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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 Hapsedilen, Tc-99m İşaretli Çok-Fonksiyonlu Lipozomların Formülasyonu

MB, Tc-99m Labeled Liposomes for SLN Imaging and Therapy
SLN Görüntüleme ve Tedavisi için MV, Tc-99m İşaretli Lipozomlar

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INTRODUCTION: Methylene blue (MB) is a commonly used dye which 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 the poor penetration through the cell membranes. Its decreased cellular uptake can be prevented by encapsulating in drug delivery systems such as liposomes. Additionally, EPR effect of tumor gives chance to enhance accumulation of nanocarriers in target site.
METHODS: In this study, 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 SLN 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, in vitro release profile of MB and physical stability were also evaluated for six months at determined time intervals by measuring mean particle size, zeta potential, encapsulation efficiency and phospholipid content of liposomes kept at room temperature (25℃) and 4℃.
RESULTS: Tc-99m radiolabeled, nanosized Lipoid S PC: PEG2000-PE: Chol: DTPA-PE and DPPC: PEG2000-PE: Chol: DTPA-PE liposomes designated proper particle size (around 100 nm), zeta potential (-9 - -13 mV), encapsulation efficiency (around 10%), phospholipid efficiency (around 85-90%) and release profile. Additionally, liposomes were observed stable for 3 months especialy when kept at 4°C.
DISCUSSION AND 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 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 give a final desicion and our studies are continuing.
Keywords: Sentinel Lymph Node, Methylene blue, Theranostic Nanomedicine, Diagnosis.

GİRİŞ ve AMAÇ: Metilen mavisi (MV) yakın infrared (NIR) görüntüleme ve ışığa maruziyet sonrası reaktif oksijen radikalleri üreterek apoptozu indükleyen foto-dinamik tedavi (PDT) için sıklıkla kullanılan bir boyadır. MV'sinin hücre membranlarından zayıf penetrasyonu etkinliğini kısıtlayan bir faktördür. MV'sinin azalmış hücresel tutulumu lipozomlar gibi ilaç taşıyıcı sistemlerde enkapsülasyonu ile önlenebilir. Ek olarak, tümörün artmış geçirgenlik ve tutulum (EPR) etkisi nanotaşıyıcıların hedef bölgede tutulumunu artırır.
YÖNTEM ve GEREÇLER: Bu çalışmada, nanoboyutlu, MV enkapsüle edilen, Tc-99m radyoişaretli Lipoid S PC: PEG2000-PE: Chol: DTPA-PE ve DPPC: PEG2000-PE: Chol: DTPA-PE lipozomları multifonksiyonel teranostik nanotaşıyıcıları daha kesin görüntüleme ve cerrahiye yardımcı olabilecek tedavi sağlayabilmek amacıyla; 1. NIR görüntüleme, 2. Sentinal lenf nodlarının (SLN) gama prob ile tayini ve 3. PDT'yi aynı tek lipozomal sistemde dizayn edilecek formülasyonlar hazırlanmıştır. Lipozomların karakterizasyonu partikül boyutu, zeta potansiyel, fosfolipid içeriği ve enkapsülasyon etkinliği ölçülerek yapılmıştır. Ek olarak, MV'sinin in vitro salım profili ve fiziksel stabilitesi altı aylık sürede belirli zamanlarda ortalama partikül boyutu, zeta potansiye, enkapsülasyon etkinliği ve fosfolipid içeriği oda sıcaklığı (25℃) ve 4℃'de ölçülerek değerlendirilmiştir.
BULGULAR: Tc-99m işaretli, nanoboyutlu Lipoid S PC: PEG2000-PE: Chol: DTPA-PE ve DPPC: PEG2000-PE: Chol: DTPA-PE lipozomları uygun partikül boyutu (100 nm civarında), zata potansiyel (-9 - -13 mV), enkapsülasyon etkinliği (%10 civarında), fosfolipid etkinliği (%85-90 civarında) ve salım profili göstermiştir. Ayrıca, 4°C'de saklanan MV hapsedilmiş lipozomlar 3 ay boyunca stabil bulunmuştur.
TARTIŞMA ve SONUÇ: MV enkapsüle edilen, Tc-99m işaretli, nanoboyutlu Lipoid S PC: PEG2000-PE: Chol: DTPA-PE ve DPPC: PEG2000-PE: Chol: DTPA-PE lipozomlar SLN'nın gama probla tayin edilme, NIR görüntüleme ve PDT için potansiyel olarak bulunmuştur. Daha doğru ve kesin sonuçlara ulaşmak için in vitro ve in vivo görüntüleme ve tedavi etkinliği değerlendirilmesi gereklidir ve çalışmalarımız devam etmektedir.
Anahtar Kelimeler: Sentinal lenf nodu, Metilen mavisi, Teranostik nanotıp, Teşhis.

