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ORIGINAL ARTICLE

The Formulation of Methylene Blue Encapsulated, Tc-99m Labeled Multifunctional Liposomes for Sentinel Lymph Node Imaging and Therapy

Sentinel Lenf Nodu Görüntüleme ve Tedavisi İçin Metilen Mavisi Enkapsüle Edilmiş, Tc-99m İşaretli Çok-Fonksiyonlu Lipozomların Formülasyonu

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ABSTRACT

Objectives: Methylene blue (MB) is a commonly used dye that can be used for near-infrared (NIR) imaging and photodynamic therapy (PDT) by producing reactive oxygen species after light exposure, inducing apoptosis. The limiting factor of MB is its poor penetration through cell membranes. Its decreased cellular uptake can be prevented by encapsulation in drug delivery systems such as liposomes. Additionally, the enhanced permeability and retention effect of tumors enables enhanced accumulation of nanocarriers at the target site.

Materials and Methods: Nanosized, MB encapsulated, Tc-99m radiolabeled Lipoid S PC:PEG2000-PE:Chol: DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were formulated to design multifunctional theranostic nanocarriers for: 1) NIR imaging, 2) gamma probe detection of sentinel lymph nodes (SLNs), and 3) PDT, which can provide accurate imaging and therapy helping surgery with a single liposomal system. The characterization of liposomes was performed by measuring particle size, zeta potential, phospholipid content, and encapsulation efficiency. Additionally, the *in vitro* release profile of MB and physical stability were also evaluated over 6 months at determined time intervals by measuring the mean particle size, zeta potential, encapsulation efficiency, and phospholipid content of liposomes kept at room temperature (25˚C) and 4˚C.

Results: Tc-99m radiolabeled, nanosized Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes showed suitable particle size (around 100 nm), zeta potential (-9 to -13 mV), encapsulation efficiency (around 10%), phospholipid efficiency (around 85-90%), and release profiles. Additionally, the liposomes found stable for 3 months especially when kept at 4˚C.

Conclusion: MB encapsulated, Tc-99m radiolabeled, nanosized Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were found to have potential for SLN imaging by gamma probe detection, NIR imaging, and PDT. *In vitro* and *in vivo* imaging and therapeutic efficiency should be definitely evaluated to enable a final decision and our studies on this research topic are continuing.

Key words: Sentinel lymph node, methylene blue, theranostic nanomedicine, diagnosis

ÖZ

Amaç: Metilen mavisi (MV) yakın infrared (NIR) görüntüleme ve foto-dinamik tedavi (PDT) için, ışığa maruziyet sonrası reaktif oksijen radikalleri üreterek apoptozu indükleyen sıklıkla kullanılan bir boyadır. MV'nin hücre membranlarından zayıf penetrasyonu etkinliğini kısıtlayan bir faktördür. Azalmış hücresel alımı lipozomlar gibi ilaç taşıyıcı sistemlere enkapsülasyonu ile önlenebilir. Ek olarak, tümörlerin artmış geçirgenlik ve tutulum özellikleri nanotaşıyıcıların hedef bölgede tutulumunu sağlar.

Gereç ve Yöntemler: Nanoboyutlu, MV enkapsüle edilmiş, Tc-99m radyo-işaretli Lipoid S PC:PEG2000-PE:Chol:DTPA-PE ve DPPC:PEG2000-PE:Chol:DTPA-PE multifonksiyonel teranostik lipozomları daha kesin görüntüleme ve cerrahiye yardımcı olabilecek tedavi sağlayabilmek amacıyla; 1) NIR görüntüleme, 2) sentinel lenf nodlarının (SLN) gama prob ile tayini ve 3) PDT'yi aynı lipozomal sistemde dizayn edilebilen formülasyonlar olarak hazırlanmıştır. Lipozomların karakterizasyonu partikül boyutu, zeta potansiyeli, fosfolipit içeriği ve enkapsülasyon etkinliği ölçülerek yapılmıştır. Ayrıca, MV'sinin *in vitro* salım profili ve fiziksel stabilitesi altı aylık sürede belirli zaman aralıklarında ortalama partikül boyutu, zeta potansiyeli, enkapsülasyon etkinliği ve fosfolipit içeriği oda sıcaklığında (25˚C) ve 4˚C'de ölçülerek değerlendirilmiştir.

