Location of Biological Compartments by High Resolution N.M.R. Spectroscopy and Electron Microscopy using Magnetite-containing Vesicles

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Summary The encapsulation of magnetite within single bilayer phosphatidylcholine vesicles and their use in high resolution n.m.r. spectroscopy and electron microscopy studies of intact biological systems is described.

ELSEWHERE we have described the combined use of electron microscopy (E.M.) and high resolution n.m.r. as a means of determining the distribution in space of molecules in biological compartments.[1] A test for the elucidation of the method is a preparation of phosphatidylcholine vesicles made by sonicating mixtures of the lipid in water. These vesicles have both internal and external compartments generating an excellent model for compartments within biological specimens. Introduction of a probe into one of these compartments makes it possible to map the bilayer headgroups (choline) localised around the probe. The method depends on the ability of the probe to perturb those n.m.r. resonances which lie close to it such that the different parts of space can be distinguished. Identification of the probe in real space can be attained simultaneously through E.M. microprobe *X*-ray analysis provided that probes are chosen containing atoms heavier than sodium.

One procedure we have developed is the *in situ* precipitation of the n.m.r. probe as a chemically fixed substance. Using cobalt(II) ions as the probe material, cobalt sulphide can be precipitated within the vesicles and this compound then provides sufficient contrast for imaging in the electron microscope.[1] An alternative method will be described here in which inert markers are encapsulated within the vesicles during the sonication process. We have now used this method to prepare vesicles containing single magnetite (Fe_3O_4) particles per vesicle. The magnetite is itself an n.m.r. probe.

Magnetite was made by the addition of ammonium hydroxide solution (1 M) to a solution containing equimolar ratios of ferrous and ferric sulphate (0·5 M) until a pH of 10·0 was reached. The magnetite formed as a suspension which was sonicated for 5 min to thoroughly disperse any large aggregates. Samples examined in the electron microscope showed particles 10—20 nm in size.

Phosphatidylcholine (34 mM) was sonicated in distilled water at 4 °C in the presence of such a magnetite dispersion for 5—7 mins. The final vesicle solution was transparent and pale-brown. Removal of magnetite external to the vesicles was accomplished by allowing the suspension to stand in a small flat-bottomed tube for 2 h at 4 °C on top of a bar magnet. The pale-brown supernatant was decanted off from sedimented Fe_3O_4 and left to stand on top of the magnet at 4 °C overnight. No further sedimentation was observed. A ^1H n.m.r. spectrum was recorded of the supernatant and showed extensive broadening of the vesicle spectrum. Experiments in which (i) vesicles with only external magnetite and (ii) sonicated magnetite dispersions, were left to stand on bar magnets showed complete magnetic separation within 2 h. No pale-brown supernatant was observed in either case.

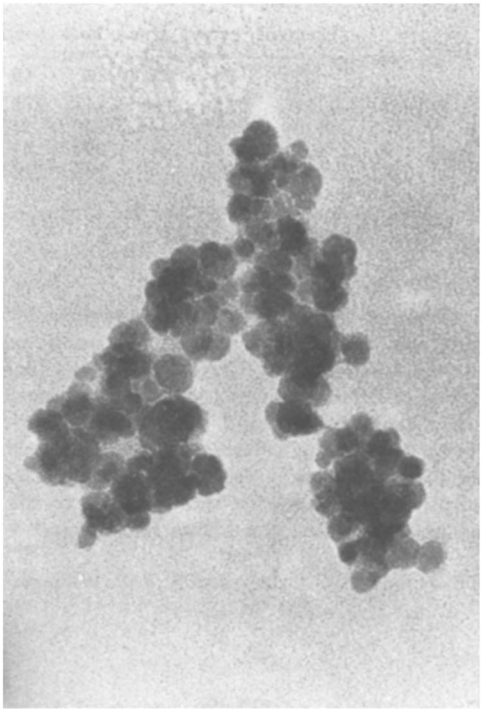

FIGURE. Magnetite-containing vesicles, average diameter 20 nm.

