Recent Developments in Negative Staining for Transmission Electron Microscopy

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INTRODUCTION

Negative staining has been a useful specimen preparation technique for biological and medical electron microscopists for almost 50 years, following its introduction as an established procedure by Robert (Bob) Horne [1]. During this period of time the technique has slowly undergone extensive modification and improvement, now extending well beyond the simple use of aqueous uranyl acetate as a negative stain for samples adsorbed to a relatively thick carbon-plastic film or to a thinner but more fragile carbon support film. Several scientists have been responsible for this progress, including Bob Horne, with the development of the mica-spreading ‘negative staining-carbon film’ procedure (reviewed in [2]), which has been useful for the preparation of twodimensional crystals of viral particles and protein molecules.

Apart from the cationic and slightly acidic 1% or 2% w/v aqueous uranyl acetate solutions of several other anionic heavy metal-containing salts have been routinely used for negative staining (e.g. tungstate, phosphotungstate, silicotungstate, molybdate and vanadate), usually at neutral pH. By varying the stain concentration, different levels of stain density can be achieved. However, it will be found that after drying, the molybdate and vanadate negative stains impart lower mass density around the biological material than uranyl acetate. The anionic negative stains generally have a finer granularity and have less direct (charge-dependent) interaction with the biological material (there are, however, notable exceptions, such as when an anionic stain produces haemocyanin dissociation). Inclusion of the disaccharide trehalose in stain solutions can provide the best negative stain density for samples adsorbed to a relatively thick carbon support film. Several scientific applications have been developed which are very useful and likely to be of increasing importance for future studies: negative staining of asexual and organic solvent polymer solutions and colloidal suspensions; dynamic or time-resolved negative staining; 2D crystallization of viruses and proteins, and formation of higher-order supramolecular assemblies during negative staining; epitope-specific antibody and site-specific affinity labelling revealed by negative staining; and negative staining of nanotechnology and nanobiology specimens.

For technical details and protocols the reader should see the literature and a recent review [27]. The impact and expansion of digital image processing and 3D reconstruction of macromolecular electron optical images at near-to-atomic resolution continues apace, but will not be expanded upon. Some negative staining approaches, such as freeze-fracture negative staining and the negative staining-carbon film technique, will also not be dealt with here.

BIOGRAPHY

Robin Harris obtained his PhD from the University of Edinburgh, Scotland. After holding a lectureship in physiology at the University of St. Andrews and a readership in biology at the North East London Polytechnic, he worked for the UK Blood Transfusion Service, at the German Cancer Research Centre in Heidelberg and at the Max-Planck-Institute for Biochemistry in Munich. Throughout his career, he has been involved with TEM, with emphasis upon the development of negative staining techniques and their application to a wide range of biological, medical and polymer science samples.

KEYWORDS

Transmission electron microscopy, negative staining, cryonegative staining, ammonium molybdate, uranyl acetate, trehalose, holey carbon support film, bacteria, viruses, particles, polymers, nanobiology

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Figure 1:

Haemoglobin from the marine annelid Nereis virens negatively stained with 3% ammonium molybdate with 1% trehalose (pH 7.0) on a carbon support film. Note the presence of intact hexameric molecules (arrowheads) and dissociating molecules, with smaller sub-components on the background. Scale bar = 100 nm.
NEGATIVE STAINING IN THE PRESENCE OF TREHALOSE

With the accumulating knowledge that trehalose is uniquely beneficial for the preservation of biological materials during dehydration, cold-storage, freezing, UV irradiation, etc., it was a natural extension to prepare negatively stained specimens in the presence of this disaccharide for TEM study. The most satisfactory combination of negative stain with trehalose is to use ammonium molybdate [3,4], in this instance with a 1% w/v trehalose and 5% w/v ammonium molybdate, neutralized with NH₄OH or NaOH, with the biological sample adsorbed to a glow-discharged continuous carbon support film. The increased concentration of ammonium molybdate, above the more usual 2% w/v, is required because of the reduction in net mass density due to the presence of 1% trehalose. The immediate benefit to be gained is that the film of dried stain and trehalose is somewhat thicker than is often the case with stain alone, allowing the biological material to be subjected to a reduced flattening force. In addition, ammonium molybdate does have a tendency to release adsorbed particles from the carbon; these can then beneficially adopt varying random orientations within the relatively deep stain-trehalose solution, prior to drying (Figure 1).