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Bulgular: Tc-99m radyo-işaretli, nanoboyutlu Lipoid S PC:PEG2000-PE:Chol:DTPA-PE ve DPPC:PEG2000-PE:Chol:DTPA-PE lipozomları uygun partikül boyutu (100 nm civarında), zeta potansiyeli (-9 mV'den -13 mV'e dek), enkapsülasyon etkinliği (%10 civarında), fosfolipit etkinliği (%85-90 civarında) ve salım profili göstermiştir. Ayrıca, 4˚C'de saklandıklarında lipozomlar 3 ay boyunca stabil kalmıştır.

Sonuç: MV enkapsüle, Tc-99m radyo-işaretli, nanoboyutlu Lipoid S PC:PEG2000-PE:Chol:DTPA-PE ve DPPC:PEG2000-PE:Chol:DTPA-PE lipozomlarının SLN'nın gama probla tayin edilebilmesi, NIR görüntüleme ve PDT için potansiyellerinin olduğu bulunmuştur. *İn vitro* ve *in vivo* görüntüleme ve tedavi etkinliklerinin daha kesin sonuca ulaşmak için değerlendirilmesi gerekmektedir ve bu konudaki çalışmalarımız devam etmektedir.

Anahtar kelimeler: Sentinel lenf nodu, metilen mavisi, teranostik nanotıp, teşhis

INTRODUCTION

The identification and mapping of sentinel lymph nodes (SLNs) for biopsy or imaging are commonly used for staging of many cancers especially breast cancer. SLNs are important as they are the first nodes draining the primary tumor to which a malignancy is likely to metastasize. Various dyes have been used for SLN identification such as isosulfan blue and methylene blue (MB). MB is a cheap and easily accessible dye that is approved by the Food and Drug Administration. Additionally, its side effects are less serious than those of isosulfan blue.[1,2] The limiting factor of MB is its poor penetration through cell membranes.

Radiopharmaceuticals have been used as an alternative or in addition to dyes for external imaging and/or radiation detector monitoring for SLN detection and mapping before surgery. Intraoperative use of gamma probe detectors enables confirmation of external sampling procedures by directly counting the various lymph nodes discovered through a small incision. By the use of these methods, various advantages can be achieved: 1) a significant reduction in the duration of the surgical procedure, 2) a significant increase in the accuracy of SLN identification, and 3) a significant decrease in morbidity due to the staging procedure.[3]

MB is used to diagnose breast tumors with near-infrared (NIR) fluorescence imaging injection after i.v. administration.[2] NIR imaging is a relatively new field for the investigation of preclinical and clinical applications in cancer imaging with its high spatial resolution and real-time display. The NIR light range (wavelength: 650-900 nm) provides tissue penetration and less autofluorescence from neighboring tissues.[4,5]

Photodynamic therapy (PDT) has been investigated as a therapeutic approach for a variety of cancers.[6-8] Its mechanism depends on photooxidation of biological matter. For this purpose, a photosensitizer is illuminated with a light having an appropriate wavelength to excite and induce photochemical reactions that generate reactive oxygen species inducing cell death.[9,10] PDT has been used as an experimental treatment approach for different cancer types in many countries[11,12] and it is generally approved as an effective therapy for some small and localized tumors.[13] Reduced long-term morbidity is the most important advantage of PDT.[9,14]

Encapsulation or modification of active pharmaceutical ingredients and/or contrast/radiocontrast agents in nanosystems is desirable due to the enhancement of bioavailability and organ/tissue accumulation by specific targeting, and decrease side effects and dose. Liposomes still attract significant attention for both diagnostic imaging[15] and therapy[16] due to their advantages of encapsulation of different kinds of drugs and modification with target specific ligands.[17-19] By the effect of reducing particle size to nanometer ranges, polymer modification of surfaces such as PEG steric stabilization can be achieved and rapid removal of liposomes can be prevented from circulation by opsonization with a reticulo-endothelial system (RES) such as plasma proteins and macrophages.[19-21] Therefore, enhanced circulation time, bioavailability, and especially for diseases related to damaged vessel integrity enhanced drug delivery accumulation and targeting in the desired disease area by enhanced permeability and retention (EPR) effect such as in tumors can be achieved at the end.[21]

