Technical Information

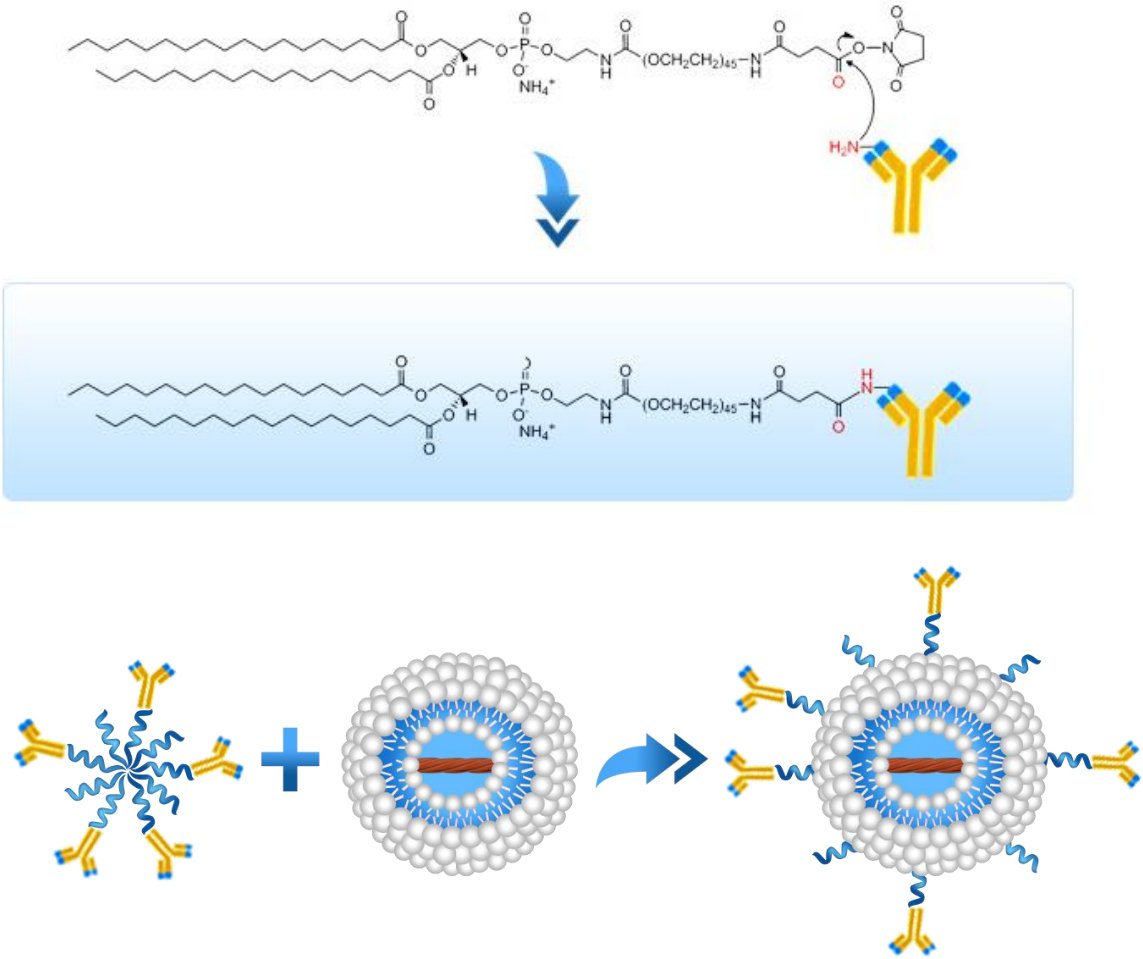
Version 1.1

Immunodox®-NHS (PEGylated) (Post-insertion)

DESCRIPTION

Numerous techniques have been developed to prepare immunoliposomes based on the nucleophilic reactivity of free amine groups of proteins or peptides. However, the most common, versatile and straightforward activation chemistry for creating reactive acylating reagents and labeling peptides/proteins is to form NHS ester with primary amines. A single step nucleophilic substitution reaction between NHS ester derivative and alpha amines at the N terminal or the beta amines of lysine side chains leads to the formation of a stable amide bond.

