CHAPTER 5

FUNCTIONALIZATION OF MICELLE FORMULATION

5.1 Introduction

The delivery of nanocarriers to the appropriate site is still being investigated. For this purpose, both active targeting and passive targeting are considered. However, conventional nanocarriers tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. (1). The development of nanocarriers containing lipid derivatives of PEG such as PEG-DSPE with micelle forming polymer has made targeted micelle therapy more feasible by reducing the uptake by the RES system and there by prolonging the circulation time(2).

Particularly, PEG is useful because of its ease manufacturing, low cost, controlled molecular weight and conjugation to polymer forming nanocarriers or proteins including the antibody by a variety of methods.

As a polymer for *in vivo* use, it should exhibit certain minimum properties, such as biocompatibility, biodegradability, non-immunogenicity and non-toxicity. Besides these advantages, it can be obtained under GMP conditions and it is FDA approved. Bioconjugation of PEG to pharmaceutical and biotechnology products provide stealth effect to biomolecule or carrier systems by shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the RES, and preventing recognition and degradation by proteolytic enzymes, increased body residence time, modification of organ disposition, drug penetration by endocytosis and new possibilities of drug targeting. In addition to these properties, PEG facilitates conjugation by providing the functional groups required for conjugation.

Now PEG derivatives are becoming available in a variety of activated and highly reactive end functional groups which need a minimum number of steps for conjugation. In a recent scenario more and more peptide and other macromolecules are delivered as a PEGylated form to overcome pharmacokinetic associated problems. Successful protein biopharmaceuticals include PEGylated interferons (PEGasys® and Intron®), PEGylated growth hormone receptor antagonist (Somavert®), PEGasparaginase (Oncospar®), adenosine deaminase (ADAGEN®), and granulocyte colony stimulating factor (Neulasta®) (3).

In our research we used lipid derivative of PEG (maleimide-PEG_{2000}-DSPE) for conjugation of stroke homing peptide to the micelle carrier and in turn avoid macrophages uptake and increase the blood circulation time.

5.2 Materials and Methods

SepharoseCL-4B and Ellman's reagent were purchased from Sigma Aldrich, Mumbai, India. Foetal bovine serum was purchased from Himedia Lab, Mumbai, India. DSPE-mPEG$_{2000}$-Maleimide was purchased from Laysan Bio In.,(Arab, Alabama USA). All other chemicals used were of analytical reagent grade and were used without further purification.

5.3 Preparation of Functionalized Micelle

5.3.1 Introduction

In bioconjugation modification, PEG has been used repeatedly as a linker. PEG remains as a spacer arm between micelle and conjugated ligand molecules (antibodies and other proteins or peptides). Various homo and hetero-bifunctional PEG derivatives, which can be employed successfully in the preparation of immune micelle using proper conjugation strategy, are well explained (3). Use of functionalized phospholipids enables researchers to conjugate protein, peptides and other biologics to the surfaces of lipid emulsions or micelle that allows some desired target features to be obtained, increase of the plasma half-life of the modified drugs with the introduction of PEG chains into the target drugs. In the present study we used homo-functional phospholipid, DSPE-mPEG$_{2000}$-Maleimide, in which the maleimide group easily reacts with –SH groups of antibody *via* covalent thioether linkage.

5.3.2 Identification of Maleimide Functional Group of DSPE-mPEG$_{2000}$-maleimide

The presence of maleimide group in the functional phospholipids, DSPE-mPEG2000-Maleimide, purchased from Laysan Bio In., (Arab, Alabama USA) was confirmed using Fourier transform infrared spectrophotometer (Bruker, Germany).

Procedure for Functionalized Micelle

The functionalized micelle were prepared as per the early reported methods with slight modifications (4). 1 mol% of DSPE-mPEG$_{2000}$-Maleimide was used to prepare functionalized micelle. Prior to experiments, micelle formulation to be functionalized were taken in a round bottomed flask, attached to Rota evaporator and allowed to rotate in water bath maintained at 65±2 ^{0}C. At the same time, concentrated micelle DSPE-mPEG$_{2000}$-Maleimide (1 mol%; 7.5mg dissolved in 0.2mL of distilled water) solution was equilibrated to 65±2 ^{0}Cand added slowly drop by drop with constant mixing to round bottomed flask containing micelle to be functionalized. The flask was then re-attached and allowed to rotate at 50rpm in water bath

maintained at 65±2 ^{0}C for period of 1.5 to 2hr (4). The collected functionalized micelle were pooled and analyzed for mean particle size, zeta potential and % Nicergoline.