Recently, NIR imaging has been used with PDT for a theranostic approach by using appropriate photosensitizer dyes. MB, a cheap and safe dye, has both NIR fluorescence (excitation: 668 nm, emission: 688 nm) and photosensitizer properties. To evaluate SLN detection, MB, isosulfan blue, radioisotopes, and nanocarriers have been used alone or in combination in some previous studies for lymphoscintigraphy and SLN biopsy as a promising approach.[3,22-24] However, the poor penetration of MB through cell membranes is a limiting factor. Its decreased cellular uptake can be prevented by encapsulating MB in delivery systems such as liposomes. Combining a radionuclide and photosensitizer dye (MB) in one injection can allow both lymphoscintigraphy and lymphatic mapping by nuclear imaging and NIR imaging and also PDT by application of a light giving suitable illumination, which can help in SLN surgery.

The aim of the present study was to formulate MB encapsulated, Tc-99m radiolabeled, PEGylated liposomes to design a multifunctional theranostic nanocarrier for NIR imaging and detection by gamma probe of SLNs and PDT that can be combined with surgery with a single vesicular system. The characterization of liposomes was performed by measurement of particle size, zeta potential, phospholipid content, radiolabeling efficiency, and encapsulation efficiency. The *in vitro* release profile of MB was determined. The *in vitro* stability of the liposomes was also evaluated over 6 months, which were stored at room temperature (25˚C) and 4˚C.

MATERIALS AND METHODS

Materials

Lipoid S phosphatidylcholine [(PC) from soybean (98%)] and dipalmitoylphosphatidylcholine (DPPC) were kind gifts from Lipoid GmbH (Germany) and Phospholipon GmbH (Germany), respectively. Cholesterol (Chol) was obtained from Sigma-Aldrich (USA)."1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000-DSPE) and 1,2-dioleoiyl-sn-

glicero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (USA). Diethylenetriaminepentaacetic acid anhydride (DTPA) and MB were obtained from Sigma-Aldrich (Germany). Dimethyl sulfoxide (DMSO) was obtained from Merck (Germany).

DTPA-PE synthesis

DTPA-PE is used for Tc-99m radiolabeling. For its synthesis, 30 µL of triethylamine was added to 0.1 mM of DOPE in 4 mL of chloroform. This solution was added to 1 mM of DTPA anhydride in 20 mL of DMSO slowly. It was then mixed for 3 h at 25˚C under argon gas. Afterwards, the mixed solution was dialyzed against water at 4˚C for 2 days and freeze-dried.[25-29]

Preparation of MB encapsulated, PEGylated, DTPA-PE containing liposomes

MB encapsulated, nanosized liposomes were prepared by film hydration.[30] Lipoid S PC:PEG2000-PE:Chol:DTPA-PE (60:0.9:39:0.1% molar ratio) were used. For this purpose, Lipoid S PC, PEG2000-PE, Chol, and DTPA-PE were dissolved in chloroform. The chloroform was then evaporated and the dry lipid film was hydrated with 0.5 mM MB solution in HEPES (1 M, pH 7.4) buffer at 35˚C. The liposomes were then extruded twice through polycarbonate membranes having 0.6, 0.4, and 0.2 µm pore sizes. Afterwards, the liposomes were dialyzed through a cellulose membrane (3500 Da cut-off size) for 12 h.[1]

For the preparation of DPPC as phospholipid containing liposomes DPPC:PEG2000-PE:Chol:DTPA-PE (60:0.9:39:0.1% molar ratio) were used for the mixture. The same procedure was also used for the preparation of MB encapsulated DPPC:PEG2000-PE:Chol:DTPA-PE liposomes; the only difference was that the hydration procedure was performed at 60˚C.

Characterization of MB encapsulated, PEGylated, DTPA-PE containing liposomes

The characterization of MB encapsulated, liposomal formulations was performed by measuring their mean particle size, zeta potential, phosphate content, and encapsulation efficiency %.

