OptiPrep™ Mini-Review MC11

Density gradient media for the purification of cells – a perspective

1. Iodinated density gradient media - a historical perspective

1a. Metrizoate and diatrizoate

In the nineteen-sixties, Nyegaard (later Nycomed) in Oslo began producing X-ray imaging agents that were derivatives of tri-iodobenzoic acid. Metrizoate (produced under the trade name Isopaque™) was one of the early versions of these tri-iodobenzoic acid derivatives (Figure 1). At about the same time, cell biologists were trying to transfer the density gradient technology developed by Christian de Duve, Albert Claude and George Palade for the purification of subcellular organelles, to the fractionation of different cell types from blood and from tissues. Many reagents were investigated. Sucrose, the compound favoured for membrane organelle purifications (and still widely used for certain subcellular particles) is toxic to most mammalian (and non-mammalian) cells; moreover all its solutions above approx. 1.03 g/ml are hyperosmotic. Other polyhydric alcohols such as mannitol and glycerol were much more cell-friendly than sucrose, but the osmolality of their solutions was even higher. High molecular weight polymers (e.g. polysaccharides, plant glycans and proteins) solved the osmolality problem, but made the viscosity problem far worse; furthermore these compounds tend to adhere to the surface of cells. Solution formation of some of these polymers is also not easy.

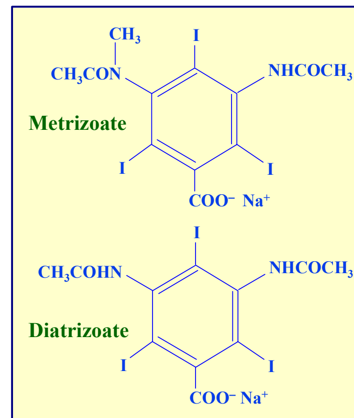

Figure 1: Molecular structure of metrizoate and diatrizoate

Against this unsatisfactory background, Arne Bøyum, working in the Division of Toxicology at the Norwegian Defence Research Establishment in Oslo, recognized that the three iodine atoms in the metrizoate molecule, which made it X-ray opaque, would also make its solutions dense and reduce their osmolality (compared to those of other small molecules). Moreover the use of metrizoate (and its derivatives), as X-ray imaging agents, requires that they undergo exhaustive clinical testing, thus emphasizing their lack of interaction with mammalian cells. Bøyum established their eminent suitability as reagents for fractionating cells and in 1968 [1] he published his seminal paper on the fractionation of leukocytes from human blood. The density barrier that he devised for the isolation of mononuclear cells from human blood comprising a solution of 9.6% (w/v) sodium metrizoate and 5.6% (w/v) polysucrose; density 1.077 g/ml and osmolality 290 mOsm. This was first manufactured commercially in 1973 as Lymphoprep™ by Nyegaard, later by Nycomed Pharma and currently by Alere Technologies in Oslo. The only minor change in the composition of the modern medium is that metrizoate has been replaced by diatrizoate (Figure1) and the modern version contains 9.1% (w/v) sodium diatrizoate and 5.7% (w/v) polysucrose. Diatrizoate is also used in "Polymorphprep™", a unique medium for the isolation of polymorphonuclear leukocytes from whole human blood.

1b. Metrizamide and Nycodenz®

In 1974 the first of the non-ionic X-ray imaging agents (metrizamide) became commercially available as a density gradient medium (Figure 2). The free carboxyl group of the benzoic acid nucleus is linked to the amine group of glucosamine. Its molecular mass is 789 and its high water solubility meant that it was easy to prepare solutions of up to ρ = 1.326 g/ml (60%, w/v) and solutions below ρ = 1.21 g/ml (approx. 40% w/v) could be made isoosmotic. Metrizamide was however found to be slightly toxic to cells and it is no longer commercially available from Alere Technologies.

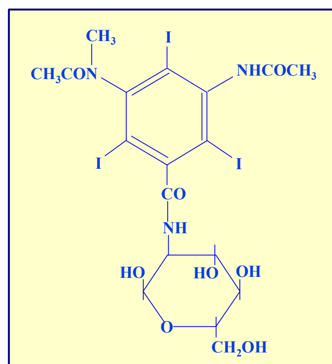

Figure 2: Molecular structure of metrizamide

In the early nineteen-eighties an alternative non-ionic medium, iohexol (commercial name, Nycodenz®) was produced (Figure 3) and both *in vivo* [2,3] and *in vitro* testing [4] demonstrated that the reagent was non-toxic and well-tolerated by mammalian cells; it neither bound to the surface of, nor entered cells. Its molecular mass is 821. A 60% (w/v) solution has a density of approx. 1.32 g/ml and solutions below 1.15 g/ml (approx. 28% w/v) can be made isoosmotic. It was, and still is, widely used in the purification of mammalian and non-mammalian cells. Non-ionic media are the media of

choice since they do not bind to cell surfaces, nor can the influence the Gibbs-Donnan distribution of ions across the surface membrane.

