Technical Information

Version 1.1
Revision Date: 03/21/2018

ImmunoFluor™-Dodecanyl (Non-PEGylated)

DESCRIPTION

Numerous techniques have been developed to prepare immunoliposomes based on the nucleophilic reactivity of free amine groups of proteins or peptides. One of the most popular and commonly used methods is to covalently couple free carboxylic groups to primary amines through activation of the carboxyl groups with EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide). EDC, which is a so-called zero-length crosslinking agent, reacts with the carboxyl to form an amine reactive intermediate (O-acylisourea). The produced O-acylisourea can be easily displaced by nucleophilic attack from the primary amino groups in the reaction mixture. However, this intermediate is unstable and hydrolyzed in aqueous solutions. In order to prevent the intermediate hydrolysis, sulfo-NHS (N-hydroxysulfosuccinimide) is added to EDC to produce a significantly more stable and more soluble active intermediate (NHS ester).

Consequently, the immunoliposomes are prepared by a two-step coupling procedure: first, activating the free carboxyl group of the linker lipid incorporated in the liposomes with EDC and sulfo-NHS, and then covalently conjugating the antibodies to the lipids through displacement of sulfo-NHS groups by antibody amines, as depicted below. EDC/sulfo-NHS coupling reactions are highly selective and highly efficient, and the biological activity of the protein or peptide is preserved.

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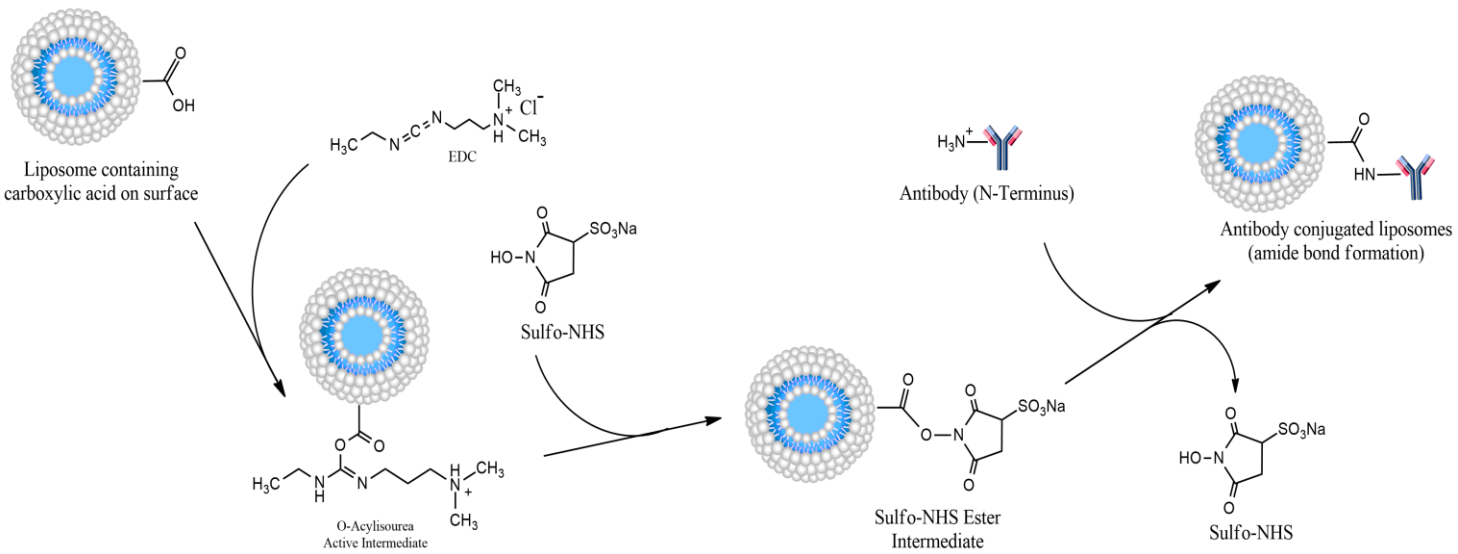

Conjugation reaction between N-terminus of antibody and carboxyl group-containing liposome.