Mean particle size and zeta potential

It was determined by using a Zetasizer Nano ZS (Malvern Instruments UK).[31]

Encapsulation efficiency (%)

After the removal of unencapsulated MB by dialysis, the liposomes were lysed with ethanol. Encapsulated MB amount was determined spectrophotometrically at 660 nm. Encapsulation efficiency (%) was calculated with the help of the standard line and line equation obtained previously. The percentage of entrapped drug was determined by applying Equation 1:

$$\text{Entrapment efficiency (\%)} = (D_E \times 100)/(D_I), \quad (1)$$

where D_E is the amount of entrapped drug and D_I is the initial amount of drug.

Liposomal phospholipid amount

The phosphate content of MB encapsulated liposomes was obtained by a modified method.[32] This method depends on the detection of phosphorus content after perchloric acid destruction of liposomes at 797 nm spectrophotometrically (Shimadzu UV-1280, Germany).

Physical stability of liposomes

Any alterations in the mean particle size, zeta potential, and encapsulation efficiency of liposomes were evaluated. The liposomes were kept at two different temperatures (4˚C and 25˚C) for 6 months and changes in these parameters were evaluated at fixed time intervals (0, 3, 5, 7, 14, 21, 30, 60, 90, 120, 150, 180 days) over 6 months.[29]

In vitro release studies

The *in vitro* release profile of MB was evaluated by dialysis.[27] For this purpose, 1 mL samples of the liposomes were put in dialysis bags (MW cut-off: 2000) in 10 mL of HEPES (1 M, pH 7.4) buffer, and agitated in a shaking water bath (SWB 5050, Labnet International, Canada) (100 oscillations.min^{-1}), which was incubated at 37˚C. Then 1 mL of formulations was taken for the measurement of MB concentrations at specific time points. Release of MB in HEPES (1 M, pH 7.4) buffer was determined spectrophotometrically (Shimadzu UV-1280, Germany).

Radiolabeling of MB encapsulated, PEGylated, DTPA-PE containing liposomes

MB encapsulated, DTPA-PE containing liposomes were radiolabeled by tin reduction. For this, 0.5 mL of $SnCl_2.2H_2O$ (1 mg.mL^{-1}) and 1.5 mCi of Tc-99m were mixed with liposomes for 30 min by shaking to allow transchelation.[28,29] After incubation, the liposomes were dialyzed against HEPES (1 M, pH 7.4) buffer for 5 h at 4˚C to remove free Tc-99m by dialysis membrane (3500-Da cut-off size). After removal of unchelated Tc-99m, the radioactivity of liposomes was measured by a gamma counter.

Statistical analysis

All data are given as mean ± standard deviation. To evaluate the statistical significance of differences among the results, statistical analysis was performed. Depending on the numbers of data, which are less than 30, nonparametric test methods were applied for evaluation. Due to the group number, the Mann-Whitney U test and Kruskal-Wallis test were applied for comparison of two groups and three or more groups, respectively. The significance level was accepted as $p \leq 0.05$.

RESULTS

Tc-99m labeled, MB encapsulated liposomes were formulated for the purpose of SLN detection by gamma probe, NIR imaging, and also PDT of SLNs to perform better detection, mapping, and also a treatment option helping during surgery.

MB encapsulated liposomes were characterized and the results are given in Table 1.

The mean particle sizes of all liposomes were about 100 nm. DPPC containing liposomal dispersions had smaller particle size than Lipoid S PC liposomes ($p>0.05$). The MB

Table 1. Characterization of MB encapsulated liposomal dispersions (n=6)

Liposome formulation	Mean particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Phospholipid efficiency (%)
Lipoid S PC:PEG2000-PE:Chol:DTPA-PE	116±0.14	0.17	-13.75±0.21	9.51±0.01	86.8±2.1
DPPC:PEG2000-PE:Chol:DTPA-PE	121±0.19	0.12	-9.81±0.16	11.84±0.02	91.7±3.2

MB: Methylene blue, PC: Phosphatidylcholine, Chol: Cholesterol, DTPA: Diethylenetriaminepentaacetic acid anhydride, DPPC: Dipalmitoylphosphatidylcholine

encapsulation amount of liposomes was higher than that of Lipoid S PC liposomes (p>0.05). The liposomes' phosphate content was high, about 85-90%. DPPC containing liposomes had higher phosphate content than Lipoid S PC containing ones did (p>0.05).

