Technical Information

Version 1.1
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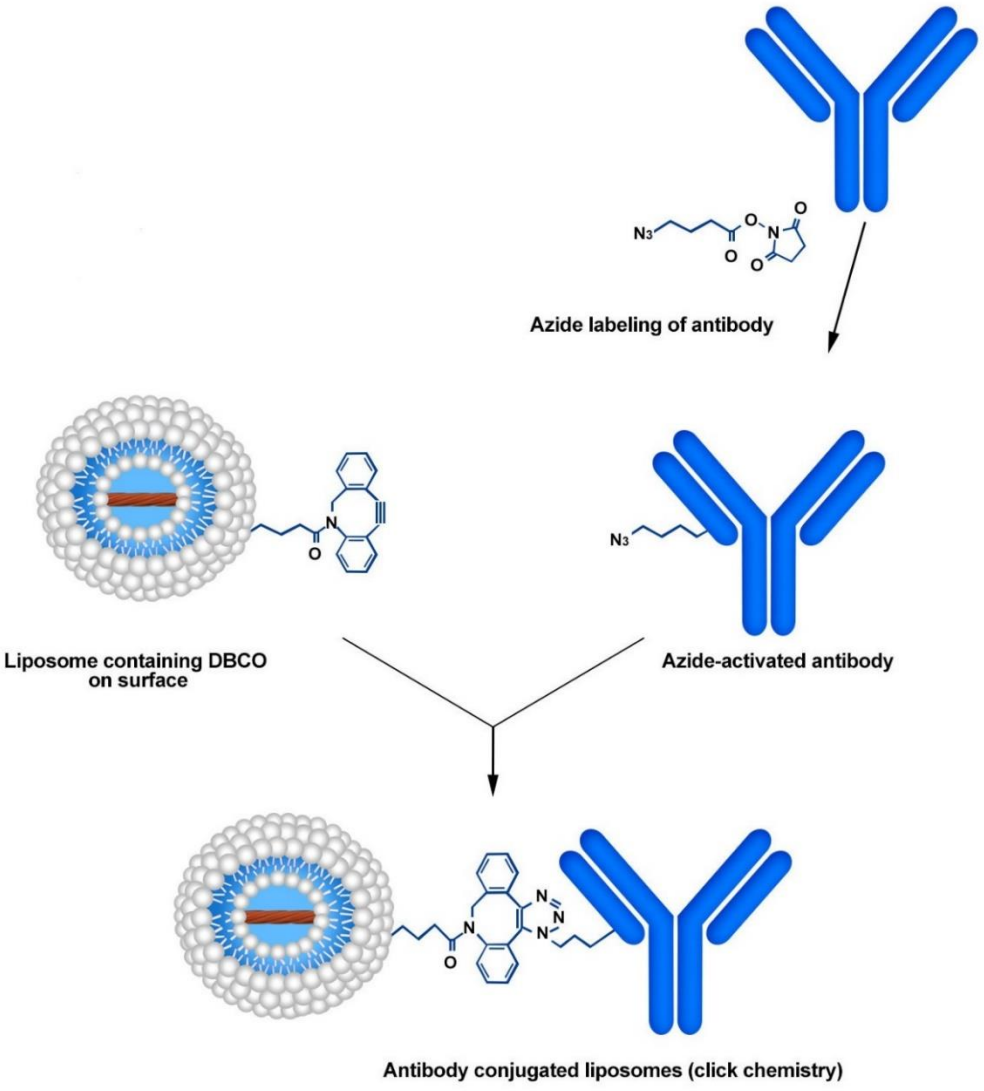
Immunodox®-DBCO (PEGylated)

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.

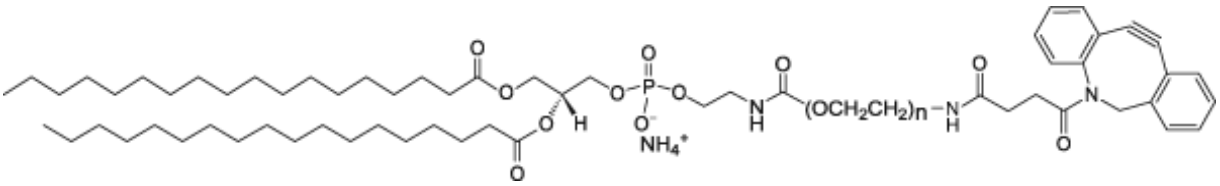
Copper-free click chemistry is a fairly new chemistry that has been commercialized during the past few years. More and more click chemistry-based reagents are becoming available commercially which makes the formulation development much easier for scientists. The great advantage of this chemistry is biocompatibility since no cytotoxic copper catalyst is required. By far, click chemistry is the most efficient and easiest conjugation chemistry available for coupling of antibodies and other reactive ligands to the surface of the liposomes. The conjugation chemistry is based on the reaction of the dibenzocyclooctyne (DBCO) reagent with an azide linker to form a stable triazole. DBCO moiety can be on the antibody and azide moiety can be on liposomes and vice versa. This conjugation protocol is based on the reaction of the dibenzocyclooctyne (DBCO) group of the liposomes with an azide linker on the antibody, peptide or proteins.



Click Chemistry: Conjugation reaction between DBCO-containing doxorubicin liposome and azide-tagged antibody

FORMULATION INFORMATION

Immunodox®-DBCO (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)	2.5	0.89	4
DSPE-PEG(2000)-DBCO	0.68	0.22	1
			
Total	**15.95 mg/ml**	**21.58 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

You need the following materials and equipment in order to use the kit.

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®-DBCO is 22.45 mM. 1% mol of the lipid in liposomes contains DBCO group and only half of them are exposed to the outside of the liposomes, which is equal to 0.11 mM of reactive conjugable lipid. For 2 ml volume liposomes, this is equal to 2.20×10^{-7} mol, and for 5 ml volume liposomes, this is equal to 5.50×10^{-7} mol of DBCO. Add 2.5 mol equivalents of DBCO-lipids in liposomes to 1 mol equivalent of azide containing protein. Incubate the mixture of liposome and antibody at room temperature for 4 h followed by overnight incubation at 4 °C in a refrigerator.
2. 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

- Before starting the conjugation process, please make sure to avoid buffers that contain azides, which can react with DBCO.
- DBCO group is known to be hydrophobic and it buries itself in the lipid bilayer of the liposomes. Direct conjugation of a ligand to the liposomes containing DBCO has produced immunoliposomes with yield of above 60% which is quite acceptable and much higher than many other conjugation chemistries. Post-insertion of DBCO lipid conjugated ligands into the liposomes increases the yield to above 80%. For more information see reference 11.
- Reactions of DBCO and azides are more efficient at high concentrations and temperatures (*i.e.*, up to 37 °C). In order to avoid denaturation of proteins, peptides and antibodies, it is recommended to incubate molecules with liposomes at room temperature followed by refrigeration (see step 1).
- Typical reaction times are less than 12 h; however, incubating for longer can improve efficiency.

- Spin columns can be used for the immunoliposome separation, and they are very fast method for purification. However, a large quantity of the samples is lost on the column. Dialysis is a slower process with minimal sample loss and therefore, we recommend dialysis over spin columns.
- 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®-DBCO 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®-DBCO 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

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