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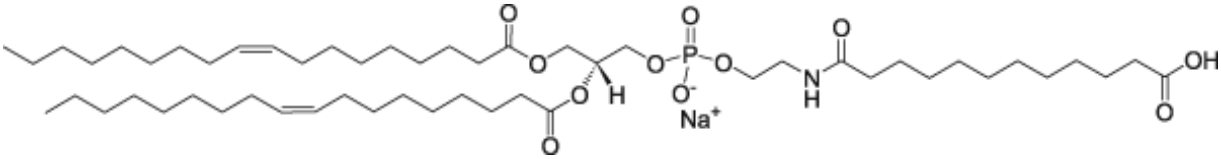
FORMULATION INFORMATION

ImmunoFluor™-Dodecanyl (Non-PEGylated)

Lipid Composition	Concentration (mg/ml)	Concentration (mM)	Molar Ratio Percentage
L-α-Phosphatidylcholine	~12	~15.5	~69
Cholesterol	2.6	6.73	30
1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(dodecanyl)	0.22	0.22	1
			
Fluorescent Lipid (see below)	Varies based on the dye	Varies based on the dye	Varies based on the dye
Total	**~14.82 mg/ml**	**~22.45 mM**	**100**

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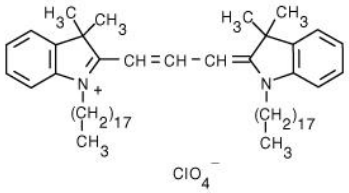
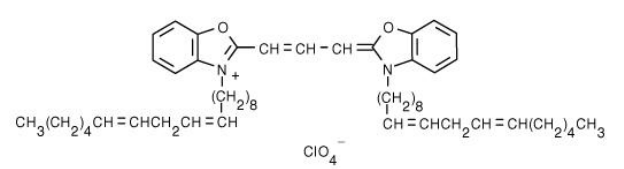
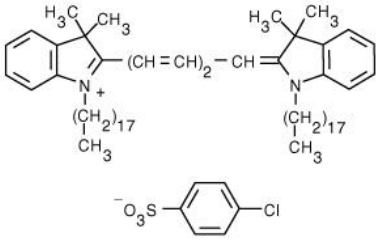
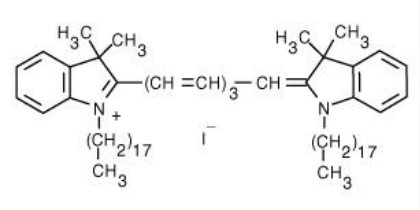
Fluorescent Dye	Excitation/ Emission (nm)	Molecular Structure
1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)	549/565	
3,3'-Dilinoleyloxacarbocyanine perchlorate (DiO)	484/501	
1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD)	644/665	
1,1'-Dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR)	750/780	

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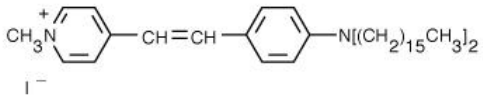
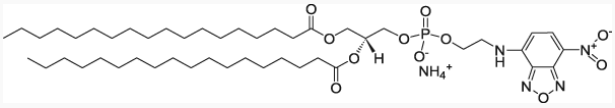
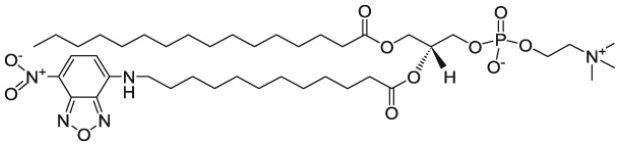
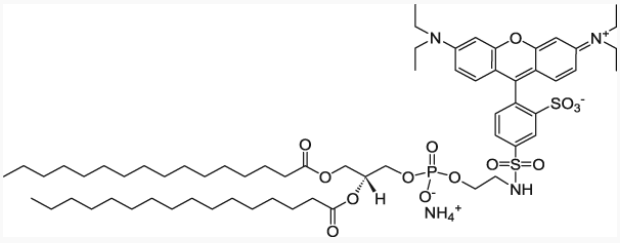
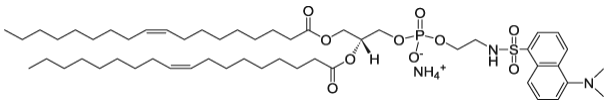
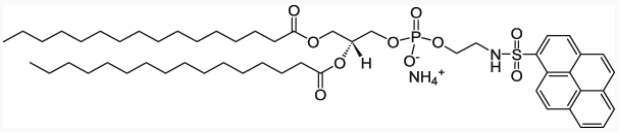
Fluorescent Dye	Excitation/ Emission (nm)	Molecular Structure
4-(4-(Dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA)	456/590	
1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD on head group)	460/535	
1-Palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (NBD on fatty acid tail)	460/534	
1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhodamine lipid)	560/583	
1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (ammonium salt) (Dansyl lipid)	336/513	
1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(1-pyrenesulfonyl) (ammonium salt) (Pyrene lipid)	351/379	

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Buffer and Liposome Size	Specification
Buffer	Phosphate Buffered Saline
pH	6 *
Liposome Size	100 nm

* In order to have a highly efficient activation reaction with EDC and Sulfo-NHS, pH of PBS buffer was adjusted to 6.

