OptiPrep™ Mini-Review MS19

Preparation of synaptosomes, synaptoneurosomes, neuromelanin granules and synaptic vesicles – a methodological survey

- This short Mini-Review summarizes the presently available published papers that describe the use of iodixanol gradients for the purification of a small range of membranous particles that are unique to neural tissues.

1. Synaptosomes

Kiebler et al [1] was the first group to describe the use of a discontinuous iodixanol gradient in the isolation of dendritic spines from mouse hippocampus. The gradient comprised four layers of 9%, 12.5%, 15% and 25% (w/v) iodixanol (equivalent to densities of 1.076, 1.095, 1.105 and 1.152 g/ml); the densest solution contained the material pelleted from the hippocampal homogenate at 900 *g* for 10 min. The gradient was centrifuged at 18,000 *g* for 20 min and it was described as a velocity flotation separation. The primary aim of the gradient was to isolate dendritic spines and the material that banded at the 9%/12.5% interface was further fractionated in a secondary Percoll-sucrose gradient.

The basic methodology has subsequently been adapted to the isolation of synaptosomes. Bagni et al [2] were the first group to report this approach and they reversed the order of the Percoll and the iodixanol flotation gradients. Material at the 15-23% Percoll interface has generally been used for subsequent purification in the iodixanol gradient, which is generally modified to include a denser layer of 35% (w/v) iodixanol and the centrifugation conditions changed to 10,000 *g* for 20 min. Synaptosomes band at the 15%/25% iodixanol interface. Essentially the same methodology has been used by other workers [3-10].

Rather fewer papers have reported the use of the original gradient sequence of iodixanol then Percoll [11-14]. Troca-Marín et al [14] underlayered the gradient with the crude fraction in 40% iodixanol to purify **synaptoneurosomes** and this was also used more recently by Heintz et al [15].

Yuan et al [16] used only a 0-30% (w/v) iodixanol gradient in their analysis of hippocampal tissue, which showed that neurofilament proteins banded with synaptosomes. Tenga et al [17] using a similar 2.5-30% (w/v) iodixanol gradient (approx 120,000 *g* for 18 h) fractionated murine pre-frontal cortex material to establish that UBXN2A co-fractionated with an ER/*cis*-Golgi compartment rather than in synaptosomes.

2. Neuromelanin granules

A 1000 *g*/20 min pellet was resuspended in 20% (w/v) iodixanol and layered over discontinuous gradient of 26%, 31%, 36% and 50% iodixanol and centrifuged for 3 h at 81,000 *g* [18,19]. The neuromelanin granules banded at the 26%-31% iodixanol interface.

3. Synaptic vesicles

3a. Discontinuous flotation gradients

Hu et al [20] was the first group to describe the use of iodixanol gradients for the purification of synaptic vesicles prepared from synaptosomes, which had been isolated by differential centrifugation. The synaptosomes were osmotically lysed and the more rapidly sedimenting vesicles removed by centrifugation at 34,000 *g* for 20 min. The light membrane suspension was then mixed with an equal volume of OptiPrep™ (final iodixanol concentration of 30% w/v) and overlaid by 24% iodixanol in 140 mM potassium gluconate, 4 mM $MgCl_2$, 20 mM HEPES, pH 7.3. The SVs were recovered from the top of the 24% iodixanol layer after centrifugation at 500,000 *g* for 1 h in a small volume (5 ml) fixed-angle rotor. This method was also used by Ferraci et al [21]. It was scaled up by Richards et al [22] to a Beckman SW28 swinging-bucket rotor centrifuged at 95,000 *g* for 17 h; the 24% iodixanol

was prepared in HEPES-buffered saline (HBS) and a layer of HBS was included on top. In a final concentration step the SV suspension was mixed with an equal volume of 80% Nycodenz®; overlaid with HBS and centrifuged for 8 h at 200,000 *g* [22]. Importantly Holt et al [23] compared the simple discontinuous iodixanol flotation gradient (as in ref 16) with the earlier sucrose gradients + size-exclusion controlled pore glass bead method and found the two preparation gave very similar data in fusion experiments between synaptic vesicles and proteoliposomes.

Discontinuous flotation methods similar to that introduced by Hu et al [20] have been used to monitor the synaptobrevin content of the synaptic vesicles [24] and the role of synaptotagmin in their exocytosis and endocytosis [25].

- A detailed protocol of the flotation method is provided in ref 26.

3b. Discontinuous sedimentation gradients

Low density membrane vesicles fraction from lysed synaptosomes have also been purified on similar iodixanol gradients top-loaded with the sample rather than bottom loaded [27]. The low-density membrane fraction in 145 mM NaCl, 1 mM EDTA, 25 mM HEPES-KOH, pH 7.4 was layered over 5%, 10%, 15% and 20% iodixanol in the same buffer. After centrifugation at 140,000 *g* for 16 h (Beckman SW28 rotor) the SVs banded at the 5%/10% iodixanol interface.

