemo-immunotherapy approach mediated by synergistic CD326 targeted porous silicon nanovectors. Nano Res. 2015;8:1505–21.
7. Tokonami S, Yamamoto Y, Shiigi H, Nagaoka T. Synthesis and bioanalytical applications of specific-shaped metallic nanostructures: a review. Anal Chim Acta. 2012;716:76–91.
8. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev. 2013;65:36–48.
9. Gregoriadis G. Liposomology: delivering the message. J Liposome Res. 2018;28:1–4.
10. Slingerland M, Guchelaar H-J, Gelderblom H. Liposomal drug formulations in cancer therapy: 15 years along the road. Drug Discov Today. 2012;17:160–6.
11. Barenholz Y. Doxil®–the first FDA-approved nano-drug: lessons learned. J Control Release Off J Control Release Soc. 2012;160:117–34.
12. Anselmo AC, Mitragotri S. Nanoparticles in the clinic. Bioeng Transl Med. 2016;1:10–29.
13. Bulbake U, Doppalapudi S, Kommineni N, Khan W. Liposomal formulations in clinical use: an updated review. Pharmaceutics. 2017;9:1–33.
14. Kastner E, Kaur R, Lowry D, Moghaddam B, Wilkinson A, Perrie Y. High-throughput manufacturing of size-tuned liposomes by a new microfluidics method using enhanced statistical tools for characterization. Int J Pharm. 2014;477:361–8.
15. Belliveau NM, Huft J, Lin PJ, Chen S, Leung AK, Leaver TJ, et al. Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. Mol Ther Nucleic Acids. 2012;e37:1.
16. Kastner E, Verma V, Lowry D, Perrie Y. Microfluidic-controlled manufacture of liposomes for the solubilisation of a poorly water soluble drug. Int J Pharm. 2015;485:122–30.
17. Song Y, Hormes J, Kumar CSSR. Microfluidic synthesis of nanomaterials. Small Weinh Bergstr Ger. 2008;4:698–711.
18. Carugo D, Bottaro E, Owen J, Stride E, Nastruzzi C. Liposome production by microfluidics: potential and limiting factors. Sci Rep. 2016;6:25876.
19. Mijajlovic M, Wright D, Zivkovic V, Bi JX, Biggs MJ. Microfluidic hydrodynamic focusing based synthesis of POPC liposomes for model biological systems. Colloids Surf B Biointerfaces. 2013;104:276–81.
20. Wibroe PP, Ahmadvand D, Oghabian MA, Yaghmur A, Moghimi SM. An integrated assessment of morphology, size, and complement activation of the PEGylated liposomal doxorubicin products Doxil®, Caelyx®, DOXOrubicin, and SinaDoxosome. J Control Release Off J Control Release Soc. 2016;221:1–8.
21. Petre CE, Dittmer DP. Liposomal daunorubicin as treatment for Kaposi's sarcoma. Int J Nanomedicine. 2007;2:277–88.
22. Leonard RCF, Williams S, Tulpule A, Levine AM, Oliveros S. Improving the therapeutic index of anthracycline chemotherapy: focus on liposomal doxorubicin (Myocet). Breast Edinb Scotl. 2009;18:218–24.
23. Rodriguez MA, Pytlik R, Kozak T, Chhanabhai M, Gascoyne R, Lu B, et al. Vincristine sulfate liposomes injection (Marqibo) in heavily pretreated patients with refractory aggressive non-Hodgkin lymphoma: report of the pivotal phase 2 study. Cancer. 2009;115:3475–82.
24. Silverman JA, Deitcher SR. Marqibo® (vincristine sulfate liposome injection) improves the pharmacokinetics and pharmacodynamics of vincristine. Cancer Chemother Pharmacol. 2013;71:555–64.
25. Drummond DC, Noble CO, Guo Z, Hong K, Park JW, Kirpotin DB. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res. 2006;66:3271–7.