CONJUGATION PROTOCOL

Materials and Equipment

In order to conjugate the amine on your antibody, protein or peptide to ImmunoFluor™-Dodecanyl (Non-PEGylated) liposomes you will need:

1. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). The solution should be made fresh moments before use.
2. Sulfo-NHS (N-hydroxysulfosuccinimide). The solution should be made fresh moments before use.
3. Sephadex® spin column. Sephadex size exclusion spin column can be used for separation of liposomes form free EDC (MW: 191.70). Since EDC is being separated from large liposome particles then any sizes of Sephadex® spin column such as G-10, G-15, G-25, G50 can be used. However, keep in mind that you will lose a large percentage of your liposomes on the spin column. Alternatively, instead of removing the EDC by spin column you can quench it by using 2-mercaptoethanol.

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4. 2-Mercaptoethanol. To quench the unreacted EDC, 2-mercaptoethanol is added to form a stable complex with the remaining carbodiimide. The 2-mercaptoethanol might not be necessary if you prefer to clean up your liposome from free EDC using a spin column.
5. 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

The two-step protocol includes the activation of carboxyl group-containing liposomes with EDC/sulfo-NHS, and subsequent conjugation with the amine group on the proteins, peptides or antibodies:

1. In order to activate the carboxyl groups on the liposomes, EDC and sulfo-NHS should be added to the liposomes. The total lipid concentration in ImmunoFluor™-Dodecanyl (Non-PEGylated) is 22.45 mM. 1% mol of the lipid in liposomes contains COOH 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 liposome, this is equal to 2.20×10^{-7} mol of COOH. Add 10-fold molar excess of EDC and 25-fold molar excess of sulfo-NHS to ImmunoFluor™-Dodecanyl (Non-PEGylated). To aid in aliquoting the correct amount of these reagents, they may be quickly dissolved in the PBS buffer at a higher concentration, and then a proper volume immediately pipetted into the protein solution to obtain the proper molar quantities.
2. Mix well and allow the reaction to proceed for 15 min at room temperature.

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3. Before adding the protein, peptide or antibody, remove the excess EDC either using a size exclusion spin column, such as Sephadex® spin column or through quenching by 2-mercaptoethanol at a 20 mM final concentration. Addition of 2-mercaptoethanol will not impact the liposomes.
4. Dissolve the protein, peptide or antibody at 1-10 mg/ml, depending on the antibody, protein or peptide, in PBS or other amine-free, carboxylate free buffer, pH 7-8.
5. Add the protein, peptide or antibody to the EDC/Sulfo-NHS activated ImmunoFluor™-Dodecanyl (Non-PEGylated) liposomes. The molar ratio of the reactive carboxyl lipid to protein, peptide or antibody is preferred to be around 10:1. The total lipid concentration in our liposomes is 22.45 mM. 1% mol of the lipid in liposomes contains COOH 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 liposome, this is equal to 2.20×10^{-7} mol of COOH. You will need to calculate the total mol of your peptide, protein or ligand in your solution and add 1:10 molar ratio of ligand to lipid.
6. Mix well and allow to react for 2 h at room temperature.
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.

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TECHNICAL NOTES

- EDC and sulfo-NHS should be prepared immediately before use and kept at room temperature.
- The activation reaction with EDC and Sulfo-NHS is most efficient at pH 4.5-7.2, and EDC reactions are often performed in at pH 4.7-6.0. For this reason, we have formulated the liposomes in PBS buffer and adjusted the pH to 6.
- Reaction of Sulfo-NHS-activated molecules with primary amines is most efficient at pH 7-8, and Sulfo-NHS-ester reactions are usually performed in phosphate-buffered saline (PBS) at pH 7.2-7.5.
- Tris buffer should never be used in any step of the process since it contains amine.
- 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.

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APPEARANCE

ImmunoFluor™-Dodecanyl formulation is colored, and the color depends on the type of the fluorescent dye that is used. 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

ImmunoFluor™ 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

ImmunoFluor™-Dodecanyl is made on daily basis. The batch that is shipped is manufactured on the same day. It is advised to use the products within 2 months of the manufacturing date.

REFERENCES AND BACKGROUND READING

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