3c. Continuous sedimentation gradients

Weible et al [28] used a sciatic nerve homogenate as a source of synaptic vesicles and after an initial centrifugation at 1000 *g* for 10 min, cytosolic proteins were removed from the supernatant by a second centrifugation over a cushion of 10% (w/v) sucrose at 100,000 *g* for 1 h. The pellets were resuspended in buffer. Continuous iodixanol gradients were generated by three cycles of freeze-thawing of either 15% or 30% (w/v) iodixanol. So long as the freeze-thawing conditions are well controlled this can be an effective and simple means of creating a continuous gradient (see OptiPrep™ Application Sheet S03 for details). The resuspended membranes were layered over the gradients and centrifuged at 200,000 *g* for 3 h. The gradients were very effective in discriminating between low-density neurotrophin-containing vesicles and denser synaptic vesicles.

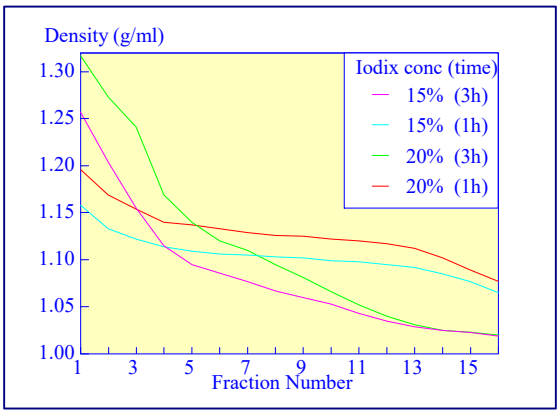
In a study to determine the physical parameters of synaptic vesicles Takamori et al [29] loaded the vesicles on to a 5-35% (w/v) iodixanol gradient; centrifugation at 180,000 *g* for 5 h demonstrated a median density of 1.09 g/ml.

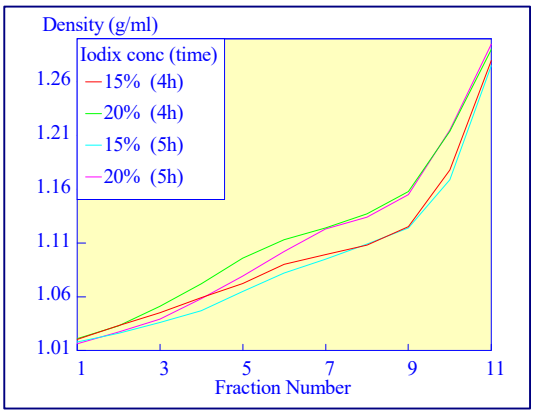
3d Self-generated gradients

The big advantages of the use of any self-generated gradient are the ease of sample handling (the sample is simply adjusted to the required starting concentration of iodixanol) and the great reproducibility of the gradient density profile under a particular set of centrifugation parameters. Iodixanol is able to form useful self-generating gradients in 1-4 h depending on the centrifugation speed and the rotor. The most widely used rotors are vertical or near-vertical rotors with tube sizes of approx. 3, 5 or 13 ml, which are capable of generating forces of at least 265,000 g_{av}. Near-vertical rotors have an advantage over vertical ones inasmuch as any material that pellets is confined to the bottom of the tube in a near-vertical rotor while in a vertical rotor such a pellet is distributed over the entire length of the tube at its outermost point. The shape of the gradient is determined principally by the time of centrifugation and the *g*-force. A couple of examples are given in Figures 1and 2

As a general rule, the gradients after a short period of centrifugation (1 h) are S-shaped and are very shallow in the middle part of the tube; as the centrifugation time is increased the gradients become more linear, although the gradients always become steeper in the high-density region (Figure 1). After 4-5 h (Figure 2) the gradients reach in equilibrium position in which the tendency of the iodixanol molecules to sediment is counterbalanced by their diffusion down the gradient.

A self-generated gradient was first used by Hashiramoto and James [30] in their studies on glucose transport vesicles in adipocytes; they were able to isolate two distinctive populations of GLUT-4 containing vesicles from a low-density membrane vesicle preparation. Ferguson et al [31]





Figures 1 (left) and 2 (right) Figure 1: Effect of cntrifugation time and iodixanol (in buffered saline) concentration; Beckman TLN100 rotor (3.9 ml) at 353,000 *g*; Figure 2: Effect of time and iodixanol (in 0.25 M sucrose) concentration; Beckman VTi65.1 rotor (11.2 ml) at 353,000 *g*

used the same strategy in which a synaptic vesicle fraction was prepared from synaptosomes. The fraction was adjusted to 14% (w/v) iodixanol and centrifuged at 265,000 *g* in a vertical rotor for 4 h and a low-density fraction rich in choline transporter was identified. Similar self-generated iodixanol gradients have also been used in the analysis of vesicles from Canton-S *Drosophila* heads. In the gradient Syt-1 synaptic vesicles were distinctly banded from the remaining synaptotagmins [32]. Although similar information was given by sedimentation velocity gradients, the ease of executing self-generated gradients makes their use very attractive alternative. Darios et al also reported the use of a self-generated gradient [33].

Iodixanol gradients for the purification of synaptic vesicles have also been reported by Herrera et al [34]

- For more information on the formation of self-generated gradients see Application Sheet S04, which can be found on the OptiPrep™ Applications flash-drive or via the following website: www.axis-shield-density-gradient-media.com. For a general description of the methodology using these gradients see Application Sheet S46.

4. References

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