OptiPrep™ Application Sheet M10

Separation of proteoliposomes from proteins and liposome-encapsulated macromolecules from liposomes

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- **To access other Application Sheets** referred to in the text: return to the **2020Macroapp** file and select the appropriate M-number.
- The **OptiPrep™ Reference List "Proteoliposomes" (RM02)** provides a comprehensive bibliography of all the published papers reporting the use of OptiPrep™: to access **RM02** return to the initial list of Folders and select "**Reference Lists**"
- Most of this Application Sheet describes the purification of proteoliposomes; **Section 6** deals briefly with the new technology of encapsulating nucleic acids within liposomes

1. Background

After protein has been incorporated into some form of liposome, it is necessary to resolve the proteoliposomes from any unincorporated protein. The most widely used strategy is described in Figure 1: the sample is adjusted to a density of 1.17-1.22 g/ml by mixing with a high-density stock solution and layered beneath two lower density solutions (the top layer is sometimes the isolation buffer). During centrifugation the proteoliposomes float upwards to band at the top interface. The big advantage of this strategy is that the unincorporated protein remains in the sample zone and will even tend to sediment in the opposite direction (Figure 1). If the sample is on top of a density barrier the proteoliposomes and the free proteins sediment in the same direction; separation of the two types of particle may be less clear; such separations also require longer centrifugation times (see Section 5b).

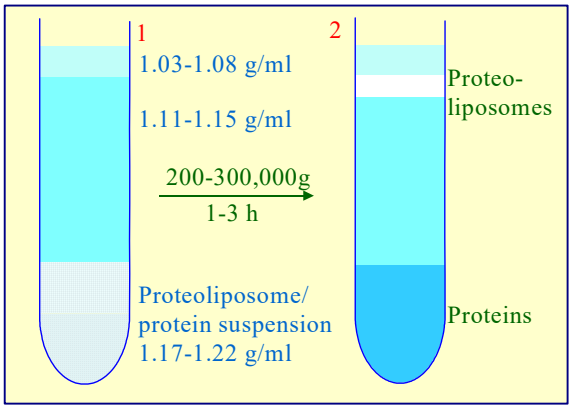

Figure 1: Diagrammatic representation of proteoliposome flotation strategy, before (1) and after (2) centrifugation

2. Density gradient format

Nycodenz® was first used by Weber et al [1] and it was considered by Scott et al [2] to be "by far the best gradient medium" for separation of proteoliposomes from the unbound protein. Small scale separations comprising 300 µl of the sample in 40% (w/v) Nycodenz®, 250 µl of 30% Nycodenz® and 50 µl of buffer were run in 0.8 ml tubes for a Beckman SW55Ti rotor, centrifuged at approx. 280,000 *g* for 4 h. A similar format (shown in Figure 1) has been used by many workers (e.g. refs 3-6). Volumes can be increased more or less proportionately and may be as much as 2 ml, 2ml and 1 ml respectively for the SW55Ti rotor [7]. More recently the use of OptiPrep™ was introduced and it is used in a similar manner [8]. Use of OptiPrep™ is much easier and is the recommended option.

3. Solution preparation

3a. Buffer composition

In the published methods there is no consistent use a particular buffer for the gradient and it can vary from a simple buffered salt solution [8,9] or buffered sucrose solution containing 1 mM EDTA [10] to solutions containing 100 mM KCl, 10% glycerol, 1 mM DTT, 25 mM HEPES-KOH, pH 7.4 [11] or 140 mM potassium gluconate, 4 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.3 [12]. The operator should use whatever medium is most suitable for the stability of the proteoliposomes, which may vary with the type of lipids used for the liposomes or the protein to be incorporated; for convenience simple buffered salt and sucrose solutions are given in this Application Sheet.

3b. Solutions required (see box on next page)
Choose either Solution D or Solution E

A. OptiPrep™ (shake the bottle gently before use)
B. OptiPrep™ diluent: 200 mM HEPES-NaOH, pH 7.4 (include 10 mM EDTA if Solution E is chosen)
C. Working solution (54% w/v iodixanol): Mix 9 vol. of OptiPrep™ with 1 vol. of Solution B
D. Liposome buffer 1: 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.4
E. Liposome buffer 2: 0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4

Keep the following stock solutions at 4°C;
1 M HEPES (free acid) 23.8 g per 100 ml water
100 mM EDTA (Na_2•$2H_2O$) 3.72 g per 100 ml water

Solution B: Dilute 20 ml of HEPES stock solution (± 10 ml of EDTA stock solution) to 80 ml with water; adjust to pH 7.4 with NaOH and make up to 100 ml