Although some electron-beam sensitivity of the stain film may be encountered, conventional electron doses at minimal conventional beam intensity do not rapidly influence the trehalose, unlike the situation with glucose and sucrose that more rapidly bubble in the electron beam. If, however, a low electron-dose system is available on the TEM, it should be used. Uranyl acetate and trehalose mixtures have been found to be workable, but the granularity of this stain and some increased sensitivity of the sample to the electron beam is more apparent that with the ammonium molybdate-trehalose combination. Other negative staining salts, such as the phosphotungstate and silicotungstate can also be used successfully in combination with trehalose.

AIR-DRY NEGATIVE STAINING ON HOLEY CARBON SUPPORTS

Although many researchers may have appreciated the fact that to study biological samples that are suspended in a thin layer of negative stain alone (i.e. without an underlying carbon support) could offer technical advantages, until fairly recently this was not established as a routine procedure. The chance spreading of an aqueous stain and sample film across the corner of a grid square undoubtedly provided the first indications that this approach can succeed, but considerable reproducibility and mechanical stability problems were usually encountered. Significant progress came when samples were spread across glow-discharged holey carbon support films and stained with negative stain and trehalose [5]. Although 5% w/v ammonium molybdate with 1.0% w/v trehalose is acceptable, some instability in the electron beam will be encountered unless low electron doses are used. However, in the pres-

Figure 2a:
Micronemes from Cryptosporidium parvum negatively stained with 5% ammonium molybdate with 1% trehalose after spreading across a holey carbon support film. Note the clustering of the micronemes to the edge of the hole. Intact micronemes appear as electron transparent rods, whereas micronemes with a damaged surface membrane allow stain entry. Scale bar = 100 nm.

Figure 2b:
The metalloendopeptidase meprin-α negatively stained on a holey carbon support film with 5% ammonium molybdate in 0.1% trehalose (pH 7.0). Scale bar = 100 nm.

Figure 3:
(a) Measles virus ribonucleoprotein imaged in 2% w/v ammonium molybdate adsorbed to a continuous carbon support film. (b) Similar sample imaged in ammonium molybdate cryonegative stain suspended across a hole in the carbon support. Note the superior image detail and specimen preservation. Scale bars = 100 nm. (c) 3D reconstruction of Echovirus type 12 bound to a two-domain fragment of its cellular receptor, CD55, calculated at 16 Å resolution from cryonegative stain TEM data. Docking of crystallographic coordinates for component molecules to the EM map produces a quasi-atomic resolution model of the virus-receptor complex.
ence of 5% w/v ammonium molybdate and 0.1% w/v trehalose, the stability is superior; particularly when the holes contain an even, thinly spread film of sample embedded in negative stain. For negative staining across holes, a relatively high sample concentration should be applied to the grid (~1.0 mg ml$^{-1}$) as subsequent washing to remove salts and addition of negative stain reduces the final concentration. The strict maintenance of sample and stain on one side only of the grid is critical for the success of this technique.

A representative example of the successful use of the holey carbon negative staining technique, showing isolated micronemes [6] from the apicomplexan parasite Cryptosporidium parvum, is given in Figure 2a. Some clustering of the micronemes towards the edge of the hole, within a slightly thicker film of stain, is characteristic of this technique, which indicates the freedom of the organelles immediately prior to drying of the stain. A macromolecular example is given in Figure 2b. Here the endopeptidase nephrin-a is shown; the protein particles, some of which form curving chains, are freely spread in the ammonium molybdate-trehalose film.

When samples are spread across holes in the presence of 1% w/v trehalose alone, the thin film of dried trehalose is remarkably stable in the electron beam [5]. This approach, which avoids the use of negative stain, has potential for biological, polymer science and nanotechnology samples where the inherent sample density is greater than that of the surrounding trehalose layer.

CRYONEGATIVE STAINING

Computerised 3D image reconstruction was initially developed as a technique for the analysis of negatively stained biological macromolecules [7,8]. Recently, however, the use of negative stain has largely been superseded by cryoelectron microscopy: imaging of unstained hydrated specimens embedded in vitreous ice [9]. The advent of cryoelectron microscopy combined with developments in TEM technology, such as the field-emission gun (FEG), has permitted microscopists to attain close-to-atomic resolution data. Imaging of unstained vitreous specimens does present significant difficulties however, not least that image data are very low contrast, requiring often significant levels of defocus and consequent contrast transfer function correction, to image smaller macromolecules (300-500 kDa). Vitreous specimens are also highly susceptible to radiation damage and furthermore require specialised and expensive electron microscopes to achieve high resolution. The recent development of cryonegative staining by Adrian et al. [10] abrogates some of these difficulties while retaining the enhanced specimen preservation obtained through imaging material embedded in vitreous ice.