Introduction

The identification and mapping of sentinel lymph nodes (SLN) for biopsy or imaging are commonly used for staging of many cancers especially breast cancer. Therefore, SLN has an importance as being the first node draining the primary tumor to which a malignancy is likely to metastasize. Various dyes have been used for SLN identification such as isosulphane blue and methylene blue (MB). MB is a cheap and easily accessible dye which is approved by FDA. Additionally, its side effects are less serious than isosulphane blue.[1,2] The limiting factor of MB is its poor penetration through the cell membranes and some allergic reactions.

Radiopharmaceuticals have been used as an alternative or in addition to dyes for external imaging and/or radiation detector monitoring for the SLN detection and mapping before surgery. Intraoperative use of gamma probe detectors permits confirming 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. significant time reduction of surgical procedure, 2. a significant increase in the accuracy of SLN identification, and 3. a significant decrease in the morbidity due to the staging procedure.[3] Although some researchers reported that sentinel lymph node (SLN) biopsy can be performed by using only radioactive colloid injection due to preventing some allergic reactions caused by the blue dye such as MB[4], it is adviced to use dye together with the radioisotope for the accurate detection of SLN at early stage breast cancer patients especially. This combined technique provides detection of SLN about 99%.

Recently, MB was also confirmed to detect the 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 both preclinical and clinical applications in cancer imaging due to having a high spatial resolution, portability and real-time display. NIR light range (wavelength: 650–900 nm) provides tissue penetration and less autofluorescence from surrounding tissues.[5]

Currently, Photodynamic therapy (PDT) has been investigated as an alternative treatment option for a variety of cancers.[6-8] Its mechanism is based on the photooxidation of biological matters. The uptake of a photosensitizer followed by illumination with light having an appropriate wavelength that is able to excite photosensitizer and induce photochemical reactions that generate reactive oxygen species (such as singlet oxygen ($^1O_2$), and radicals) 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 approach in 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 kept significant attention for both diagnostic imaging[15] and therapy[16] due to the suitable properties such as ability to encapsulate drugs having different physicochemical properties and modify with target specific ligands.[17-19] By the effect of reducing particle size to nanometer ranges, surface modification with proper polymers such as PEG steric stabilization can be achieved and rapid removal of liposomes from blood-circulation by opsonization with reticulo-endothelial system (RES) such as plasma proteins and macrophages can be prevented.[20, 21] Therefore, enhanced blood-circulation time, bioavailability and especially in the case of diseases related with damaged vessel integrity to enhance drug delivery accumulation and targeting in the desired disease area by enhanced permeability and retention (EPR) effect such as tumor can be achieved finally. The use of liposomes for the delivery of MB has potential advantages due to their properties. Encapsulation of MB in liposomal formulations can reduce some undesired side effects of MB such as allergic reactions, the volume of distribution by increasing accumulation at the desired site and the dose significantly. The poor penetration of MB through the cell membranes and its slight side effects such as allergic reactions can be decreased by encapsulating in liposomal systems.

Recently, near-infrared (NIR) imaging can be used with PDT for theranostic approach by using proper photosensitizer dyes. MB, as a cheap and safe dye, has both NIR fluorescence (excitation: 668 nm, emission: 688 nm) and photosensitizer properties. To evaluate sentinel lymph node detection, MB, isosulphane blue, radioisotopes and nanocarriers have been used as single 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 the cell membranes is the limiting factor. Its decreased cellular uptake can be prevented by encapsulating MB in drug delivery systems such as liposomes. Combining radionuclide and photosensitizer dye (MB) in one injection can provide both lymphoscintigraphy or lymphatic mapping by nuclear imaging and NIR imaging and also PDT by application of a light having a proper light which can help SLN surgery.

In this study, it was aimed to formulate MB encapsulated, Tc-99m radiolabeled, PEGylated liposomes to design a multifunctional theranostic nanocarrier for NIR imaging, detection by gamma probe of SLN and PDT which can be combined with surgery with a single vesicular system. The characterization of liposomes was performed by measuring particle size, zeta potential, phospholipid content, radiolabeling efficiency and encapsulation efficiency. In vitro release profile of MB was determined. In vitro stability of liposomes was also evaluated for six months which were stored at room temperature (25℃) and 4℃.