N-Hydroxysuccinimide (NHS) esters of DSPE-PEG-NHS liposomes react with the primary amine groups on the peptides, proteins, antibodies or other functional ligands for targeted drug delivery applications. The nucleophilic attack of the amine groups on the NHS-activated carbonyl group of the DSPE-PEG-NHS results in the elimination of the NHS group and formation of amide linkage.



Conjugation reaction between NHS-activated DSPE-PEG lipid with the amine group of the ligand and formation of stable amide linkage. Micelles formed from lipid conjugated ligand and non-reactive PEG lipids are mixed together and the PEGylated lipids are post-inserted into the liposomes in order to form PEGylated ligand surface conjugated liposomes.

FORMULATION INFORMATION

Immunodox®-NHS (PEGylated) (Post-insertion)

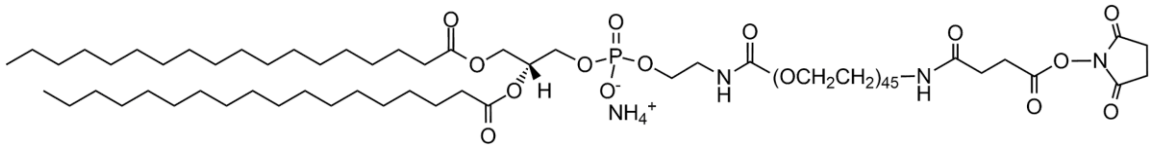
Post-insertion Kit (3 Vials)	Specification
Vial 1	Preformed Liposomes composed of HSPC and Cholesterol (60:40 molar ratio)
Vial 2	DSPE-PEG(2000)-NHS lipid (reactive PEGylated lipid) in powder form
Vial 3	DSPE-PEG(2000) lipid (non-reactive PEGylated lipid) in powder form

Lipid Composition for Vial 1*	Concentration (mg/ml)	Concentration (mM)	Molar Ratio Percentage
Hydrogenated Soy PC	11.5	14.66	60
Cholesterol	3.83	9.9	40
Total	**15.33**	**24.56**	**100**

* For the 5-ml kit, the volume of vial 1 is 4 ml. 1 ml of micelle solution that are formed using vials 2 and 3 will be added to this vial to make the final volume of 5 ml in the final product. For the 2-ml kit, the volume of vial 1 is 1.6 ml. 0.4 ml of micelle solution that is formed using vials 2 and 3 will be added to this vial to make the final volume of 2 ml in the final product.

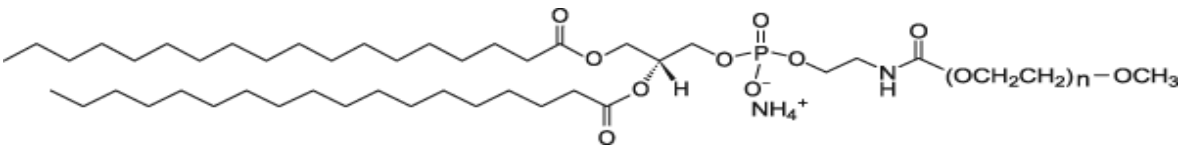
Buffers, Liposome Size and Encapsulated Drug Concentration for Vial 1	Specification
Inside Buffer	Ammonium Sulfate
Outside Buffer	Phosphate Buffered Saline
pH	7.4
Liposome Size	100 nm
Encapsulated Doxorubicin	2 mg/ml (3.45 mM)

Vial 2*	Specification
DSPE-PEG(2000)-NHS Lipid	This vial contains reactive DSPE-PEG(2000)-NHS lipid in powder form. This lipid is conjugated to a reactive protein, peptide or ligand containing amine and then mixed with non-reactive DSPE-PEG(2000) lipid in aqueous solution to form micelles. The PEGylated lipid micelles are incubated with preformed liposomes in vial 1 and PEG lipids will post-insert themselves into the liposomes.



* The amount of the powdered PEG(2000)-NHS lipid for the 2-ml kit is 1.34 mg and for the 5-ml kit is 3.34 mg.