A carbon-coated copper grid was immersed in the supernatant and allowed to dry in the air on filter paper before introduction into the electron microscope. The supernatant was observed to be one of uniform spheres of *ca.* 20 nm (see Figure). E.M. *X*-ray microprobe analysis on areas of the dark spheres, 100 × 100 nm, showed phosphorus and iron counts in the ratio Fe/P of *ca.* 7·5 (Table I).

TABLE 1. *X*-ray microprobe analysis for magnetite vesicles

Sample	Element, counts above background/100 s		
	P	Fe	Fe/P
1	774	6762	8·75
2	1400	10356	7·4
3	510	3652	7·16

Single vesicles could not be analysed. Diffraction patterns of the particles were identified as crystalline magnetite. We need to show that these spheres have complete spherical phospholipid membranes. This was done through the incorporation of other probe solutions within the vesicles.

Two internal probes were used: (a) Vesicles were prepared in the presence of magnetite dispersed in 500 mM $MnCl_2 \cdot 4H_2O$ solution. External magnetite was removed as before. Subsequent cation ion-exchange chromatography removed the Mn^{II} ions from the external phase of the vesicles. (b) Magnetite-vesicles were prepared as before in the presence of 25 mM K_2PtCl_4 solution. Removal of external $PtCl_4^{2-}$ was accomplished by anion ion-exchange chromatography.

TABLE 2. *X*-ray microprobe analysis for magnetite vesicles with internal probes

Sample		Element, counts above background/100 s					
		P	Fe	Mn	K	Cl	Pt
Mn		1181	7395	694	—	1023	—
K_2PtCl_4	1	640[a]	2951	—	160	253	119
	2	492[a]	1745	—	259	370	111
	3	209[a]	781	—	108	63	41

[a] The relatively high P counts to Fe is due to the unavoidable contribution from the shoulder peak of Pt-M_α.

Results from *X*-ray analysis of areas similar to those of the Figure are shown in Table 2. In each case areas analysed showed P, Fe, Mn, Cl (or Pt, Cl, K) which locates the presence of intact vesicles containing magnetite. The contribution from background counts was determined in each case by completing a 'blank' run, *i.e.*, analysing on an area close to the vesicles. *Note:* analysis on the dark areas for a sample where magnetite was external to the vesicles shows no phosphorus counts above background.

We conclude that the encapsulation of magnetite particles of the correct size is possible within intact phosphatidylcholine vesicles of diameter *ca.* 20 nm. The high contrast obtained in the electron microscope provides easy location of the vesicles but it must be stressed that, without the aid of *X*-ray microprobe analysis, complete identification of the vesicular nature of the preparation cannot be established. In this way no staining or fixing methods are needed and hence the possibility of introducing artifacts through external stains is diminished.

One important use of magnetite vesicles could be in their ability to act as magnetic drug carriers. The increase in specificity of a drug through its localisation using external magnetic fields would be of great value in decreasing unwanted systemic distribution of drugs and also in eradicating localised disease. Experiments are in progress to determine the magnetic responsiveness of these magnetite vesicles. We have shown that incorporation of a water soluble molecule, such as K_2PtCl_4 which is obviously closely related to the anti-cancer drug *cis*-$[Pt(NH_3)Cl_2]$, in magnetite vesicles is possible. Also, the marked contrast of such probes in E.M. samples of biological material will lead to information concerning the distribution of drugs within a whole body. Finally the vesicles could be used to carry radio-active contrast elements.

There may also be scope for the use of magnetite probes in the study of n.m.r. of whole organs. The perturbation effects caused by the ferromagnetic iron oxide may well provide a means of mapping biological space; resonances sited near to the probe being more greatly affected than those at some distance.

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[1] A. J. Skarnulis, P. J. Strong, and R. J. P. Williams, *J.C.S. Chem. Comm.*, 1978, 1030.