Technical Information

Version 1.1
Revision Date: 03/21/2018

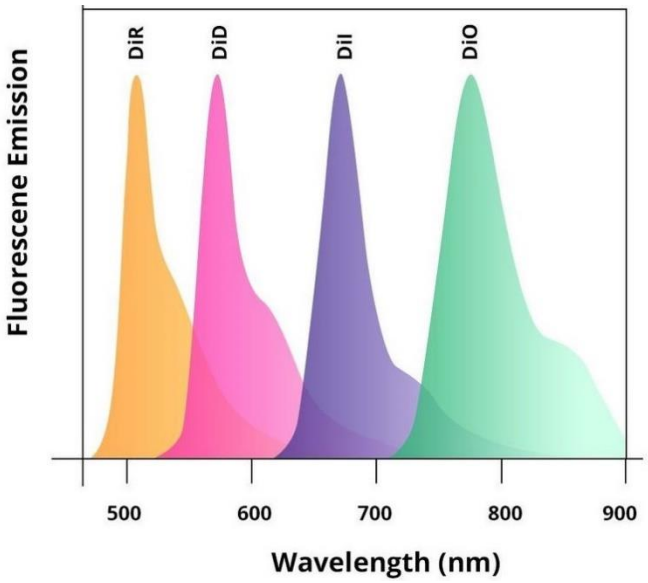
Fluorescent-DiI Macrophage Depletion Kit

DESCRIPTION

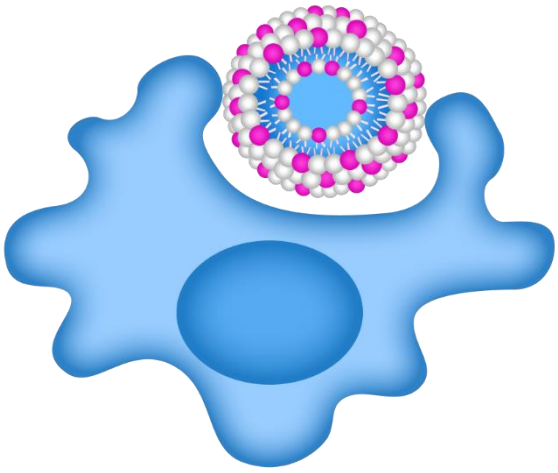
Macrophage depletion kits are composed of two vials; one vial of Clodrosome® (Clodronate liposomes) and one vial of Encapsome® (control liposomes containing no drug). The volume of the macrophage depletion kit represents the volume of each reagent individually. For example, the 5-ml macrophage depletion kit means 5 ml of Clodrosome® and 5 ml of Encapsome®. Each reagent in the kit can also be purchased individually.

Clodrosome® is a multilamellar liposome suspension in which clodronate is encapsulated in the aqueous compartments of the liposomes. Encapsome® is formulated and prepared identically to Clodrosome® except that clodronate is not added to the liposomes. The liposomes are filtered through 2 μm polycarbonate membranes to ensure that the larger particles, which may be toxic to animals, are removed from the suspension. Both are prepared and packaged under sterile conditions. When animals or cells are treated with Clodrosome®, phagocytic cells recognize the liposomes as invading foreign particles and proceed to remove the liposomes from the local tissue or serum via phagocytosis. The liposomes then release clodronate into the cytosol, resulting in cell death. Non-encapsulated clodronate cannot cross the cell membrane to initiate cell death.

Control liposomes (Encapsome®) are recognized and phagocytosed by the same mechanism as Clodrosome®. Since the control liposomes do not contain clodronate, the phagocytic cells are not killed. However, phagocytes do respond to the ingestion of control liposomes by cytokine secretion, temporary suspension of phagocytic activity and other responses described in the literature.



Normalized fluorescence emission spectra of DiD, DiI, DiO and DiR.



Macrophage uptake of fluorescent liposome containing DiI.