Solution D: Dissolve 0.584 g NaCl in 50 ml water; add 2 ml of HEPES stock solution; adjust to pH 7.4 with NaOH and make up to 100 ml

Solution E: Dissolve 8.55 g sucrose in 50 ml water; add 2 ml and 1 ml respectively of HEPES and EDTA stock solutions; adjust to pH 7.4 with NaOH and make up to 100 ml

Note. The preparation of the 54% (w/v) iodixanol working solutions permits the concentration of buffer, and that of the EDTA (if Solution E is chosen), to be constant in the gradient solutions and in the sample. If other reagents are considered important for the stability of the proteoliposomes, such as 1 mM DTT [11] or 4 mM $MgCl_2$ [12], then these too may be included at 10x the required concentration in Solution B. This solution strategy avoids the 50% reduction in the concentration of these reagents if the proteoliposome suspension were simply diluted with an equal volume of neat OptiPrep™. It is not normal practice to include the osmotic balancer, NaCl, KCl, sucrose or glycerol in the Working Solution. For more information on preparing gradient solutions see **Application Sheet M01**.

4. Ultracentrifuge rotor requirements

Swinging bucket rotors with or 0.8-5 ml tubes (e.g. Beckman SW55Ti) are commonly used. Small-volume Beckman fixed-angle rotors have also been used with both Nycodenz® and iodixanol gradients: MLA130 [13], TLA100.2 [14] and TLA100.4 [9,12].

5. Gradient centrifugation

5a. Flotation in iodixanol gradients (adapted from ref 8, for variations and comments see Section 5b)
Carry out all operations at 4°C.

1. Take 2.4 vol. of the proteoliposome suspension and mix well with 3 vol. of Solution C to adjust its density to approx 1.16 g/ml.

2. Make up two solutions of 25% and 5% (w/v) iodixanol by diluting Solution C with Solution D OR E using volume ratios of 2.5:2.9 and 0.5:4.9 respectively.

3. In tubes for the chosen rotor layer the proteoliposome suspension, 25% iodixanol and 5% iodixanol solutions in using a volume ratio of approx 1.0:2.5:0.1 respectively.

4. Centrifuge at 200,000 *g* for 3 h. If using a fixed-angle rotor use a slow acceleration program.

5. Allow the rotor to decelerate from 2000 rpm without the brake or use a slow deceleration program if this is available on the ultracentrifuge and harvest the banded proteoliposomes from the 25%/5% iodixanol interface. The gradient may alternatively be unloaded in a series of fractions. For more information see **Application Sheet M04**.

5b. Other gradient options

In the protocol given in Section 5a the top layer of buffer, commonly used in Nycodenz® gradients, is replaced with the 5% iodixanol layer. In bottom-loaded gradients the iodixanol concentration in the proteoliposome preparation is normally either 30% (w/v) or 40% (w/v) although occasionally a lower concentration (24%) is used [15]. Variations in the gradient format are: (a) 20% and 0% [11], 24%, 18% and 3% [16]; (b) omission of the topmost layer, e.g. sample in 30% iodixanol overlaid with 18% iodixanol [9,12] and (c) sample is adjusted to approx 33% (w/v) iodixanol [17], or

30% iodixanol [18], by mixing with OptiPrep™ and overlaid with buffer. The relative volumes of sample and density layers are probably not very critical but the layer(s) above the sample should be sufficiently large enough to allow the proteoliposomes to be well separated from the unincorporated proteins.

Increased resolving power has also been reported for top-loaded 5%, 15%, 30%, 40% iodixanol gradients run in larger approx. 14 ml tube rotors run for 18 h at 150,000 *g* [10]. Separations have also been carried out in fixed-angle rotors at 100,000 *g* for 5 h [15]; while if the small-volume TLA100.4 is used only 1 h is required at 500,000 *g* [9,12].

6. Nucleic acid-containing liposomes

Modern strategies for the targeting of DNA nanostructures in biomedical applications have become a very active area of research. Encapsulating nucleic acids in lipid bilayer-containing structures such as exosomes or artificial polyethylene-glycolated liposomes (PEG-liposomes). Perrault, S.D. and Shih [19] used PEG-liposomes, which mimic the morphology of enveloped virus particles, provide protection against nuclease digestion and reduce immune activation. A discontinuous gradient of 28, 18, and 8% (w/v) iodixanol, layered over the crude sample in 35% iodixanol, was centrifuged for 16 h at 4°C. The gradient permitted the total removal of excess PEG-liposomes from the preparation, which floated to the top of the gradient.

7. References

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