Methods

Materials

Lipoid S PC (Phosphatidylcholine from Soybean (98%)) and Dipalmitoylphosphatidylcholine (DPPC) were kind gifts of Lipoid GmbH (Ludwigshafen, Germany) and Phospholipon GmbH (Cologne, Germany), respectively. Cholesterol (Chol) was obtained from (Sigma-Aldrich, St. Louis, Missouri, 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, Inc., Alabaster, Alabama, USA. Diethylenetriaminepentaacetic acid anhydride (DTPA) and Methylene blue were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from Merck, Darmstadt, Germany.

Synthesis of DTPA-PE

DTPA-PE, is used for Tc-99m radiolabeling. For the synthesis, 0.1 mM of DOPE in 4 mL of chloroform was supplemented with 30 µL of triethylamine. This solution was added dropwise to another solution of 1 mM of DTPA anhydride in 20 mL of DMSO. It was then incubated for 3 hours at room temperature (25°C) under argon gas. Afterwards, the mixed solution was dialyzed against 6 L of water at 4°C for 48 hours. Purified DTPA-PE was freeze-dried and stored frozen at −80°C.[25, 26]

Preparation of MB Encapsulated, PEGylated, DTPA-PE Containing Liposomes

MB encapsulated, nanosized liposomes were formulated by film hydration method (Bangham et al. 1965). 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, cholesterol and DTPA-PE were dissolved in chloroform at 35°C. Chloroform was then evaporated and the dry lipid film was hydrated with 0.5 mM MB solution (5 mL) in HEPES (1 M, pH 7.4) buffer at 35°C. Multilamellar vesicles were extruded 2 times through polycarbonate membranes having 0.6 µm, 0.4 µm and 0.2 µm pore sizes, respectively. Afterwards, liposomes were dialyzed through a regenerated cellulose membrane (3,500 Da cut-off size) for 12 hours to remove unencapsulated MB.

For the prepartion of DPPC as phospholipid containing liposomes DPPC:PEG2000-PE:Chol:DTPA-PE (60:0,9:39:0,1% molar ratio) were used for mixture. The same procedure was also used for the preparation of MB encapsulated DPPC:PEG2000-PE:Chol:DTPA-PE liposomes, the only difference is that the hydration procedure was performed at 60°C.

Characterization of MB Encapsulated, PEGylated, DTPA-PE Containing Liposomes

The characterization of MB encapsulated, DTPA-PE containing, PEGylated nanosized Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposome formulations was determined by measuring mean particle size, zata potential, phosphate content and encapsulation efficiency (EE%).

- **Mean Particle Size and Zeta Potential**

Mean particle size and polydispersity index (PDI) and zeta potential of liposome formulations were determined using the Nano-ZS (Malvern Instruments, Malvern, UK) by dynamic light scattering method.

- **Encapsulation Efficiency (%)**

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

Entrappment Efficiency (%)= $(D_E \times 100)/(D_I)$……Eq. (1)

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

- **Liposomal Phospholipid Amount**

Phosphate content of MB encapsulated, PEGylated, DTPA-PE containing liposome formulations was obtained by uing modified Rouser method (Rouser et al. 1970). This method depends on the determination of phosphorus content after perchloric acid destruction of liposomes at 797 nm using an ultraviolet spectrophotometer (Schimadzu UV-1280, Germany).

Physical Stability of Liposomes

Any alterations in mean particle size, zeta potential and MB leakage of MB containing Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were measured to evaluate *in vitro* physical stability of liposomes. For this purpose, liposomes were kept at two different temperatures (4°C and 25°C) for 6 months and the alterations of these parameters were determined at fixed time intervals (0, 3, 5, 7, 14, 21, 30, 60, 90, 120, 150, 180 days) for 6 months.

In Vitro Release Studies

In vitro release profile of MB was assessed by the dialysis method.[27] For this purpose, 1 mL of the liposomes were put in dialysis bags (MW cut-off: 2,000) in 10 mL of HEPES (1 M, pH 7.4) buffer, and agitated in a shaking water bath (SWB 5050, Labnet International, Canada) at a rate of 100 oscillations per minute which was incubated at 37°C. 1 mL of the dialysate was removed for the measurement of MB concentrations and 1 mL of HEPES (1 M, pH 7.4) buffer was added at specific time points. Release of MB in HEPES (1 M, pH 7.4) buffer was determined spectrophotometrically (Shimadzu, UV-1280, Duisburg).

Radiolabeling of MB Encapsulated, PEGylated, DTPA-PE Containing Liposomes

MB encapsulated, DTPA-PE containing, Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were labeled with Tc-99m by tin-reduction method. Briefly, 0.5 mL of

$SnCl_2.2H_2O$ (1 mg.mL^{-1}) and 1.5 mCi of Tc-99m were incubated with liposomes for 30 minutes by shaking to allow transchelation.[28, 29] After incubation, liposomes were dialyzed against HEPES (1 M, pH 7.4) buffer for 5 hours at 4°C to remove unchelated Tc-99m by cellulose ester dialysis membrane (3,500-Da cut-off size). After removal of free Tc-99m, the radioactivity of liposomes was measured by a gamma counter.