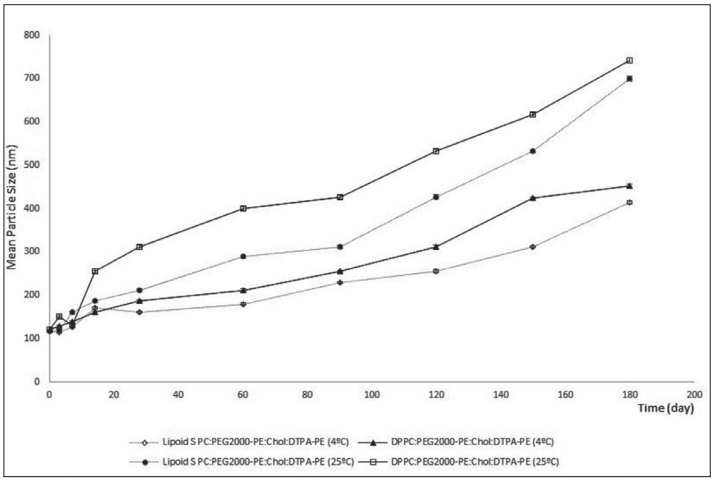

Figure 1. Alterations in mean particle size of MB encapsulated, nanosized liposomes over 6 months of storage at both 4˚C and 25˚C
MB: Methylene blue, Chol: Cholesterol, DTPA: Diethylenetriaminepentaacetic acid anhydride

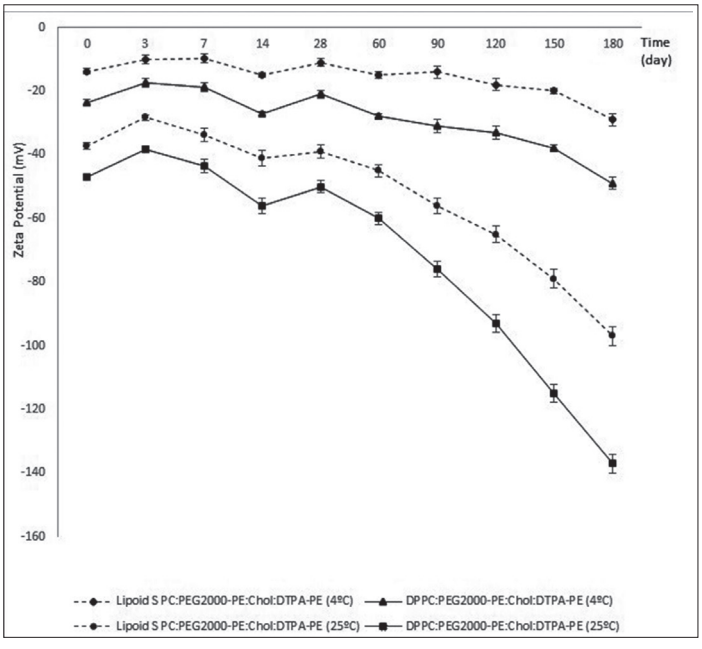

Figure 2. Alterations in zeta potential of nanosized, MB encapsulated liposomes over 6 months of storage at both 4˚C and 25˚C
MB: Methylene blue, Chol: Cholesterol, DTPA: Diethylenetriaminepentaacetic acid anhydride

Alterations in mean particle size, zeta potential, and MB encapsulation efficiency of liposomes stored at both 4˚C and 25˚C for 6 months are given in Figures 1-3, respectively. Only slight increases were observed in these parameters of both formulations stored at 4˚C at the end of 3 months (p>0.05). Higher alterations were observed in these parameters of both liposomes stored at 25˚C when compared with those stored at 4˚C at the end of 3 months (p<0.05). Significant increases were observed in these parameters for all formulations stored at both 4˚C and 25˚C for 6 months (p<0.05). On the other hand, MB encapsulated liposomes were stable due to mean particle size, zeta potential and MB encapsulation efficiency when stored at 25˚C for 1 month. When the effect of phospholipid was