Vial 3*	Specification
DSPE-PEG(2000) Lipid	This vial contains non-reactive DSPE-PEG(2000) lipid in powder form. This lipid in mixed with DSPE-PEG(2000)-NHS lipid which is already conjugated to a ligand (protein, peptide, *etc.*) in aqueous solution to form micelles. The PEGylated lipid micelles are incubated with preformed liposomes in vial 1 and PEG lipids will post-insert themselves into the liposomes.



* The amount of the powdered PEG(2000)-DSPE lipid for the 2-ml kit is 5 mg and for the 5-ml kit is 12.5 mg.

CONJUGATION PROTOCOL (POST-INSERTION)

Materials and Equipment

The 3-vial post-insertion kit contains preformed liposomes (vial 1), non-reactive PEGylated lipid in powder form (vial 2) and DSPE-PEG(2000)-NHS lipid in powder form (vial 3). In order to use the post-insertion kit, you will need:

1. Laboratory vortex mixer is recommended to have.
2. Laboratory magnetic stirrer is needed for dialysis.
3. Two small 10-ml round bottom flasks or two small glass vials.
4. A rotary evaporator. We understand that many labs might not have a rotovap. Alternatively, you can use a nitrogen tank connected to a thin hose for creating a stream of nitrogen flow to dry the lipid and make a thin film.

5. A small amount of a solvent such a chloroform or methylene chloride (you will only need a few milliliters).
6. Phosphate buffered saline (PBS).
7. Ammonium sulfate buffer.
8. A Sonicator. It is better to have a bath sonicator. If you do not, that is fine. You still can follow the protocol. You may also use a vortex instead of the sonicator for agitation of the solution as well.
9. Float-A-Lyzer® with a proper MWCO that easily allows the cleanup of your liposome conjugated ligand from free and non-conjugated protein, peptide or antibody. You need to make sure that the MWCO is below 1,000,000 dalton. At 1,000,000 dalton, the pore size on the dialysis membrane gets close to 100 nm and therefore your liposomes can be dialyzed out. You cannot use dialysis cassettes blindly. Please understand the technique before using either spin column or dialysis cassette. If you do not use the correct MWCO, you can lose your entire prep. For this protocol, we recommend MWCO of 300,000 dalton.

Preparation Method

1. The post-insertion kits come in two sizes; 2 ml and 5 ml. For the 2-ml kit size, dissolve the content of vial 3 in 100 µl of chloroform or methylene chloride. For the 5-ml kit size, the content of vial 3 should be dissolved in 250 µl of chloroform or methylene chloride. Transfer the solution to a 10 ml round bottom flask. Dry the chloroform using a rotary evaporator or under a stream of nitrogen and make a dried lipid film.
2. For the 2-ml kit, add 100 µl of PBS buffer to the dried lipid film. For the 5-ml kit, the amount of the added buffer is 250 µl. It is preferred to sonicate the hydrated lipid film using a bath sonicator and sonicate the micelle solution for 5 minutes. If you do not have a bath sonicator then hydrate the dried lipid film with PBS for at least 1 hour and constantly rotate the solution in the round bottom flask using a rotavap (not connected to vacuum) or by hand to make sure that all the dried lipid on the wall of the round bottom

flask will go to the solution and form micelles. Alternatively, you can use a vortex to agitate the solution. The goal is to have all the dried lipid on the wall of the round bottom glass to go the micelle solution. Cover the mouth of the round bottom flask with parafilm. Refrigerate the micelle solution of non-reactive PEG lipids until it is ready to be mixed with micelles formed in step 4.