5.3.3 Confirmation of DSPE-mPEG$_{2000}$-Maleimide Insertion in Micelle

The insertion of functional phospholipid, DSPE-mPEG$_{2000}$-Maleimide, on micelle was confirmed by Ellman's sulphydryl group estimation assay(thermo scientific protocol with slight modifications) and as per the reported method with slight modifications (5).(Indirectly, maleimide groups can be assayed by first reacting them (prepared functionalized micelle) with a known amount of thiol, present in excess, and then assaying the remaining unreacted thiol using Ellman's reagent (6). The amount of maleimide is calculated as the difference between the initial amount of thiol and the amount of un-reacted thiol after complete reaction of all maleimide groups. The assay involves the reaction of maleimide (prepared functionalized micelle) with an excess of cysteine and quantization of the remaining cysteine using Ellman's reagent. The amount of maleimide is calculated as the difference between the amounts of initial cysteine and assayed remaining cysteine.

Prepared a set of test tubes (Blank, positive control and sample tubes) in triplicate and 1.5mM cysteine hydrochloride solution in 0.1M sodium phosphate (pH 8) containing 1mM EDTA. For positive control tubes we have added 200µL non-functional micelle and 250µL of 1.5mM cysteine solution. In blank test tubes added 200µL non-functional micelle, 250µL 0.1M sodium phosphate solution (pH 8) and no cysteine solution. For sample test tubes we have added 200µL of prepared functional micelle and 250µL of 1.5mM cysteine. To all prepared blank, positive control and sample test tubes added 2.3ml of 0.1M sodium phosphate solution (pH 8) containing 1mM EDTA, mixed well, purged with nitrogen gas, covered the mouth completely and kept overnight in a cold room with constant mixing. 50µL of Ellman's reagent (4mg in 1mL 0.1M sodium phosphate solution, pH 8) was added to all test tubes, mixed well and kept at room temperature for 15minutes. The unreacted cysteine was then determined by measuring the absorbance of the above solutions (yellow colored solution) at 412nm using UV-visible spectrophotometer. The amount of cysteine remain unreacted was calculated as per the thermo scientific protocol.

5.4. Results and Discussion

5.4.1. Preparation of Functionalized Micelle

The presence of maleimide group in the functional phospholipids, DSPE-mPEG2000-Maleimide (**Figure 5.1**), purchased from Laysan Bio In.,(Arab, Alabama USA) was tested using Fourier transform infrared spectrophotometer (Bruker, Germany).The FTIR spectrum (**Error! Reference source not found.**) confirms the presence of maleimide group.