Statistical Analysis

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

Results and Discussion

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

MB encapsulated, Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were characterized. Mean particle size, PDI, EE%, and phospholipid amount of MB encapsulated, PEGylated, DTPA-PE containing liposomes are given in Table 1.

Mean particle sizes of all liposomes were observed to be approximately 100 nm. DPPC containing liposome dispersions have smaller particle size than Lipoid S PC containing ones ($p > 0.05$). MB encapsulation amount of DPPC containing liposomes was found to be higher than Lipoid S PC liposomes ($P > 0.05$). Phosphate content of liposomes found high about 85-90%. DPPC containing liposomes had higher phosphate content than Lipoid S PC containing ones ($P > 0.05$).

Alterations in mean particle size, zeta potential and MB encapsulation efficiency of MB encapsulated Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes stored at both 4°C and 25°C for 6 months are given in Figure 1-3, respectively. Only slight increase was observed in mean particle size, zeta potential and encapsulation efficiency of both formulations stored at 4°C at the end of 3 months ($p>0.05$). Higher alteration was observed in these parameters of both liposomes stored at 25°C when compared with that of stored at 4°C at the end of 3 months ($p<0.05$). A significant increase in these parameters was observed for all formulations stored at both 4°C and 25°C for 6 months ($p<0.05$). On the other hand, Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were observed 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 compared, no significant difference was obtained between Lipoid S PC containing liposomes and DPPC containing ones stored at the same temperature and time durations ($p>0.05$). These results are in agreement with the previous studies.[28-30]

In vitro release of MB encapsulated, nanosized Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were evaluated by dialysis method.[27] Lipoid S PC containing liposomes designated slightly faster release of MB when compared with DPPC containing ones ($p > 0.05$). As shown in Figure 4, both formulations were exhibited similar release profiles. A number of studies were found in the literature supporting our data.[31, 32]

Discussion

Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes were formulated to combine radionuclide and photosensitizer dye (MB) in one injection to achieve both lymphoscintigraphy by nuclear and NIR imaging and also PDT which can help surgery.

All formulations were 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.[33] Lipoid S PC containing liposomes have smaller particle size than DPPC containing ones ($p > 0.05$). This may be due to liquid cristaline structure of Lipoid S PC which may easily be reduced by extrusion because of more elasticity than gel state phospholipids. Also, PDI values, which is a significant indicator for the homogenity of particle size distribution in a dispersion, of MB encapsulated, Lipoid S and DPPC containing liposomes were observed very small around approximately 0,1. Surface modification was performed by PEG2000-DSPE coating for all formulations for passive targeting of SLN and surrounding tumor tissue by EPR effect which prevents opsonization by RES cells and increasing blood circulation time.

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

Zeta potential exhibits the magnitude of the electrostatic repulsion/attraction between particles. Therefore, it affects stability and shelf-life of formulations and provides detailed information about dispersion, aggregation, or flocculation. The zeta potentials of liposomes are proper (-9 - -13 mV) to prevent aggregation.[32] The zeta

potential of Lipoid S PC:PEG2000-PE:Chol:DTPA-PE liposomes was slightly higher when compared with DPPC:PEG2000-PE:Chol:DTPA-PE liposomes ($p > 0.05$) due to different nature of the phospholipids. MB encapsulation efficiency of DPPC containing liposomes was slightly higher than Lipoid S PC containing ones ($p > 005$) which is due to gel state of DPPC phospholipid providing less leakage of encapsulated drug through the vesicles. Although, the ability to encapsulate hydrophilic drugs within the liposomal vesicles is very limited (5–10%),[33] both formulations exhibited about 10% encapsulation efficiency. The phosphate content of all liposomes was found very high (around 85-90%) which is very essential for obtaining intact liposome vesicles. The phosphate content loss was observed lesser in DPPC liposomes than Lipoid S PC liposomes, but this difference was not statistically significant ($p > 005$). This may be 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 mean particle size, zeta potential and MB encapsulation efficiency of Lipoid S PC and DPPC liposomes were evaluated for 6 months of storage at both 4°C and 25°C. Change in these parameters is essential to evaluate physical stability of liposomes, because instability may result in phase seperation. Alteration in zeta potential is also a crutial 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 protect encapsulated drug during storage. Only a small amount of increase was observed for each measurement in mean particle size, zeta potential and encapsulation efficiency of both MB encapsulated liposomes stored at 4°C at the end of 3 months ($p>0.05$). Higher alteration was observed in these parameters of both liposomes stored at 25°C when compared with that of stored at 4°C at the end of 3 months ($p<0.05$). Lipoid S PC and DPPC liposomes were observed 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 at the same temperature and time durations ($p>0.05$). This may be because of the liquid crystalline state structure of Lipoid S PC and its low phase transition temperature.[33] As a result, leakage of MB from Lipoid S PC liposomes was found to be higher than DPPC liposomes which is compatible with previous studies.[28-30]

