

LIPOSOMES AS ULTRASOUND IMAGING CONTRAST AGENTS AND AS ULTRASOUND-SENSITIVE DRUG DELIVERY AGENTS

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Ultrasound imaging contrast agents that exhibit an affinity for specific tissue types, and especially, disease sites, would have considerable clinical value. Liposomal dispersions can be prepared such that they both reflect diagnostic ultrasound [1] and, when conjugated to an appropriate antibody, target themselves to thrombi in the vascular circulation [2].

The procedure for preparation of ultrasound-reflective liposomes, consisting of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cholesterol (CH), requires sonication to hydrate the lipid thoroughly, addition of an excipient, lyophilization, and reconstitution. These steps were examined to generate an optimal preparation [3]. Ultrasound reflectivity was assessed using a 20 MHz intravascular ultrasound catheter and computer-assisted videodensitometry. Ultrasound reflectivity was found to be maximal at a CH concentration of 10 mol %. Variation in PG had little effect, although in the total absence of PG, aggregation was undesirably high. Optimal acoustic stability (resistance to loss of reflectivity upon standing following the reconstitution step) was observed with CH concentrations of 10-15 mol % and with PG concentrations greater than 4 mol %. Preparations made with 0.2 M mannitol present during lyophilization were much more ultrasound reflective than those made with similar concentrations of lactose, trehalose, or sucrose. The preparations were stable when stored in the lyophilized state, but became acoustically inactive a few hours after reconstitution at room temperature. Such preparations could be rejuvenated by lyophilizing a second time. Careful attention to formulation conditions produced preparations that could be diluted to 10-50 :g/ml and still produce strong ultrasound reflection. An indication of the basis of the acoustic activity came from measurements of effects of variations in ambient pressure; echogenicity was greatly reduced by exposure to 0.5 atm vacuum or 1.5 atm pressure for 10 s [4]. (Pressure changes of the magnitude that are present in the arterial circulation had little effect on echogenicity.) Such a response suggested that the active lipid preparations contained small amounts of highly dispersed air. Indeed, application of vacuum resulted in the release of approximately 100 :L of air from a standard preparation of 10 mg lipid in 1 mL of buffer.

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Maximum ultrasound reflectivity was critically dependent upon the conditions of lyophilization. Mannitol (0.1-0.2 M) was required during the lyophilization step and high echogenicity was associated with large-volume freeze-dried cakes *and* fusion of liposomes (10X increase in size). Lyophilization from water caused liposome fusion but the cakes were small and ultrasound reflectivity was weak. Lyophilization from cryoprotectants (e.g., trehalose) produced large cakes but little liposome fusion and also resulted in weak ultrasound reflectivity. These findings indicate that lyophilization from 0.2 M mannitol solution generates a disrupted array of lipid bilayers that, upon rehydration, fuse and trap small amounts of air distributed among liposome-size particles. The peculiar requirement for mannitol rather than a cryoprotective excipient evidently relates to the unusual freezing behavior of mannitol solutions; extensive crystallization occurs upon freezing, evidently generating bilayer defects which subsequently account for both bilayer fusion during freezing and air entrapment during reconstitution. 50% of the echogenicity originated from particles smaller than 1 micrometers.

The phosphatidylcholine component could be replaced by *O*-ethylphosphatidylcholine, a cationic derivative of PC that is an effective DNA transfection agent [5], so it became possible to develop a liposome preparation that could both provide imaging of and gene delivery to a target site. During the development of such lipid dispersions it was found that transfection of DNA into cells *in vitro* was markedly enhanced by irradiation of cells with ultrasound in the presence of acoustically-active lipid preparations. The procedure was to first apply DNA-cationic lipid complexes to rat arterial smooth muscle cells in 6-well plates and then, after 30 min, add a larger portion of cationic, acoustically-active lipids (without DNA) and immediately subject the cells to 1-MHz ultrasound (0.5 W/cm^2) for a few seconds. Transfection efficiency (and DNA uptake) was significantly enhanced (up to 5+ fold) by ultrasound exposure.

Ultrasound in conjunction with ultrasound-reflective lipoplexes appears to have considerable promise for improving gene transfer. Since acoustically-active liposomes conjugated to antibodies target themselves to vascular disease sites *in vivo*, it thus becomes feasible (by using ultrasound) to both identify a disease site and activate a therapeutic agent *in situ*.

REFERENCES

1. Alkan-Onyuksel, H., Demos, S.M., Lanza, G.M., Vonesh, M.J., Klegerman, M.E., Kane, B.J., Kuszak, J. and McPherson, D.D. Development of inherently echogenic liposomes as an ultrasonic contrast agent. **J. Pharm. Sci.** 85 (1996) 486-490.
2. Demos, S.M., Alkan-Onyuksel, H., Kane, B.J., Ramani, K., Nagaraj, A., Greene, R., Klegerman, M. and McPherson, D.D. *In vivo* targeting of acoustically reflective liposomes for intravascular and transvascular ultrasonic enhancement. **J. Am. Coll. Cardiol.** 33 (1999) 867-875.

3. Huang, S.L., Hamilton, A.J., Nagaraj, A., Tiukinhoy, S.D., Klegerman, M.E., McPherson, D.D. and Macdonald, R.C. Improving ultrasound reflectivity and stability of echogenic liposomal dispersions for use as targeted ultrasound contrast agents. **J. Pharm. Sci.** 90 (2001) 1917-1926.
4. Huang, S.-L., Hamilton, A.J., Pozharski, E.V., Nagaraj, A., Klegerman, M.E. and McPherson, D.D. Physical correlates of the ultrasonic reflectivity of lipid dispersions suitable as diagnostic contrast agents. **J. Ultrasound Med.** (2002) in press.
5. MacDonald, R.C., Ashley, G.W., Shida, M.M., Rakhmanova, V.A., Tarahovsky, Y.S., Pantazatos, D.P., Kennedy, M.T., Pozharski, E.V., Baker, K.A., Jones, R.D., Rosenzweig, H.S., Choi, K.L., Qiu, R. and McIntosh, T.J. Physical and biological properties of cationic triesters of phosphatidylcholine. **Biophys. J.** 77 (1999) 2612-2629.