26. Rasch MR, Rossinyol E, Hueso JL, Goodfellow BW, Arbiol J, Korgel BA. Hydrophobic gold nanoparticle self-assembly with

phosphatidylcholine lipid: membrane-loaded and janus vesicles. Nano Lett. 2010;10:3733–9.
27. Witzigmann D, Sieber S, Porta F, Grossen P, Bieri A, Strelnikova N, et al. Formation of lipid and polymer based gold nanohybrids using a nanoreactor approach. RSC Adv. 2015;5:74320–8.
28. Kulkarni JA, Tam YYC, Chen S, Tam YK, Zaifman J, Cullis PR, et al. Rapid synthesis of lipid nanoparticles containing hydrophobic inorganic nanoparticles. Nanoscale. 2017;9:13600–9.
29. Balbino TA, Aoki NT, Gasperini AAM, Oliveira CLP, Azzoni AR, Cavalcanti LP, et al. Continuous flow production of cationic liposomes at high lipid concentration in microfluidic devices for gene delivery applications. Chem Eng J. 2013;226:423–33.
30. Huang Z, Li X, Zhang T, Song Y, She Z, Li J, et al. Progress involving new techniques for liposome preparation. Asian J Pharm Sci. 2014;9:176–82.
31. Balbino TA, Azzoni AR, de la Torre LG. Microfluidic devices for continuous production of pDNA/cationic liposome complexes for gene delivery and vaccine therapy. Colloids Surf B Biointerfaces. 2013;111:203–10.
32. Jahn A, Stavis SM, Hong JS, Vreeland WN, DeVoe DL, Gaitan M. Microfluidic mixing and the formation of nanoscale lipid vesicles. ACS Nano. 2010;4:2077–87.
33. Correia MG, Briuglia ML, Niosi F, Lamprou DA. Microfluidic manufacturing of phospholipid nanoparticles: stability, encapsulation efficacy, and drug release. Int J Pharm. 2017;516:91–9.
34. Leung AKK, Tam YYC, Chen S, Hafez IM, Cullis PR. Microfluidic mixing: a general method for encapsulating macromolecules in lipid nanoparticle systems. J Phys Chem B. 2015;119:8698–706.
35. Zhigaltsev IV, Tam YK, Leung AKK, Cullis PR. Production of limit size nanoliposomal systems with potential utility as ultra-small drug delivery agents. J Liposome Res. 2016;26:96–102.
36. Gregoriadis G, Davis C. Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. Biochem Biophys Res Commun. 1979;89:1287–93.
37. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem J. 1980;186:591–8.
38. Briuglia M-L, Rotella C, McFarlane A, Lamprou DA. Influence of cholesterol on liposome stability and on in vitro drug release. Drug Deliv Transl Res. 2015;5:231–42.
39. Arvizo R, Bhattacharya R, Mukherjee P. Gold nanoparticles: opportunities and challenges in nanomedicine. Expert Opin Drug Deliv. 2010;7:753–63.
40. Dreaden EC, Alkilany AM, Huang X, Murphy CJ, El-Sayed MA. The golden age: gold nanoparticles for biomedicine. Chem Soc Rev. 2012;41:2740–79.
41. Dreaden EC, Mackey MA, Huang X, Kang B, El-Sayed MA. Beating cancer in multiple ways using nanogold. Chem Soc Rev. 2011;40:3391–404.
42. Nikfarjam A, Rezayan AH, Mohammadkhani G, Mohammadnejad J. Label-free detection of digoxin using localized surface plasmon resonance-based nanobiosensor. Plasmonics. 2017;12:157–64.
43. Liyun F, Xianggui K, Kefu C, Yajuan S, Qinghui Z, Youlin Z. Efficient phase transfer of hydrophobic CdSe quantum dots: from nonpolar organic solvent to biocompatible water buffer. Mater Chem Phys. 2005;93:310–3.
44. Al-Jamal WT, Al-Jamal KT, Tian B, Lacerda L, Bomans PH, Frederik PM, et al. Lipid−quantum dot bilayer vesicles enhance tumor cell uptake and retention in vitro and in vivo. ACS Nano. 2008;2:408–18.