FORMULATION INFORMATION

Clodrosome® Liposomal Clodronate Suspension

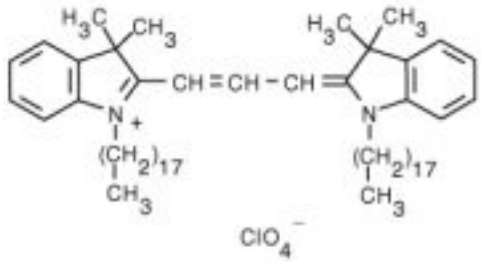
Lipid Composition	Concentration (mg/ml)	Concentration (mM)	Molar Ratio Percentage
L-α-Phosphatidylcholine	18.8	24.3	70
Cholesterol	4.2	10.9	30
Total	**23 mg/ml**	**35.1 mM**	**100**

Encapsulated Drug	Concentration
Clodronate ((Dichloro-phosphono-methyl)phosphonate), Disodium Salt	18.4* mM
* Depending on the type of the clodronate salt, its concentration (mg/ml) varies. If tetra hydrate salt is used, the concentration of the encapsulated drug will be ~7 mg/ml, and if a non-hydrated salt is used, the concentration will be ~5 mg/ml.	

Fluoroliposome®-DiI

Lipid Composition	Concentration (mg/ml)	Concentration (mM)	Molar Ratio Percentage
L-α-Phosphatidylcholine	18.8	24.3	70
Cholesterol	4.2	10.9	30
Total	**23 mg/ml**	**35.1 mM**	**100**

Fluorescent Dye	Excitation/ Emission (nm)	Concentration (mg/ml)	Concentration (mM)
1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI)	549/565	0.0625	0.065



Buffer and Liposome Size	Specification
Buffer	Phosphate Buffered Saline
pH	7.4
Liposome Size	1.5-2 µm

TECHNICAL NOTES

- The issue with fluorescent Clodrosome® has to do with the potential for inaccurate and/or uninterpretable data being generated by labelled Clodrosome®. When Clodrosome® induces macrophage apoptosis, the fluorescent lipid incorporated into the Clodrosome® that is disrupted and metabolized in the phagolysosome will be dispersed among the residual apoptotic bodies which are subsequently phagocytosed by other

macrophages. Therefore, fluorescent lipid may be detected in phagocytic cells which never phagocytosed Clodrosome® especially when FACS or fluoroscopy are utilized to detect fluorescent cells (FACS) or fluorescence levels in a tissue homogenate (fluoroscopy). Another potential artifact arises from fluorescent lipid remaining in the extracellular "garbage", which has not yet been cleared by other phagocytes, generating a high background fluorescence. However, experienced confocal microscopists may be able to differentiate between the punctate fluorescence resulting from fluorescent intact liposomes versus the more diffuse fluorescence characteristic of disrupted liposomes and some have successfully used fluorescent clodronate liposomes to visualize the cellular location of these liposomes by confocal microscopy *in vivo* [1]. A further complicating factor is that published data varies widely as to exactly when clodronate liposomes begin to induce apoptosis in macrophages. Mönkönnnen *et al.* show that macrophage death is measurable within the first hour after clodronate liposome treatment on RAW264 cells *in vitro* [2], while many report no signs of macrophage apoptosis until several hours after treatment *in vivo*. The variability in the data is likely due to different liposomal formulations of clodronate as well as the vastly different experimental conditions. Therefore, as with most biological studies, especially those involving liposomes, the amount of time between treating the animal or cells with clodronate liposomes and the onset of apoptosis will need to be established in each experimental model. If the nature of the research demands that Clodrosome® be tracked rather than the control, Encapsula can provide DiI-labelled Clodrosome® upon request, and assuming that the Clodrosome® distribution can definitively be assessed prior to the onset of apoptosis, clear and valid data on the biodistribution of fluorescent Clodrosome® should be obtainable. Still, for most purposes, Fluoroliposome® (fluorescent control liposomes) will provide the required data with far fewer potential artifacts.