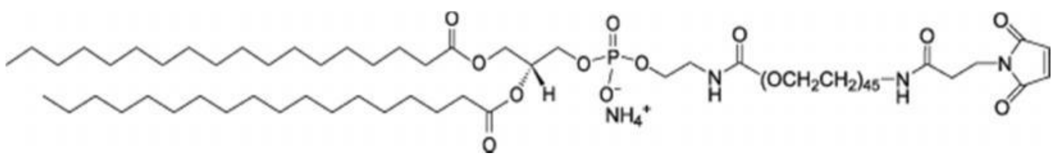

Figure 5.1 Chemical structure of DSPE-mPEG$_{2000}$-Maleimide

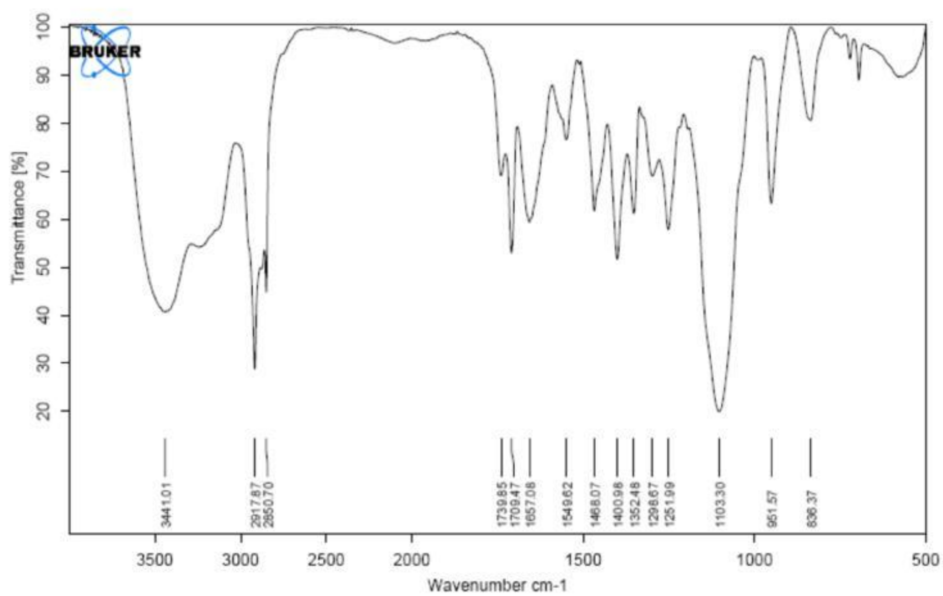

Figure 5.2 FTIR spectrum of DSPE-mPEG$_{2000}$-Maleimide

The spectrum depicts (3441cm^{-1}: N-H bending; 2917 cm^{-1}: C-H stretching; 1739 cm^{-1}:C=O stretching ester; 1709 cm^{-1}:C=O stretching imide; 1657 cm^{-1}: C=O stretching amide; 1103 cm^{-1}:C=O stretching imide;836 cm^{-1}:C=O imide. When the functionalized micelle were prepared by co-dissolving DSPE-mPEG$_{2000}$-Maleimide along with TPGS and PF-127 (pre-insertion method), the maleimide functional groups are distributed on the surface of the micelle, are available for coupling to ligand molecules.

This post-insertion technique is a logical progression from the work of Uster et al. (7) and involves the coupling of ligands to the terminus of polyethylene glycol (PEG)-lipid derivatives followed by the time and temperature dependent transfer of the ligands-coupled PEG-lipids into the pre-formed drug-loaded micelle during a simple incubation step. As the antibodies (specially monoclonal and other proteins) are more heat sensitive (they may lose their biological activity) and this method involves time and temperature dependent transfer, we can make some slight modifications in the above method. We can first transfer the DSPE-$mPEG_{2000}$-Maleimide (functional lipid derivatives) in a micelle and then we can incubate the functionalized micelle with ligand at room temperature or in a cold room.

Hence, in our study the functionalized micelle were first prepared as per the early reported methods with slight modifications(8)and then we used these micelle to prepare immune-micelle. Solution of 1mol% of DSPE-$mPEG_{2000}$-Maleimide was then inserted into the preformed Nicergoline loaded functionalized micelle as described in method. The micelles were slightly diluted after separation but no change in total drug content and mean particle size were observed.

5.4.2 Confirmation of Presence of Maleimide Groups over Micelle

The insertion of functional phospholipids, DSPE-$mPEG_{2000}$-Maleimide, on micelle was confirmed by Elman's sulphydryl group estimation assay (thermo scientific protocol with slight modifications) and as early the reported method with slight modifications (5). The amount of maleimide is calculated as the difference between the initial amount of thiol(cysteine) and the amount of unreacted thiol(cysteine) after complete reaction of all maleimide groups **(Table 5.1**). Prepared a set of test tubes (Blank, positive control and sample tubes) (yellow product) analyzed using UV-visible spectrophotometer at 412nm. The absorbance positive control tubes and sample tubes were subtracted from blank sample absorbance. Using the absorbance values we have calculated the amount thiol (cysteine) remained unreacted in samples as below mentioned.