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.[31, 32] Liposome formulations containing DPPC as the phospholipid showed 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.[30, 33]

Study Limitations

Further in vitro and vivo imaging and therapy studies are surely needed to perform to obtain more certain decisions which are still continuing.

Conclusion

Methylene blue encapsulated, nanosized, PEGylated, Tc-99m radiolabeled liposomes were designed as potential formulations to accumulate in SLN due to EPR effect by PEGylation for prolonged blood circulation time and nanosize to obtain imaging and therapy. Methylene blue 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 promising multifunctional carriers for SLN imaging, mapping by gamma probe, NIR imaging and therapy by PDT which may help surgery from vesicular particle characteristics (i.e. particle size, EE%, phospholipid amount) release profile and physical stability perspectives. Encapsulation of MB in liposomal formulations may reduce some undesired side effects of MB such as allergic reactions, the volume of distribution by increasing accumulation at the desired site and the dose. The poor penetration of MB through the cell membranes and its slight side effects such as allergic reactions may be decreased by encapsulating in liposomal systems. Further in vitro and vivo imaging and therapy studies are needed to give more certain decisions which are still continuing. This study may lead to design more specific radiocontrast agents for both imaging and therapy of many cancers.

References

1. Özdemir A, Mayir B, Demirbakan K, Oygür N. Efficacy of methylene blue in sentinel lymph node biopsy for early breast cancer, J Breast Health. 2014;10:88-91.
2. Zhang C, Mao Y, Kun Wang, Jie Tian. The identification of breast cancer by Near-Infrared fluorescence imaging with methylene blue. J Clin Oncol. 2018;36:15-17.
3. Hinkle GH. Lymphoscıntıgraphy and Sentinel Node Biopsy in The Staging of Cancer. Correspondence Continuing Education Courses for Nuclear Pharmacists and Nuclear Medicine Professionals. (11th ed). The Unıversity of New Mexico Health Sciences Center College of Pharmacy Albuquerque, New Mexico;2005,1-43.
4. Sevinç Aİ, Aksoy SÖ, Durak MG. Adverse Reactions to Blue Dye Injections During Sentinel Lymph Node Biopsy. 9 Eylül Tıp Dergisi. 2017;31,81-85.
5. Silindir-Gunay M, Sarcan ET, Ozer AY. Near-infrared imaging of diseases: A nanocarrier approach. Drug Dev Res. 2019;1:1–14.