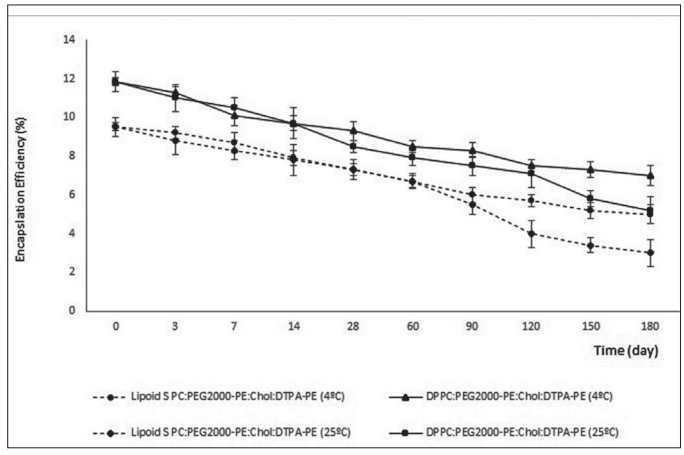

Figure 3. Alterations in MB encapsulation efficiency of nanosized liposomes over 6 months of storage at both 4˚C and 25˚C
MB: Methylene blue, Chol: Cholesterol, DTPA: Diethylenetriaminepentaacetic acid anhydride

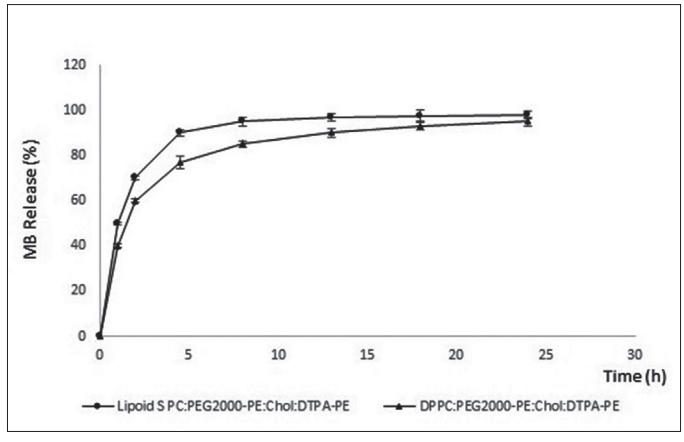

Figure 4. *In vitro* release of nanosized, MB encapsulated liposomes at 37˚C
MB: Methylene blue, Chol: Cholesterol, DTPA: Diethylenetriaminepentaacetic acid anhydride

compared, no significant difference was seen between Lipoid S PC containing liposomes and DPPC containing ones stored in the same conditions (p>0.05). These results are compatible with those of previous studies.[28,29,33]

The *in vitro* release of both MB encapsulated, nanosized liposomes were evaluated by dialysis.[27] Lipoid S PC containing liposomes showed slightly faster release of MB when compared with DPPC containing ones (p>0.05). As shown in Figure 4, the two formulations exhibited similar release profiles. A number of studies were found in the literature supporting our data.[34,35]

DISCUSSION

Both liposomes were formulated to combine a radionuclide and photosensitizer dye (MB) in one injection to achieve both lymphoscintigraphy by nuclear and NIR imaging and also PDT, which can help in surgery.

All formulations exhibited nanosize (around 100 nm) significant for long-circulation and passive targeting by PEGylation to obtain "stealth" formulations, which is essential for EPR effect in tumor accumulation.[36] Lipoid S PC containing liposomes have smaller particle size than DPPC containing ones (p>0.05). This may be due to the liquid crystalline structure of Lipoid S PC, which may easily be reduced by extrusion because of greater elasticity than gel state phospholipids. Furthermore, PDI values, which are a significant indicator for the homogeneity of particle size distribution in a dispersion, of MB encapsulated, Lipoid S and DPPC containing liposomes were very small, around approximately 0.1. Surface modification was performed by PEG2000-DSPE coating for all formulations for passive targeting of SLNs and surrounding tumor tissue by EPR effect, which prevents opsonization by RES cells and increasing blood circulation time.