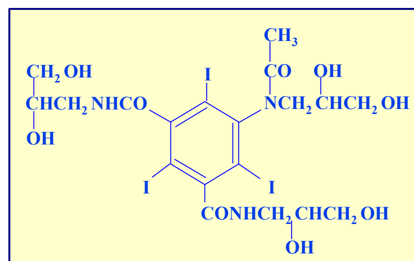

Figure 3: Molecular structure of Nycodenz®

- Bøyum et al [5,6] published important papers, on the use of Nycodenz® gradients for the fractionation of blood leukocytes, which considered, amongst other parameters, how small changes in density and osmolality can influence the separation of lymphocytes, monocytes and polymorphonuclear leukocytes.
- Based on the work of Bøyum et al customized media containing, a buffered solution of Nycodenz®, for the purification of human peripheral blood mononuclear cells (NycoPrep™ 1.077), rodent and rabbit blood mononuclear cells (NycoPrep™ 1.077A) and human peripheral blood monocytes (NycoPrep™ 1.068) were produced commercially. Of these only NycoPrep™ 1.077 remains available from Alere Technologies, however solutions of the same properties as NycoPrep™ 1.077A and NycoPrep™ 1.068 can be easily prepared from OptiPrep™ (see Section 1c).

1c. Iodixanol

- OptiPrep™ is the trademark of a sterile solution of 60% (w/v) iodixanol (ρ = 1.32 g/ml)

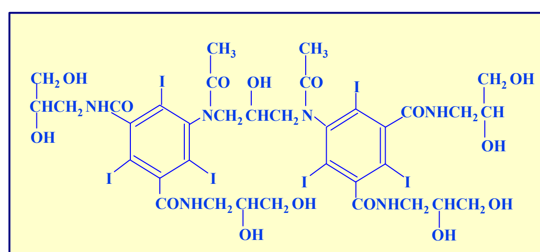

Figure 4: Molecular structure of iodixanol

In the early nineteen-nineties a new X-ray imaging agent, iodixanol, was introduced by Nycomed Pharma. It is more or less a dimer of Nycodenz® and has a molecular mass of 1550 (Figure 4). Iodixanol has been shown to have an extremely low acute toxicity in rodents and the LD_{50} was higher than for any other X-ray contrast medium tested [7]. Upon injection it is rapidly excreted by the kidneys in an unchanged form [8] and in clinical trials iodixanol showed a lower frequency of adverse effects, compared to other media [9,10]. Non-ionic media such as iodixanol induce markedly less abnormalities (cytoplasmic vacuole formation) in cultured renal epithelial cells [11] compared to ionic media. In rat glial cell studies only a transient and small reduction in viability was observed [12]. No teratogenic potential of iodixanol has been observed in rats or rabbits, nor any genotoxicity (four separate standard tests) or antigenic potential in passive cutaneous anaphylactic or active systemic anaphylactic tests in guinea pigs [13].

Studies have shown that iodixanol does not bind to proteins in human plasma [8] and neither iodixanol [14] nor Nycodenz® [15] have significant effects on cell morphology or on cell growth, nor are they metabolized by cells. MOLT-4 T cells have been grown as monolayers in a standard RPMI medium supplemented with 5% (wlv) iodixanol. After 1-72 h the increase in viable cell number, as judged by the MTT Test was identical to control cells grown in the absence of the medium [14]. Confluent monolayers of human embryo lung fibroblasts can be exposed to 30% (wlv) iodixanol in culture medium for up to 3 days without any change in cell viability or subsequent plating efficiency.

- No other density gradient media have been studied so exhaustively in order to establish their compatibility with mammalian cells.
- When OptiPrep™ is diluted with a buffered saline solution, balanced salt solution or routine culture medium all the solutions produced will be isoosmotic. Iodixanol is unique amongst all of the iodinated density gradient media in being able to provide isoosmotic solutions up to a density of 1.32 g/ml.

2. Quality assurance of iodinated density gradient media

All Alere Technologie's density gradient media are produced under FDA and EU cGMP compliance. Density and osmolality specifications are within ± 0.001 g/ml and ± 15 mOsm of the defined values. The endotoxin levels, as defined by the European Pharmacological Standards, are < 1.0 endotoxin units/ml. **Actual measured levels on individual batches are routinely < 0.13 endotoxin units/ml**. Certificates of Analysis describing these three parameters for each batch are available from Alere Technologies.