6. Rizvi I, Celli JP, Evans CL, Abu-Yousif AO, Muzikansky A, Pogue BW, Finkelstein D, Hasan T. Synergistic enhancement of carboplatin efficacy with photodynamic therapy in a three-dimensional model for micrometastatic ovarian cancer. Cancer Res. 2010;70:9319–28.
7. Ahn TG, Lee BR, Choi EY, Kim DW, Han SJ. Photodynamic therapy for breast cancer in a BALB/c mouse model. J Gynecol Oncol. 2012;23:115-119.
8. Montazerabadi AR, Sazgarnia A, Bahreyni-Toosi MH, Ahmadi A, ShakeriZadeh A, Aledavood A. Mitoxantrone as a prospective photosensitizer for photodynamic therapy of breast cancer. Photodiagnosis Photodyn Ther. 2012;9:46–51.
9. Acedo P, Stockert JC, Cañete M, Villanueva A. Two combined photosensitizers: a goal for more effective photodynamic therapy of cancer. Cell Death Dis. 2014;5:1122-1134.
10. Sarcan ET, Silindir-Gunay M, Ozer AY. Theranostic polymeric nanoparticles for NIR imaging and photodynamic therapy. Int. J. Pharm. 2018;551:329–338.
11. Agostinis P, Berg K, Cengel K a, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowiz D, Piette J, Willson BC, Golab J. Photodynamic therapy of cancer : An update. Am Cancer Soc. 2011;61:250–281.
12. Simone CB, Friedberg JS, Glatstein E, Stevenson JP, Sterman DH, Stephen M, Cengel KA. Photodynamic therapy for the treatment of non-small cell lung cancer. J Thorac Dis. 2012;4:63-75.
13. Marchal S, Dolivet G, Lassalie HP, Guillemin F, Bezdetnaya L. Targeted photodynamic therapy in head and neck squamous cell carcinoma: heading into the future. Lasers Med Sci. 2015;30:2381–2387.
14. dos Santos AF, Terra LF, Wailemann RAM, Oliveira TC, de Morais Gomes V, Mineiro MF, Meotti FC, Bruni-Cardoso A, Baptista MS, Labriola L. Methylene blue photodynamic therapy induces selective and massive cell death in human breast cancer cells. BMC Cancer. 2017;17:1-15.
15. Guenoun J, Koning GA, Doeswijk G, Bosman L, Wielopolski PA, Krestin GP, Bernsen MR. Cationic Gd-DTPA liposomes for highly efficient labeling of mesenchymal stem cells and cell tracking with MRI. Cell Transplant. 2012;21:191–205.
16. Nisini R, Poerio N, Mariotti S, De Santis F, Fraziano M. The multirole of liposomes in therapy and prevention of ınfectious diseases Front Immunol. 2018;9:1-23.
17. Seleci M, Ag Seleci D, Scheper T, Stahl F. Theranostic liposome–nanoparticle hybrids for drug delivery and bioimaging. Int J Mol Sci. 2017;18:1415-1426.
18. Silindir M, Erdoğan S, Özer AY, Maia S. Liposomes and their applications in molecular imaging. J. Drug Target. 2012;20:401–415.
19. Silindir-Gunay M, Ozer AY. Liposomes and Micelles as Nanocarriers for Diagnostic and Imaging Purposes. In: Grumezescu AM, eds. Design of Nanostructures for Theranostics Applications 1st ed. USA, Elsevier;2018,305-340.
20. Kwon GS, Suwa S, Yokoyama M, Okano T, Sakurai Y, Kataoka K. Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxideaspartate block copolymers-andriamycin conjugates. J Control Release. 1994;29:17-23.
21. Maruyama, K. Peg-immunoliposomes. Biosci Rep. 2002;22:251-266.
22. Osborne MP, Richardson VJ, Jeyasingh K, Ryman BE. Potential applications of radionuclide-labelled liposomes in the detection of the lymphatic spread of cancer. Int J Nucl Med Biol. 1982;9:47-51.
23. Phillips WT, Klipper R, Goins B. Use of 99mTc-labeled liposomes encapsulating blue dye for indentification of the sentinel lymph node. J Nucl Med. 2001;42:446-451.
24. Bao A, Goins B, Klipper R, Negrete G, Mahindaratne M, Phillips WT. A novel liposome radiolabeling method using ^{99m}Tc-"SNS/S" complexes: In vitro and in vivo evaluation. J Pharm Sci. 2003;92:1-13.
25. Grant CW, Stephen K, Florio EA. Liposomal MRI contrast agent: phosphatidylethanolamine-DTPA. Magn Reson Med. 1989;11:236–243.
26. Elbayoumi TA, Torchilin V. Enhanced accumulation of longcirculating liposomes modified with the nucleosome-specific monoclonal antibody 2C5 in various tumours in mice: Gamma imaging studies. Eur J Nucl Med Mol Imaging. 2006;33:1196–1205.
27. Zheng J, Perkins G, Kirilova A, Allen C, Jaffray DA. Multimodal contrast agent for combined computed tomography and magnetic resonance imaging applications. Invest Radiol. 2006;41:339–348.
28. Silindir M, Erdoğan S, Özer A Y, Doğan L, Tuncel M, Ugur O, Torchilin V P. Nanosized multifunctional liposomes for tumor diagnosis and molecular imaging by SPECT/CT. J Liposome Res. 2013;23:20-27.
29. Silindir-Gunay M, Karpuz M, Ozturk N, Ozer AY, Erdogan S, Tuncel M. Radiolabeled, folate-conjugated liposomes as tumor imaging agents: Formulation and in vitro evaluation. J Drug Deliv Sci Technol. 2019;50:321–328.
30. Mutlu NB, Değim Z, Yılmaz Ş, Eşsiz D, Nacar A. New perspective for the treatment of Alzheimer diseases: Liposomal rivastigmine formulations. Drug Dev Ind Pharm. 2011;37,775–789.

31. Wu PT, Lin CL, Lin CW, Chang NC, Tsai WB, Yu J. Methylene-blue-encapsulated liposomes as photodynamic therapy nano agents for breast cancer cells. Nanomaterials. 2019;9,1-12.
32. NonoComposix. Zeta potential analysis of nanoparticles. 2012;1:1–6.
33. Shashi K, Satinder K, Bharat P. A complete review on: liposomes. Int. Res. J. Pharm. 2012;3:10-17.