As with negative staining of biological material embedded in the presence of trehalose, cryonegative staining requires a higher concentration of stain to attain adequate contrast, typically a solution of 16-20% w/v ammonium molybdate at neutral pH is used. Prepara-

Figure 4: A liquid crystalline 2D array of the amphiphilic poly(dimethylsiloxane)-b-poly(ethylene oxide) diblock copolymer spread across a hole and negatively stained with 5% ammonium molybdate in 0.1% trehalose (pH 7.0). Scale bar = 100 nm.

Figure 5: Cholesterol microcrystals with attached Vibrio cholerae cytolytin (VCC) oligomers. (a) After 15 min. incubation the pore-like oligomers are attached only at the blayer edges of the planar cholesterol crystals. (b) Following 1h incubation the whole of the surfaces of the microcrystals are coated with oligomers [21]. The samples were negatively stained with 2% ammonium molybdate following adsorption to a carbon support film.
tried, leading to improved alignment of the tilt series, cryonegative stain would seem to have much to offer in this field.

NEGATIVE STAINING OF POLYMERS AND COLLOIDS

Despite the fact that polymer chemists use a variety of physical techniques to assess the size and shape of their synthetic particles, the exploitation of TEM negative staining has been rather slow, even though aqueous polymer solutions behave in an essentially similar manner to biological macromolecules and subcellular organelles. Aqueous suspensions of gas-filled n-butyl-2-cyanoacrylate microcapsules, termed cavisomes, have been studied using negative staining on carbon support films [14]. In this instance the globular surface of the cavisomes was revealed, an interpretation supported by metal shadowing. Further application of negative staining to copolymer particles, in both aqueous and organic solvents [15,16] showed that uranyl acetate can be utilized as dimethylformamide, tetrahydrofuran and dimethylsulfoxide solutions, selected for miscibility with the copolymer solutions. Furthermore, the aqueous polymer solutions and colloidal suspensions can also be usefully imaged by negative staining across holes, with ammonium molybdate and trehalose, as shown in Figure 4, or in trehalose alone [5]. Others are gradually appreciating the potential and simplicity of air-dry negative staining for the study of polymers and it can be predicted that it will be increasingly used for polymer samples, alongside cryonegative staining and unstained cryo-electron microscopy.

DYNAMIC AND TIME-RESOLVED NEGATIVE STAINING

The use of transmission electron microscopy for the study of slow and rapid time-dependent events has always presented considerable possibilities. With negative staining, the drying of the thin layer of stain solution occurs over 1-2 minutes, and this might generally be thought to impose a minimum time period for dynamic studies, but this is not the case under conditions where the direct action of the negative stain or a fixative has the ability to rapidly trap biological material in a defined metabolic state. This latter approach has been particularly successful for the dynamic study of flexible molecules and myosin filaments [17,18]. For any system where the time-dependent changes occur over a period in excess of a few minutes, conventional negative staining on a continuous carbon support film or across holey carbon films can be successfully utilized. With cryonegative staining, sample pretreatment would again generally be somewhat slow, but the possibility to treat a sample immediately before plunge freezing, such as by suddenly changing the pH, adding a metabolite or drug, or exposure to a temperature change, lighting conditions or gaseous environment, could reduce the interaction time to the millisecond range.

There is great interest in the many peptides that spontaneously form fibres in aqueous and physiological solutions, in particular the amyloid-β and tau peptides involved in Alzheimer’s disease. Dynamic negative staining performed over a period of minutes, hours and days provides a system by which peptide oligomerization, prototribil and fibre formation, and fibre aggregation can be assessed. When combined with studies on potentiating compounds and drugs that inhibit fibrillogenesis this negative staining approach can immediately be readily seen to have even further possibilities [19,20].

The time-dependent interaction of bacterial pore-forming toxins with biomembranes and artificial lipid systems can likewise be investigated using negative staining. We found that over a period of a few minutes the cytolysin from Vibrio cholerae formed oligomers attached to the bilayer edges of cholesterol microcrystals, but over a longer period of time (1 h) the planar surfaces of the microcrystals also became coated with oligomers [21], as shown in Figure 5.