5.4.3 Calculation of the Free Sulfhydryl Concentration

A 250µl aliquot of the 1.5mM standard cysteine solution mixed with 2.3 ml of reaction buffer (0.1M sodium phosphate of pH 8), 200µl non functionalized micelle and 50µl of Ellman's reagent solution gave an absorbance of 0.6703 (after subtracting the blank; 0.283) using a

1cm spectrophotometric cuvette. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB in this buffer system at 412 nm is 14,150(9).

Molar absorptivity, 'E' is defined as follows:

$$E = A/bc$$

Where A = absorbance,

b = path length in centimeters,

c = concentration in moles/liter (=M)

Solving for concentration gives the following formula: C=A/bE

A=0.6703, b=1cmand E = 14,150 $M^{-1}cm^{-1}$

Therefore, C= 0.6703/1(14,150) M

$= 4.737\times10^{-5}$M / litre

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is

2.3 ml of Reaction Buffer
+ 0.2ml non-functionalized micelle
+ 0.25 ml of standard cysteine solution
+ 0.05 ml of Ellman's Reagent Solution
2.80 ml of solution

The concentration of the positive control solution is 4.737×10^{-5} M (1000ml contains 4.737×10^{-5} M), then 2.80 ml of that solution contains

$(2.8 \times 4.737\times10^{-5})/1000 = 1.326\times10^{-7}$ moles

These 1.326×10^{-7} moles of sulfhydryls are present in the positive control solution and in similar way we have calculated the sulfhydryls concentration of test samples. The amount of

sulfhydryls reacted with functionalized micelle ($0.6457\times10^{-7}\pm0.256\times10^{-7}$ moles) was calculated by subtracting test sample values from positive control sample values (Table 5.1). The obtained value of sulfhydryls reacted with functionalized micelle indicate the availability of sufficient maleimide for conjugation of ligand.

Table 5.1 Estimated cysteine (thiol) concentrations using Elman's assay

Concentration of thiol (positive control samples)	$1.326\times10^{-7}\pm0.166\times10^{-7}$ moles
Concentration of thiol remain unreacted (functionalized micelle)	$0.6602\times10^{-7}\pm0.127\times10^{-7}$ moles
Concentration of thiols reacted with functionalizedmicelle (0.2ml)	$0.6221\times10^{-7}\pm0.149\times10^{-7}$ moles

Values are Mean ± SD, n=3.

5.5 Reference

1. Oku N. Anticancer therapy using glucuronate modified long-circulating liposomes. Advanced drug delivery reviews. 1999;40(1):63-73.

2. Lundberg BB, Griffiths G, Hansen HJ. Specific binding of sterically stabilized anti-B-cell immunoliposomes and cytotoxicity of entrapped doxorubicin. International journal of pharmaceutics. 2000;205(1):101-8.

3. Manjappa AS, Chaudhari KR, Venkataraju MP, Dantuluri P, Nanda B, Sidda C, et al. Antibody derivatization and conjugation strategies: application in preparation of stealth immunoliposome to target chemotherapeutics to tumor. Journal of Controlled Release. 2011;150(1):2-22.

4. Allen T, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly (ethylene glycol) show prolonged circulation half-lives in vivo. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1991;1066(1):29-36.

5. Singh R. A sensitive assay for maleimide groups. Bioconjugate chemistry. 1994;5(4):348-51.

6. Yoshitake S, Yamada Y, Ishikawa E, Masseyeff R. Conjugation of Glucose Oxidase from Aspergillus niger and Rabbit Antibodies Using N-Hydroxysuccinimide Ester of N-(4-Carboxycyclohexylmethyl)-Maleimide. European Journal of Biochemistry. 1979;101(2):395-9.

7. Uster PS, Allen TM, Daniel BE, Mendez CJ, Newman MS, Zhu GZ. Insertion of poly (ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. FEBS letters. 1996;386(2):243-6.

8. Moreira JN, Ishida T, Gaspar R, Allen TM. Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. Pharmaceutical research. 2002;19(3):265-9.

9. Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman's reagent. Methods in enzymology. 1982;91:49-60.