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

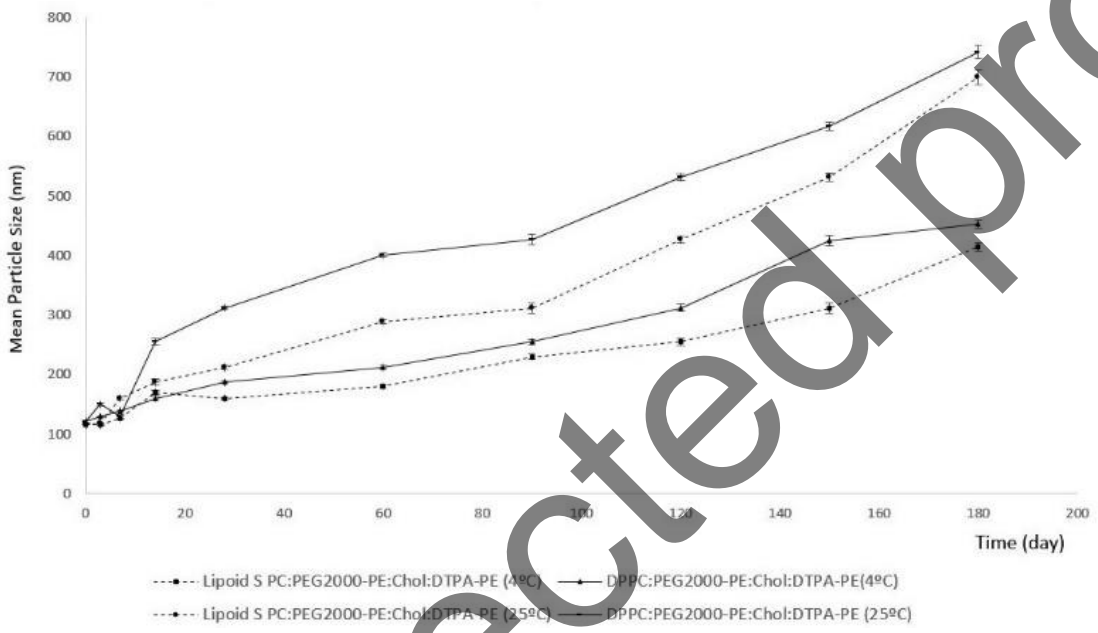

Figure 1. Alterations in mean particle size of MB encapsulated, Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes for 6 months of storage at both 4°C and 25°C.

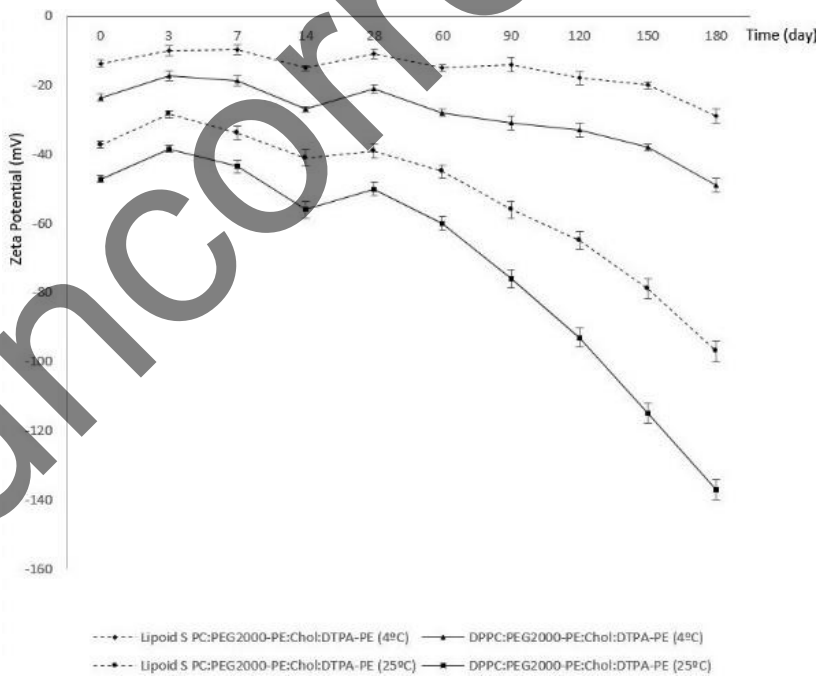

Figure 2. Alterations in zeta potential of MB encapsulated Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes for 6 months of storage at both 4°C and 25°C.