- When monitoring monocyte uptake *in vivo* in normal animals, the circulating monocytes may "disappear" or show reduced counts within the first 2 h post-injection due to margination of the monocytes post-liposome phagocytosis. These cells will re-enter the circulation within a few hours. Sunderkötter *et al.* demonstrate this phenomenon and discuss the behavior in detail. Also consider that circulating monocytes have a lifetime of

about 24 h so labeled monocytes will be continually leaving the circulation, even in normal animals, due to aging of the monocytes [3].

- When animals or cells are treated with Clodrosome®, phagocytic cells recognize the liposomes as invading foreign particles and proceed to remove the liposomes from the local tissue or serum via phagocytosis. The liposomes then release clodronate into the cytosol resulting in cell death. Unencapsulated clodronate cannot cross the cell membrane to initiate cell death.
- Encapsome® control liposomes are recognized and phagocytosed by the same mechanism as Clodrosome®. Since the control liposomes do not contain clodronate, the phagocytic cells are not killed. However, phagocytes do respond to the ingestion of the control liposomes by cytokine secretion, temporary suspension of phagocytic activity and other responses described in the literature.
- The product must be removed from the vial using sterile technique. Do not use if sterility is compromised. This is particularly important if a single vial is accessed multiple times over several weeks. The product should not be used more than 60 days after receipt, even if unopened.
- Liposomes may settle when left undisturbed for more than a few hours. Immediately prior to use, in order to ensure a homogeneous liposome suspension, slowly invert the vial several times until the suspension appears homogeneous by visual inspection. Vigorous or erratic shaking will not damage the liposomes, but may induce foaming and bubble formation making it more difficult to accurately measure the desired dosage.
- If the personnel performing intravenous injections are not experienced in or familiar with, precautions for injecting larger volumes (~10% animal weight in ml), viscous liquids or particulate suspensions, consider having extra animals available in case serious injection-related adverse events occur. Dose control animals first to become familiar with large volume injections.
- Within hours after systemic administration of Clodrosome®, animals begin to lose important components of their immune system. Standard animal handling and housing protocols are not suitable for immunocompromised animals. Even when such precautions

are taken, monitor the general health of each animal for opportunistic infections unrelated to the experimental protocol. There is no inherent toxicity to the product at the recommended dose levels.

- When dosing intravenously, use standard precautions for dosing larger volumes to animals including the following: a) warm product to room temperature prior to dosing; b) ensure that all air bubbles are removed from the syringe prior to dosing. Intravenous injection of air bubbles may result in air emboli which can kill or seriously injure animals; c) inject product at a slow, steady rate of no more than 1 ml/min; d) decrease infusion rate if animals display any atypical reactions such as unusual agitation.
- Infusion-related adverse reactions usually involve the animal gasping for air or other seizure-like movements. Animals often recover with no apparent permanent injury, but any potential effects on experimental results must be assessed by the researcher.
- Liposomes should be kept at 4 °C and **NEVER** be frozen.

APPEARANCE

Clodrosome® is a white milky suspension, and Fluoroliposome®-DiI is a pink liquid suspension, both made of large micro size multilamellar liposomes. Due to their large size, some liposomes might settle to the bottom of the vial. If left sitting idle in the refrigerator, Fluoroliposome®-DiI will phase separate and form pellets in the bottom of the vial, leaving a clear solution on top. Clodrosome® might do the same only not as severely. Therefore, both should be shaken to form a homogeneous solution prior to use.

STORAGE AND SHELF LIFE

Storage

Clodrosome® and Fluoroliposome® 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, clodronate can be released from the liposomes thus limiting its effectiveness in depleting macrophages. ENS is not responsible for results generated by frozen product.

Shelf Life

Clodrosome® and Fluoroliposome® are made on daily basis. The batch that is shipped is manufactured on the same day. It is advised to use the products within 60 days of the manufacturing date.

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