2D CRYSTALLIZATION OF VIRUSES AND PROTEINS AND FORMATION OF MACROMOLECULAR ASSEMBLIES

Induction of 2D crystal formation by viruses and protein molecules in the presence of ammonium molybdate and polyethylene glycol (PEG) is the underlying formative principle of the mica-spreading negative staining-carbon film technique [2]. Intermolecular forces at the fluid-air interface and in solution, rather than at the fluid-mica interface are considered to be of importance for the production of ordered arrays [4]. On transferring this approach to samples spread across holey carbon support films, it has been found that, again, viruses and protein molecules have a tendency to produce 2D crystals (Figure 6a) [5]. Furthermore, the time-dependent creation of higher-order macromolecular assemblies in the staining solution spread across the holes of holey carbon support films can also occur (Figure 6b), indicating that the negative staining procedure can actually be utilised to induce experimental changes. Similarly, the cryonegative stain procedure can incorporate the presence of PEG to induce 2D crystallization, prior to specimen freezing [10].

NEGATIVE STAINING AND IMMUNOLABELLING

The combination of negative staining with immunolabelling has been available since the early days of the technique, but in comparison to pre- and postembedding immunogold labelling of thin sectioned biological material, it has received relatively little attention. However, by negative staining, immunogold labelling can even reveal the location of an internalised C. parvum micrornone antigen, but only when the micromone surface membrane is damaged [22]. With the increasing availability of peptide sequence-specific poly-

Figure 6:
(a) 2D arrays of the ring-like decameric peroxiredoxin from Thermus aquaticus (courtesy of Stephen G. Mayhew) spread across a holey carbon support film in the presence of 5% ammonium molybdate and 1% trehalose (pH 7.0).
(b) Dodecahedral supramolecular assemblies formed from the decameric erythrocyte peroxiredoxin-2 in the presence of 5% ammonium molybdate, 0.1% trehalose and 0.2% PEG (M, 1000) (pH 6.5), when spread across a holey carbon support film.

Figure 7:
Decamers, didecamers and multidecamers of keyhole limpet hemocyanin type 2 (KLH2), linked with a monoclonal IgG specific for epitope on the functional unit h, located at the collar edge of the decamers (smaller arrowheads). Decamers (larger arrowheads) are always located at the ends of the antibody-linked molecular chains. Negatively stained with 5% ammonium molybdate in 1% trehalose (pH 7.0) after adsorption to a carbon support film.
clonal and monoclonal IgGs, Fab’ fragments and single-chain variable (scFv) cloned antibody fragments, it is possible to perform immunolabelling at the molecular level, in an attempt to define the location of defined and accessible epitopes on the surface of macromolecules [23]. The macromolecular linkage pattern induced by bivalent IgG can be particularly useful (Figure 7), but a higher level of definition can be achieved (albeit with greater technical difficulty) by defining the location of bound Fab’ [24].

Site-specific affinity labelling is being increasingly used in molecular studies. The biotin-streptavidin system is particularly powerful, because of the high affinity between these two reagents, and the fact that biotinylated proteins and nucleic acids can readily be produced. Histagged proteins can be labelled with his-specific antibodies or with nickel-linked gold probes. Labelling with small gold probes (e.g. 2.5 nm colloidal gold, nanogold and undecagold) can readily be performed in combination with negative staining and cryoelectron microscopy of unstained vitrified specimens. The density of the negative stain needs to be minimized, to avoid masking the small gold probes, thus sodium vanadate or a low concentration of ammonium molybdate should be employed.

Protein-protein interactions often can be studied by negative staining, significantly when one of the proteins possesses a fibrous nature and binds a soluble protein on to its surface. The amyloid-β peptide, composing the fibres within Alzheimer amyloid plaques, binds several different proteins with both salt- and peptide sequence-specific affinity. Of interest is the binding of the antioxidant enzyme catalase to amyloid fibres, as this could be of physiological significance in relation to combating the putative oxidant activity of the amyloid-β peptide. Figure 8 shows the binding of human erythrocyte catalase to fibres formed from the amyloid-β 17-32 fragment. The complimentary peptide sequences on catalase and amyloid-β have been defined biochemically, thus introducing the possibility of probing structurally the interaction site of the two proteins by TEM.

**NANOSTRUCTURES**

Alongside atomic force microscopy, TEM has much to contribute within the disciplines of nanotechnology and nanobiology. Negative staining can often be utilized, in some instances to complement the study of unstained samples. Particulate chemical and materials science samples as well as synthetic and biological polymers and lipids (nanoparticles, nanovesicles, nanotubes and nanofibres) can all be studied by negative staining, as long as aqueous or organic solvent suspensions or solutions are available at an appropriate concentration (i.e. overloading of an EM grid is worse than underloading!) [25]. The pharmaceutical industry, with its wide-ranging emphasis on drug delivery systems and biodegradable nanoparticles could have much to gain from the use of TEM during product development and quality assessment.