Endotoxin (also known as lipopolysaccharide) is secreted by gram-negative bacteria and it is particularly important that reagents used for cell isolation contain the lowest possible levels. Endotoxin is known to:

- Interact with CD14 and other receptors
- Cause cytokine, nitric oxide and eicosanoid production in dendritic cells, monocytes and macrophages
- Activate complement and coagulation cascades
- Act as a B cell mitogen

3. Comparisons with the use of colloidal silica

3a. General observations

The application of colloidal silica gradients for the purification of mammalian and non-mammalian cells was introduced in the nineteen-sixties and it was the use of polyvinylpyrrolidone (PVP) to coat the silica particles in order to render them non-toxic that resulted in the production of the density gradient medium Percoll™. Although the commercial medium contains low levels of free PVP, the latter has been shown to have a low acute oral toxicity in humans. Nevertheless Percoll™ was recently withdrawn from use in the purification of motile human sperm and replaced by an alternative material in which the colloidal silica particles are coated in silane. Endotoxin levels are not as well controlled in colloidal silica gradient media as they are in Alere Technologies products; endotoxin concentrations are not quoted for the routine Percoll™, while the levels in Percoll™ Plus are currently given as <2 endotoxin units/ml.

3b. Purification of monocytes

Percoll™ gradient strategies for monocyte purification have often relied on the use of continuous density gradients and while the creation of such gradients is very easy with Percoll™ the procedure does require the use of a high-speed centrifuge. Using a vertical rotor, gradient formation took 1 h at 26,000 *g* [16], while in a fixed-angle rotor only 13,000 *g* was used for 12 min [17]. Thus the routine sterile conical-bottomed 15 ml or 50 ml tubes used for handling cultured cells and blood samples cannot be used. The crude cell fraction is then top-loaded and the gradient recentrifuged at 400 *g*. In an alternative strategy [18] the leukocytes were loaded on to a hyperosmotic density barrier to separate the monocytes and the lymphocytes and a second isoosmotic barrier to remove the platelets. This procedure also required an additional centrifugation step to remove the aspirated liquid recovered from the first barrier - any additional manipulations are liable to lead to a loss of cell function or viability. Only the continuous gradients allowed a monocyte purity of about 90%.

Wakefield et al [19] commented that the colloidal silica particles in Percoll™ could be ingested by monocytes and Coligan et al [20] noted that the presence of these particles could lead to activation of these cells. In the discontinuous flotation iodixanol gradient developed by Graziani-Bowering et al [21], the leukcoyte-rich plasma (LRP) as adjusted to approx. 1.1 g/ml and overlaid with a discontinuous gradient (see Figure 5-1). During the centrifugation the leukocytes rapidly float to the top of the load zone, (see Figure 5-2) from which the monocytes float more rapidly than the lymphocytes while polymorphonuclear leukocytes (see Figure 5-3) remain at the load zone interface. The monocytes form a broad band at the top of the liquid column. Monocyte purity was approx. 94%, while all of the platelets remain in the load zone (Figure 5). Graziani-Bowering et al [21] commented that the iodixanol flotation gradient permitted a more effective resolution of monocytes from lymphocytes.

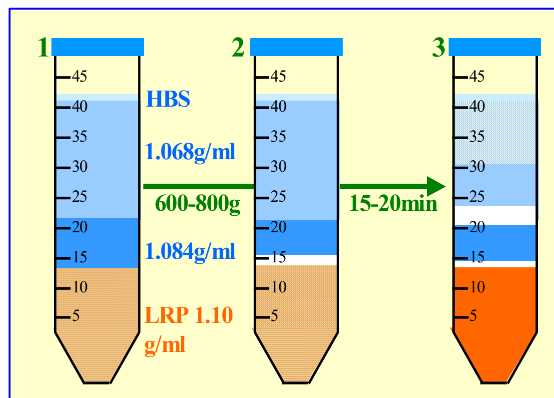

Figure 5: Isolation of human monocytes: LRP = leukocyte-rich plasma; LC =leukocytes; HBS = Hepes-buffered saline; M = monocytes, L= lymphocytes; P= polymorphs. See text for more information.