3. For the 2-ml post-insertion kit, the amount of DSPE-PEG-NHS is 1.34 mg (0.22 µmol) and for the 5-ml of post-insertion kit the amount of DSPE-PEG-NHS is 3.34 mg (0.55 µmol). For the 2-ml kit size, dissolve the content of vial 2 in 100 µl of chloroform or methylene chloride. For the 5-ml kit size, the content of vial 2 should be dissolved in 250 µl of chloroform or methylene chloride. Transfer the solution to a 10 ml round bottom flask. Dry the chloroform using a rotary evaporator or under a stream of nitrogen.
4. Add the water-soluble protein, peptide or ligand at 1:2 molar ratio of ligand to dried film of DSPE-PEG-NHS. For the 2-ml kit add 300 µl of water soluble solution of protein of ligand in PBS (pH 7.4) to the dried lipid film and for the 5-ml kit add 750 ul of water soluble solution of protein of ligand in PBS (pH 7.4) to the dried lipid film. It is preferred to sonicate the hydrated lipid film using a bath sonicator and sonicate the micelle solution for 5 minutes. If you do not have a bath sonicator then hydrate the dried lipid film with PBS for at least 1 hour and constantly rotate the solution in the round bottom flask using a rotavap (not connected to vacuum) or by hand to make sure that all the dried lipid on the wall of the round bottom flask will go to the solution and form micelles. Alternatively, you can use a vortex to agitate the solution. The goal is to have all the dried lipid on the wall of the round bottom glass to go the micelle solution. The solution is incubated at room temperature for 6 hours and in refrigerator for 24 hours. The reaction is pH sensitive. Read the technical note below for more information.
5. Mix the micelles in step 2 to the micelles in step 4. The total volume of micelles for the 2-ml kit should be 400 µl and for the 5-ml kit should be 1000 µl.
6. To conduct post-insertion, the micellar dispersion is then co-incubated with preformed plain liposomes at 60 ℃ for 30 min.
7. Remove the non-conjugated protein, peptide or antibody from the immunoliposomes by dialysis. We prefer dialysis to size exclusion columns. Dialysis is a much slower process

but there will be minimum loss of immunoliposomes after the prep is cleaned from non-conjugated protein/peptide/ligand. Spin columns are much faster; however, you can easily lose over 50% of the liposomes on the spin column. We recommend using Float-A-Lyzer® dialysis cassette from Spectrum Labs. You will need to choose a cassette with proper MWCO depending on the MW of your protein, peptide, antibody or antibody fragment. **NOTE**: If you decide to use a dialysis cassette, you will need to make sure that the MWCO is below 1,000,000 dalton. At 1,000,000 dalton, the pore size on the dialysis membrane gets close to 100 nm and therefore, your liposomes can be dialyzed out. You cannot use dialysis cassettes and spin columns blindly. They come in various sizes and you need to choose the correct size wisely. Dialyze the immunoliposome solution in 1 liter of PBS at pH 7.4 for 8 hours. Change the dialysis buffer with a fresh 1 liter of PBS and let is dialyze for another 8 hours. After this step, your cleaned up immunoliposome is ready to be used.

TECHNICAL NOTES

- Doxorubicin is a fluorescent molecule with λ_{ex} 470 nm and λ_{em} 585 nm. If you are using a fluorescent tag on your antibody or ligand, then you need to make sure that they will not interfere with each other.
- Hydrolysis of DSPE-PEG-NHS in aqueous solutions competes with the primary amine reaction, resulting in the elimination of the NHS group, which can consequently decrease the coupling yield prior to the reaction with the protein/antibody. In order to minimize the impact of the hydrolysis of NHS ester, use a high concentration of protein/antibody to increase the efficiency of the crosslinking.
- The reaction of NHS esters with amines is strongly pH-dependent: at low pH, the amino group is protonated, and no modification takes place. At higher-than-optimal pH, hydrolysis of NHS ester is quick, and modification yield diminishes. The half-life of NHS esters at pH 7 and 8 is 4-5 hours and 1 hour, respectively. Whilst NHS esters have a half-life of only 10 minutes at pH 8.6. Therefore, to avoid the hydrolysis of NHS ester, DSPE-PEG-NHS lipid **should be used immediately** for conjugation to antibodies,

proteins or peptides containing free amines. NHS ester reactions are conducted in common buffers at pH 7-8.

