Immunodox®-Folate (PEGylated)

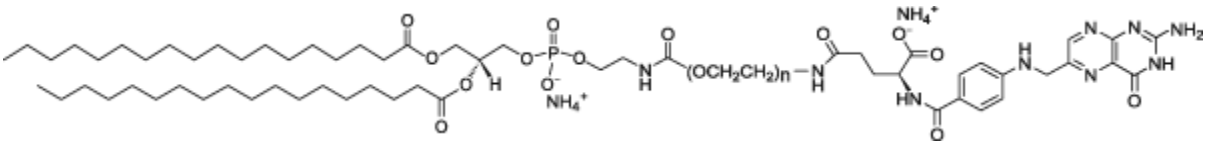
DESCRIPTION

During the past five decades, various types of chemistries have been used for conjugation of molecules such as antibodies, peptides, proteins or other reactive ligands to the surface of liposomes. In general, the conjugation can be achieved through the N-terminus, the C-terminus or the available sulfur (*e.g.* Fab' fraction or thiolated antibodies). Not all chemistries have the same yield and efficiency of conjugation and often reproducing biocompatible batches can be a challenge.

Folate binding protein (FBP) is an endogenous protein, which shows a very high affinity for folate. An antibody can be tagged by FBP and an immunoliposome can be formed by non-covalent and high affinity interaction between FBP and folate lipid on the surface of the liposomes. In this method, the antibody, which has been already covalently linked to FBP through a thioether linkage, is conjugated with folate-derivative liposomes. Having a relatively low molecular weight (MW ~40 kDa) and a single binding site prevents liposomes from crosslinking.

FORMULATION INFORMATION

Immunodox®-Folate (PEGylated)

Lipid Composition	Concentration (mg/ml)	Concentration (mM)	Molar Ratio Percentage
Hydrogenated Soy PC	9.58	12.22	57
Cholesterol	3.19	8.25	38
DSPE-PEG(2000)	2.7	0.96	4.5
DSPE-PEG(2000)-Folate	0.39	0.12	0.5
			
Total	**15.86 mg/ml**	**21.55 mM**	**100**

Buffers, Liposome Size and Encapsulated Drug Concentration	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)

CONJUGATION PROTOCOL

Materials and Equipment

In order to conjugate your antibody or protein tagged with FBP to Immunodox®-Folate liposomes you will need:

1. Laboratory vortex mixer is recommended to have.
2. Laboratory magnetic stirrer is needed for dialysis.
3. Float-A-Lyzer® with a proper MWCO that easily allows the cleanup of your liposome conjugated ligand from free and non-conjugated protein/peptide/ligand. 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 total lipid concentration in Immunodox®-Folate is 21.55 mM. 0.5% mol of the lipid in liposomes contains PEG-Folate group and only half of them are exposed to the outside of the liposomes, which is equal to 0.053 mM of reactive conjugatable lipid. For 2 ml volume liposomes, this is equal to 1.08×10^{-7} mol, and for 5 ml volume liposomes, this is equal to 2.69×10^{-7} mol of PEG-Folate. Add 1:20 molar ratio of antibody tagged with FBP to PEG-Folate lipid.
2. Incubate Immunodox®-Folate with antibody-FBP for 1 hour at room temperature.
3. 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. If you do not use the correct MWCO, you can lose your entire prep.

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, you need to make sure that they will not interfere with each other.
- Please keep in mind that the main using for liposomes containing folate on surface is for targeting folate receptors on the cells. Making immunoliposomes using antibodies/proteins or ligands tagged with FBP is a very niche application.
- The immunoliposomes may disassemble following internalization by endocytosis upon exposure to the low pH environment of the endosomal compartment, due to reduced affinity between folate and FBP under low pH. This internalization-triggered uncoupling may be beneficial to delivery of liposomal drug at the cellular level by allowing the sorting of the internalized liposomes to the appropriate subcellular compartment and/or facilitating intracellular release of drug molecules from the liposomes.
- Size exclusion spin columns such as Sepharose® CL-4B can be used instead of Float-A-Lyzer® dialysis cassette. However, a very large amount of liposomes will stick to the

column during the cleanup process and therefore we strongly suggest using dialysis than size exclusive beads.

- 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®-Folate is a red translucent liquid made of nano size unilamellar liposomes. Usually due to the small size of liposomes no settling will occur in the bottom of the vial. The liposomes are packaged in an amber vial.

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®-Folate 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.

REFERENCE AND BACKGROUND READING

Pan X, Lee RJ. Construction of anti-EGFR immunoliposomes via folate–folate binding protein affinity. International journal of pharmaceutics. 2007 May 24;336(2):276-83.