4. References

1. Boyum, A. (1968) *Isolation of mononuclear cells and granulocytes from human blood: Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1g* Scand. J. Clin. Lab. Invest., 21 (Suppl. 97), 77-89
2. Salvesen, S. (1980) *Acute intravenous toxicity of iohexol in the mouse and in the rat* Acta Radiol., Suppl., **362**, 73-75
3. Mützel, W. and Speck, V. (1980) *Pharmacokinetics and biotransformation of iohexol in the rat and the dog* Acta Radiol., Suppl. **362**, 87-92

4. Ford, T.C. and Rickwood, D. (1982) *Formation of isotonic Nycodenz gradients for cell separation* Anal. Biochem., **124**, 293-298
5. Bøyum, A., Løvhaug, D., Tresland, I. and Nordlie, E.M. (1991) *Separation of leucocytes: improved cell purity by fine adjustments of gradient medium density and osmolality* Scand. J. Immunol., **34**, 697-712
6. Bøyum, A., Brincker Fjerdingstad, H., Martinsen, I., Lea, T. and Løvhaug, D. (2002) *Separation of human lymphocytes from citrated blood by density gradient (NycoPrep) centrifugation: monocyte depletion depending upon activation of membrane potassium channels* Scand. J. Immunol., 56**, 76-84**
7. Nossen, J.Ø., Aakhus, T., Berg, K.J., Jørgensen, N.P. and Andrew, E. (1990) *Experience with iodixanol, a new non-ionic dimeric contrast medium preliminary results from the human phase I study* Invest. Radiol., **25**, S113-S114
8. Jacobsen, P.B., Blindheim, L. and Skotland, T. (1995) *Bioanalytical methods for iodixanol and their application to studies on metabolism and protein binding* Acta Radiol., **36**, **Suppl. 399**, 61-66
9. Jørgensen, N.P., Nossen, J.Ø., Borch, K.W., Kristiansen, A.B., Kristoffersen, D.T., Lundby, B. and Theodorsen, L. (1992) *Safety and tolerability of iodixanol in healthy volunteers with reference to two monomeric X-ray contrast media* Eur. J. Radiol., **15**, 252-257
10. Bolstad, B., Borch, K.W., Grynne, B.H., Lundby, B., Nossen, J.O.E., Kloster, Y.F., Kristoffersen, D.T. and Andrew, E. (1991) *Clinical trials of newer iodinated agents: Safety and tolerability of iodixanol a dimeric, non-ionic contrast medium: An emphasis on European Clinical Phases I and II* Invest. Radiol., **26**, S201-S204
11. Andersen, K-J., Vik, H., Eikesdal, H.P. and Christensen, E.I. (1995) *Effects of contrast media on renal epithelial cells in culture* Acta Radiol., **36**, **Suppl. 399**, 213-218
12. Aardal, N-P., Andersen, K.J., Christensen, E.I. and Vik, H. (1994) *The effects of non-ionic X-ray contrast media on glial cells in vitro* ATLA, **22**, 193-200
13. Heglund, I.F., Michelet, X.A., Blazak, W.F., Furuhama, K. and Holtz, E. (1995) *Preclinical pharmacokinetics and general toxicology of iodixanol* Acta Radiol., **36**, **Suppl. 399**, 69-82
14. Ford, T., Graham, J. and Rickwood, D. (1994) *Iodixanol: A non-ionic iso-osmotic centrifugation medium for the formation of self-generated gradients* Anal. Biochem., **220**, 360-366
15. Ford, T.C. and Rickwood, D. (1982) *Formation of isotonic Nycodenz gradients for cell separation* Anal. Biochem., **124**, 293-298
16. Hardin, J.A. and Downs, J.T. (1981) *Isolation of human monocytes on reorienting gradients of Percoll* J. Immunol. Methods., **40**, 1-6
17. Feige, U., Overwien, B. and Sorg, C. (1982) *Purification of human blood monocytes by hypotonic density gradient centrifugation in Percoll* J. Immunol. Methods, **54**, 309-315
18. Repnika, U., Knezevica, M. and Jeras, M. (2003) Simple and cost-effective isolation of monocytes from buffy coats J. Immunol. Methods, **278**, 283– 292
19. Wakefield, J.St.J., Gale, J.S., Berridge, M.V., Jordan, T.W. and Ford, H.C., 1982. Is Percoll innocuous to cells? Biochem. J., **202**, 795- 797
20. Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W., eds.(1994) In: Current Protocols in Immunology, Vol. 2, Section 7, Immunological Studies in Humans. John Wiley & Sons, USA, pp. 7.6.1–7.6.8
21. Graziani-Bowering, G.M., Graham, J. and Filion, L.G. (1997) *A quick, easy and inexpensive method for the isolation of human peripheral blood monocytes* J. Immunol. Methods, **207**, 157-168

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