- Primary amine buffers such as Tris should NOT be used because they compete for reaction; however, in some procedures, it is useful to add Tris or glycine buffer at the end of a conjugation procedure to quench (stop) the reaction.
- If you are using a ligand or peptide that is hydrophobic then it is recommended to solubilize it in DMSO or DMF and then add the buffer to it. It is recommended not to use more than 5% volume of DMSO or DMF in the solution. DMF and DMSO are both compatible with liposomes and they are also miscible in water. Other organic solvent such as ethanol and chloroform are not compatible with liposomes and will cause the liposomes to lyse. If you end up using DMSO or DMF then after the conjugation reaction is done, you need to remove DMSO and DMF from the liposomes. In order to do that you need to use a dialysis cassette that is made from REGENERATED CELLULOSE MEMBRANE. **NOTE**: Not all membranes are compatible with DMF and DMSO. We recommend using a Slide-A-Lyzer™ MINI Dialysis Device with MWCO of 2K made from regenerated cellulose membrane manufactured by ThermoFisher. After DMSO or DMF is removed you can use Float-A-Lyzer® dialysis device for the final step of cleaning up the prep.
- Liposomes should be kept at 4 °C and **NEVER** be frozen.

APPEARANCE

Immunodox®-NHS (PEGylated) post-insertion kit comes in three vials: vial 1 is a red translucent liquid made of nano size unilamellar liposomes which does not contain any reactive of non-reactive PEGylated lipid. Usually due to the small size of liposomes no settling will occur in the bottom of the vial. Vial 2 contains reactive DSPE-PEG(2000)-NHS lipid in white powder form. Vial 3 contains non-reactive DSPE-PEG(2000) lipid in white powder form.

STORAGE AND SHELF LIFE

Storage

Immunodox® products should always be stored at in the dark at 4 °C, except when brought to room temperature for brief periods prior to animal dosing. DO NOT FREEZE. If the suspension is frozen, the encapsulated drug can be released from the liposomes thus limiting its effectiveness. In addition, the size of the liposomes will also change upon freezing and thawing.

Shelf Life

Immunodox®-NHS is made on daily basis. The batch that is shipped is manufactured on the same day. It is advised to use the products within 4 months of the manufacturing date.

REFERENCES AND BACKGROUND READING

1. *Na K, Lee SA, Jung SH, Hyun J, Shin BC. Elastin-like polypeptide modified liposomes for enhancing cellular uptake into tumor cells. Colloids and Surfaces B: Biointerfaces. 2012 Mar 1;91:130-6.*

2. *Dai W, Fan Y, Zhang H, Wang X, Zhang Q, Wang X. A comprehensive study of iRGD-modified liposomes with improved chemotherapeutic efficacy on B16 melanoma. Drug delivery. 2015 Jan 2;22(1):10-20.*

3. *Ferreira Ddos S, Faria SD, de Araújo Lopes SC, Teixeira CS, Malachias A, Magalhães-Paniago R, de Souza Filho JD, Oliveira BL, Guimarães AR, Caravan P, Ferreira LA. Development of a bone-targeted pH-sensitive liposomal formulation containing doxorubicin: physicochemical characterization, cytotoxicity, and biodistribution evaluation in a mouse model of bone metastasis. International journal of nanomedicine. 2016;11:3737-51.*

4. *Wang D, Fu J, Shi Y, Peng D, Yuan L, He B, Dai W, Zhang H, Wang X, Tian J, Zhang Q. The modulation of tumor vessel permeability by thalidomide and its impacts on different types of targeted drug delivery systems in a sarcoma mouse model. Journal of Controlled Release. 2016 Sep 28;238:186-96.*

5. *Fei W, Zhang Y, Han S, Tao J, Zheng H, Wei Y, Zhu J, Li F, Wang X. RGD conjugated liposome-hollow silica hybrid nanovehicles for targeted and controlled delivery of arsenic trioxide against hepatic carcinoma. International journal of pharmaceutics. 2017 Mar 15;519(1):250-62.*

6. *Abuchowski A, Kazo GM, Verhoest Jr CR, Van Es T, Kafkewitz D, Nucci ML, Viau AT, Davis FF. Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates. Cancer biochemistry biophysics. 1984 Jun;7(2):175-86.*

7. *Sartore L, Caliceti P, Schiavon O, Veronese FM. Enzyme modification by MPEG with an amino acid or peptide as spacer arms. Applied biochemistry and biotechnology. 1991 Jan 1;27(1):45-54*.

8. *Shahin M, Soudy R, El-Sikhry H, Seubert JM, Kaur K, Lavasanifar A. Engineered peptides for the development of actively tumor targeted liposomal carriers of doxorubicin. Cancer letters. 2013 Jul 1;334(2):284-92*.