

Ferritin as electron dense label

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The purpose of immunohistology is the identification and characterization of cell structure and function *in situ* by suitable procedures with virtually no serious disruption of morphological structure. To this aim, sophisticated “labeling” techniques are needed which allow defined molecules and structures to be observed at the same time. A milestone for investigations at the electron microscopic levels was the conjugation of the metalloprotein ferritin with antibodies by SJ SINGER (1959) which enabled a new era of ultrastructure research. Even today and regardless of several newer developments, the immunoferritin technique is still in use in electron microscopy. This in mind and due to its many merits, the main technical steps are worthwhile to be considered.

Ferritin is the major iron-storage protein in vertebrates, plants, fungi and bacteria. The term ferritin goes back to the early observations of O SCHMIEDEBERG (1894) who was one of the first to report on iron containing proteins in animal tissues. He used the isolated iron containing proteins for experiments on their role in iron metabolism and named the main iron containing protein “Ferratin”. Thereafter, ferritin has come into focus of many scientists for multiple reasons. Because of the large amount of ferritin in horse spleen, this has been the primary source for intensive studies on ferritin molecules. Due to its high iron content (average iron content 23%) and electron scattering density, ferritin molecules can be directly observed in the electron microscope. Despite the low atomic number of iron as compared with that of mercury or uranium, the 2,000 to 3,000 atoms of iron in a ferritin molecule confer a very high electron density.

The molecular mass of horse ferritin varies depending on the organ source. It consists of a spherical protein shell surrounding an inner core of crystalline ferric hydroxide-phosphate. The molecular mass of ferritin is about 460 kDa when isolated from the spleen and 515 kDa when isolated from the heart. Apoferritin consists of 24 subunits (folded into ellipsoids and to form a hollow cage-like structure of 12 nm in diameter) of two types designated H (21 kDa) and L (19 kDa) which are responsible for generating the structural heterogeneity of ferritin from different organs of the same species. Each subunit is an individual molecule that joins to its neighboring subunits through noncovalent interactions. Iron atoms are stored as a cluster of ferric oxyhydroxide within a cavity of diameter 8 nm formed by the protein subunits.

Purification of ferritin molecules from horse spleen

For immunohistological purposes at the electron microscopic level, ferritin from horse spleen is preferred and usually conjugated with antibodies according to procedures described by SJ SINGER (1959) or RA RIFKIND et al. (1960, 1964). Horse spleen ferritin (e.g. six times recrystallized, cadmium-free) can be obtained from commercial sources, however it is advised to further purify those ferritin solutions in order to obtain optimal results in electron

microscopy. Alternatively, ferritin can be self-prepared from fresh horse spleen. This is often the best way because commercial ferritin molecules have sometimes lost its iron which is the very important part of the molecule for electron microscopy.

Ferritin molecules are usually prepared according to S GRANICK (1946) by use of cadmium sulfate which is a modification of that devised by V LAUFBERGER (1937) and R KUHN et al. (1940). It has long been recognized that bivalent cations in general can facilitate protein crystallization. In the particular case of cadmium (Cd^{2+}), its use to promote crystallization of ferritin dates back long before the advent of protein crystallography. The addition of a 10% CdSO_4 solution to horse spleen tissue juice immediately yields cubic ferritin crystals whose growth can be observed in the microscope (already described by L MICHAELIS, 1947).

Fresh horse spleen being obtainable from a slaughterhouse (in some countries and local areas difficult to find) is transported on ice (do not freeze) to the laboratory. One kilogram of spleen is ground in a meat grinder, mixed with 1.5 L of water, and heated rapidly in a water bath to 80°C with stirring. The heavy coagulum of denatured proteins is removed by filtration; a clear brownish solution will result. Ferritin is purified by repeated crystallization and precipitation. In the first step, to each 100 mL of this solution is added 35 g of ammonium sulfate with stirring, and the resulting suspension is left overnight on ice (0°C). The brown precipitate containing the ferritin molecules is centrifuged down (the supernatant liquid is discarded). The precipitate is then dissolved in a small volume of distilled water. To each 100 mL of this solution is added 25 mL of a 20% CdSO_4 solution; crystallization begins within a few minutes. After 1-2 days, the crystals are centrifuged down. Recrystallization is performed by dissolving the pellet in very small volume of 2% ammonium sulfate solution, and for each 100 mL of the clear solution 25 mL of a 20% CdSO_4 solution are added. This cadmium sulfate crystallization procedure is repeated about four to six times until microscopic examinations show only the typical orange-brown ferritin crystals.

After the repeated recrystallizations, purified ferritin crystals are dissolved in 2% ammonium sulfate, and amorphous ferritin is precipitated three times with ammonium sulfate at 50% saturation (to remove cadmium ions); a small amount of water is added to the final precipitate. The suspension is then dialysed against running cold water for at least 24 hours, and, finally overnight at 4°C against 0.05 mol/L phosphate buffer pH 7.5.

Purified ferritin is ultracentrifuged at 100,000 g for two hours. The upper three-quarters of the fluid is discarded; pellet and bottom quarter containing concentrated ferritin are taken which will completely dissolve overnight at 4°C . Finally, concentrated purified ferritin is sterilized by Millipore filtration and stored at 4°C (stable for years); ferritin must not be frozen or lyophilized. Crystallized and purified ferritin molecules can be controlled in the electron microscope. To this aim, a drop of the sample is applied on a Formvar and carbon coated copper grid (400 mesh). After 10 sec the grid is rinsed with distilled water and submitted to the electron microscope in order to observe the iron core of ferritin molecules. Another grid is prepared in the same manner but stained with freshly prepared 2% uranyl acetate in order to observe the shape of ferritin molecules.

Meta-xylylene diisocyanate, toluene 2,4-diisocyanate, difluoro-dinitrodiphenyl-sulfone, glutaraldehyde or other bifunctionale reagents may be used for conjugation of ferritin molecules with purified antibodies in two-step or one-step procedures.

Selected publications for further readings

Schmiedeberg O (1894)
Laufberger V (1937)
Kuhn R *et al.* (1940)
Michaelis L *et al.* (1943)
Granick S (1946)
Michaelis L (1947)
Farrant JL (1954)
Singer SJ (1959)
Rifkind RA *et al.* (1960)
Rifkind RA *et al.* (1964)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>