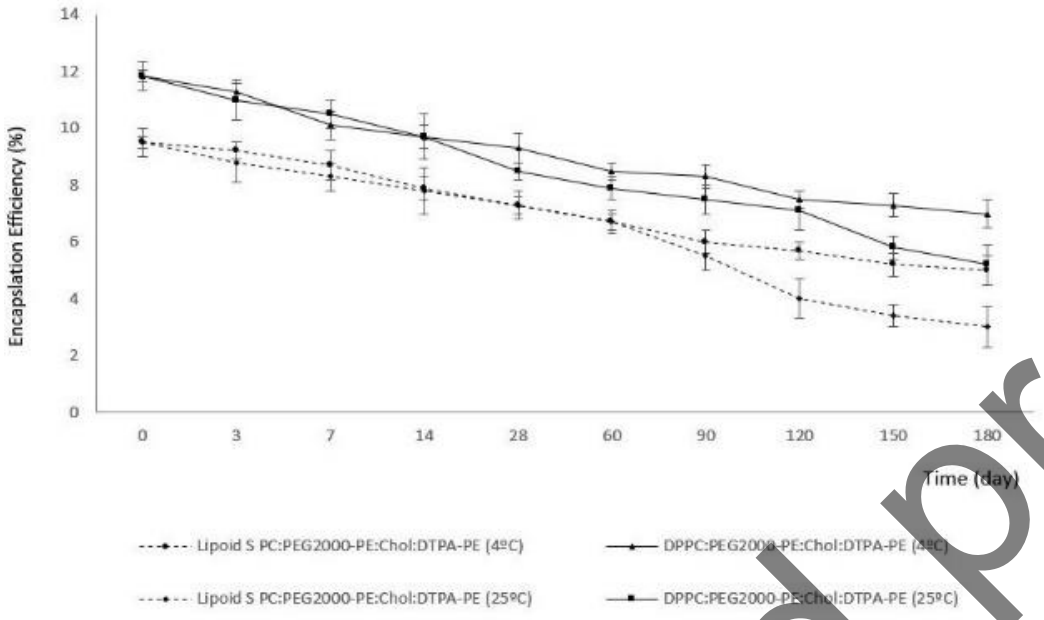

Figure 3. Alterations in MB encapsulation efficiency of Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes for 6 months of storage at both 4°C and 25°C.

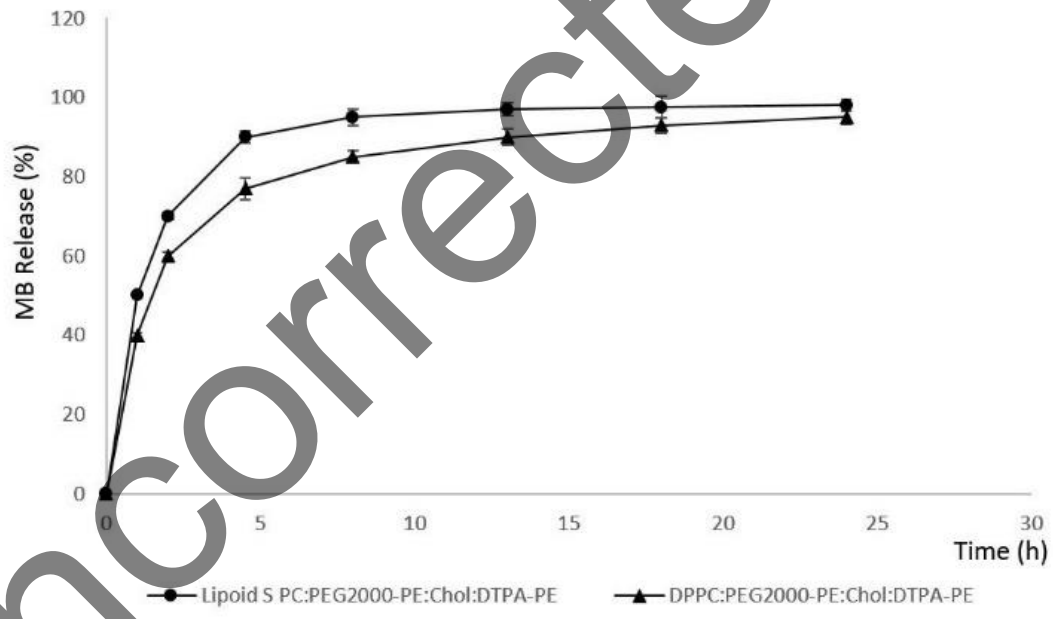

Figure 4. In vitro release of MB encapsulated Lipoid S PC:PEG2000-PE:Chol:DTPA-PE and DPPC:PEG2000-PE:Chol:DTPA-PE liposomes at 37°C.