DTPA-PE was synthesized for Tc-99m radiolabeling of formulations due to its metal chelator properties. DTPA is one of the most commonly used chelator agents nowadays for efficient radiolabeling of liposomes for imaging.[26,28,29] The chelating agent DTPA conjugates to the amino head group with a -COOH group and in this way while PE was incorporated with the lipid bilayer of liposomes, Tc-99m was incorporated with DTPA with high efficiency.[25,26]

Zeta potential exhibits the magnitude of the electrostatic repulsion/attraction between particles. Therefore, it affects the stability and shelf-life of formulations and provides detailed information about dispersion, aggregation, and flocculation. The zeta potentials of liposomes are suitable (-9 to -13 mV) to prevent aggregation.[35] The zeta potential of Lipoid S PC:PEG2000-PE:Chol:DTPA-PE liposomes was slightly higher when compared with DPPC liposomes (p>0.05) due to the different nature of the phospholipids. The MB encapsulation efficiency of DPPC liposomes was slightly higher than that of Lipoid S PC containing ones (p>0.05), due to the gel state of DPPC phospholipid providing less leakage of encapsulated drug through the vesicles. Although the ability to encapsulate hydrophilic drugs within the liposome vesicles was very limited (5-10%),[36] both formulations exhibited about 10% encapsulation efficiency. The phosphate content of all liposomes was very high (around 85-90%), which is essential for obtaining intact liposome vesicles. The phosphate content loss was smaller in DPPC liposomes than in Lipoid S PC liposomes, but this difference was statistically insignificant (p>0.05). This may have been due to the gel state phospholipid content of DPPC liposomes, which is a saturated synthetic lipid and generally forms stable and intact vesicles.

Alterations in the characterization parameters of Lipoid S PC and DPPC liposomes were evaluated over 6 months during storage at both temperatures. Changes in these parameters are essential for evaluating the stability of liposomes. Alterations in zeta potential are also crucial for physical stability, in which any alteration may cause aggregation. Leakage of encapsulated drug is essential for physical stability of liposomes during storage because vesicles should stay stable during storage. Only a slight increase was observed for each measurement in mean particle size, zeta potential, and encapsulation efficiency of both MB encapsulated liposomes stored at 4˚C for 3 months (p>0.05). Higher alterations were observed in these parameters of both liposomes stored at 25˚C when compared with those stored at 4˚C for 3 months (p<0.05). Lipoid S PC and DPPC liposomes were stable when stored at 25˚C for 1 month. When the effect of phospholipid amount was compared, no significant difference was evaluated between Lipoid S PC and DPPC containing liposomes stored in the same conditions (p>0.05). This may be because of the liquid crystalline state structure of Lipoid S PC and its low phase transition temperature.[29,36] As a result, leakage of MB from Lipoid S PC liposomes was higher than that from DPPC liposomes, which is compatible with previous studies.[28-33]

In vitro release profiles of liposomes are meaningful for predicting an efficient therapeutic effect. The release of MB is faster from liposomes composed of liquid-crystalline type phospholipids, depending on the distance within the polar head groups of liquid crystalline-type phospholipids, and is also faster than in gel state phospholipids.[34,35] Liposomes comprising DPPC exhibited slightly better *in vitro* MB release amounts than Lipoid S PC containing liposomes. However, this difference was not significant. These results were comparable with previously published results.[33,36]

Study limitations

Further studies are needed in order to reach to a clearer decision on this topic.

CONCLUSION

MB encapsulated, nanosized, PEGylated, Tc-99m radiolabeled liposomes were designed as potential formulations to accumulate in SLNs due to EPR effect by PEGylation for prolonged blood circulation time and nanosize for imaging and therapy. MB encapsulated, nanosized, PEGylated, Tc-99m radiolabeled Lipoid S PC:PEG2000-PE:Chol:DTPA-PE

and DPPC:PEG2000-PE:Chol:DTPA-PE (60:0.9:39:0.1% molar ratio) liposomes were evaluated as potential carriers for SLN imaging, mapping by gamma probe, NIR imaging, and therapy by PDT, which may help during surgery in terms of vesicular characteristics, release profile, and physical stability. Further studies should be surely performed to enable certain decisions to be made. This study may lead to the design of more specific radiocontrast agents for both imaging and therapy of many cancers.

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