New Organo-metallic Reagents for Electron Microscopy

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Summary The use of two organo-metallic reagents, located within the lipid bilayer of unilamellar phosphatidylcholine vesicles, as potential electron microscopy stains is described; the possibility of combining paramagnetic electron microscopy stains with proton n.m.r. spectroscopy is also discussed.

THE existing chemical methods for fixing and staining biological membranes use chemically aggressive reagents such as osmium tetroxide and lead(IV) citrate. Alternative milder stains such as Ln^{III}- or Pb^{II}-salts bind strongly to anionic centres, could cause damage to specimens, and are unsuitable for comparative n.m.r. studies. We have therefore looked for more gentle reagents which would dissolve directly into the lipids of biological membranes. Obviously, such stains will have to contain heavy metal atoms in order to be imaged in the electron microscope, and have no reaction with water.

$(Bu^tNH_3)_4[(n\text{-}C_{22}H_{45}PO_3)_2Mo_5O_{15}]$
(**1**)

In this paper we describe two potentially useful stains, $Gd(fod)_3$, where fod is $CF_3(CF_2)_2COCH_2COCHMe_2$, and $(Bu^tNH_3)_4[(n\text{-}C_{22}H_{45}PO_3)_2Mo_5O_{15}]$ (**1**). The first of these compounds is commercially available, whereas compound (**1**) has been prepared in our laboratory by the following method. A solution of 5 mmol of t-butylammonium molybdate[1] and 2 mmol of docosylphosphonic acid, $n\text{-}C_{22}H_{45}PO_3H_2$,[2] in 100 ml of methanol was acidified to a nominal pH of 4·5† with a few ml of glacial acetic acid. The solution was stirred at 60 °C for 30 min, filtered, cooled, and stirred with an excess of ethyl acetate at 5 °C. The solid which separated was filtered off, washed with hexane and ethanol, air dried, and recrystallized from hot methanol (yield *ca.* 55%). The product gave a satisfactory elemental analysis and had an i.r. spectrum in the Mo–O and P–O stretching region (1100—600 cm^{-1}) that is characteristic of the heteropoly-pentamolybdobisphosph(on)ate skeleton.[3]

The gadolinium complex is soluble in organic solvents such as chloroform and lipids. Compound (**1**) is soluble in solvents such as methanol, *NN*-dimethylformamide, and dimethyl sulphoxide, but not significantly in chloroform or benzene. Both compounds are insoluble in water.

† Glass electrode reading.

The structure of (**1**) is a disc-like oxometallate group with the organic residues directed along the axes in opposite directions.[4] Thus we would expect this compound to be also soluble in lipids with the long fatty acid chains aligned with the lipid bilayers.

Test experiments concerning the ability of these compounds to stain biological membranes were carried out by preparing phosphatidylcholine vesicles in the presence of $Gd(fod)_3$ or (**1**) by the following procedure. 1 ml of phospholipid solution (25 mg/ml chloroform) was added separately to a solution of 10 mg of $Gd(fod)_3$ dissolved in chloroform and to a solution of 21 mg of (**1**) dissolved in methanol. The resulting solutions were evaporated to dryness *in vacuo* using a rotary evaporator with a liquid nitrogen trap. 4 ml of distilled water (D_2O for proton n.m.r. experiments) were added to the lipid films and the milky dispersions obtained on shaking were sonicated separately at 4 °C until clear (opalescent). Drops of the vesicle solutions were transferred to carbon-coated copper electron microscope grids and allowed to dry on filter paper in the air. No other stains were added. The electron microscope used was a JEOL JEM 100 CX operating at 100 keV with an ASID attachment and a Link energy dispersive *X*-ray analytical system.

Vesicles prepared in the presence of $Gd(fod)_3$ or reagent (**1**) showed images in the electron microscope corresponding to 'hollow' spheres of diameter 25—30 nm. *X*-Ray microprobe analysis over these areas detected Gd and P, and Mo and P, respectively, (Table) indicating that the contrast observed in the electron microscope was due to the high electron scattering ability of heavy metal atoms located within the phospholipid membrane.

TABLE. *X*-Ray microprobe analysis data for membrane-loaded vesicles.

Reagent	Sample	Counts above background/100 s P-K	Gd-L_α
$Gd(fod)_3$	1	430	156
	2	578	162
	3	236	155
	4	326	226
		P-K	Mo-K_α
(**1**)	1	575	597
	2	436	525
	3	527	486
	4	429	370

The $Gd(fod)_3$-loaded vesicles were also examined by proton n.m.r. spectroscopy. The presence of the paramagnetic Gd^{III} entity within the membrane broadened the vesicle spectrum such that the resonance positions of the terminal-CH_3 and $[CH_2]_n$ chain of the fatty acids could not be resolved. The choline head group was also broadened but could still be resolved indicating that the stain is located within the hydrophobic regions of the membrane and is therefore at some distance from the polar head group.

We conclude that the new organo-metallic stains presented in this paper will penetrate lipid membranes and remain located within these regions of space without affecting the integrity of the lipid bilayer used here.

In the case of stains prepared from paramagnetic species there is the possibility of combining proton n.m.r. techniques with electron microscope imaging of biological material. For instance, $Gd(fod)_3$-loaded vesicles will behave as paramagnetic probes towards proteins binding at the vesicle surface. N.m.r. spectroscopy will then correlate the perturbations of protein regions close to the membrane binding site whilst regions further from this binding site will show unchanged resonance positions. We shall describe experiments on the binding of neurotoxins and of cytochrome c, both basic proteins, to anionic lipid surfaces in future publications.

In this work, as in all probe techniques, there is the danger that the probes will perturb the material under examination. In the work described here, on vesicles, n.m.r. studies indicate that vesicle stability and aggregation are not problems but we expect that protein binding to vesicles will be affected by the molybdate reagents. The effect of $Gd(fod)_3$ is readily followed using $La(fod)_3$.

Structural variations on compound (**1**), both in the organic and polyoxometallate portions, are obviously possible for other staining purposes.

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