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Review

Optimized negative-staining electron microscopy for lipoprotein studies

Lei Zhang, Huimin Tong, Mark Garewal, Gang Ren *

The Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley CA 94720, USA

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ABSTRACT

Background: Negative-staining (NS), a rapid, simple and conventional technique of electron microscopy (EM), has been commonly used to initially study the morphology and structure of proteins for half a century. Certain NS protocols however can cause artifacts, especially for structurally flexible or lipid-related proteins, such as lipoproteins. Lipoproteins were often observed in the form of rouleau as lipoprotein particles appeared to be stacked together by conventional NS protocols. The flexible components of lipoproteins, i.e. lipids and amphipathic apolipoproteins, resulted in the lipoprotein structure being sensitive to the NS sample preparation parameters, such as operational procedures, salt concentrations, and the staining reagents.
Scope of review: The most popular NS protocols that have been used to examine lipoprotein morphology and structure were reviewed.
Major conclusions: The comparisons show that an optimized NS (OpNS) protocol can eliminate the rouleau artifacts of lipoproteins, and that the lipoproteins are similar in size and shape as statistically measured from two EM methods, OpNS and cryo-electron microscopy (cryo-EM). OpNS is a high-throughput, high-contrast and high-resolution (near 1 nm, but rarely better than 1 nm) method which has been used to discover the mechanics of a small protein, 53 kDa cholesterol ester transfer protein (CETP), and the structure of an individual particle of a single protein by individual-particle electron tomography (IPET), i.e. a 14 Å-resolution IgG antibody three-dimensional map.
General significance: It is suggested that OpNS can be used as a general protocol to study the structure of proteins, especially highly dynamic proteins with equilibrium-fluctuating structures.

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1. Introduction

Most transmission electron microscopy (TEM) has the capability to display the atomic structure of hard materials [1]. However, when examining the structure of soft (especially biological) materials such as proteins it is challenging to obtain structure at even near one nanometer resolution [2], due to radiation damage, low contrast of images, structural collapse or flattening, and dehydration [3,4].

Cryo-electron microscopy (cryo-EM) is an advanced approach to determining protein structure at an atomic resolution under near physiological conditions [5–7]. The cryo-EM technique involves preparing the sample in vitreous ice by flash freezing the specimen, and then examining the specimen under cryogenic conditions, such as liquid nitrogen or helium temperatures [6–10]. Advantages of cryo-EM include absence of artifacts, and the ability to examine the protein with near-native state images [4]. Despite its advantages cryo-EM has many complications. The primary disadvantages involve low contrast and necessary expensive equipment that is not readily accessible to many laboratories, including specimen cryo-holders and a cryo-plunger that is needed for sample preparation apparatuses with liquid nitrogen. In addition, preparing a cyro-EM sample with perfect ice thickness and imaging under low-dose conditions requires highly intensive specialized training [4,11,12]. Namely knowledge with image processing software to enhance the poor signal-to-noise ratio (SNR) of cryo-EM [3] images which has a steep learning curve and is time consuming. In addition, the one-time usage of the cryo-specimens limits the chance for catching unexpected discoveries, especially when the sample is difficult to purify or is unstable after isolation [13].

Historically, the study of protein structure via negative-staining (NS) was initially developed with viruses by Brenner and Horne a half century ago [14]. The concept of NS began with light microscopy by submerging bacteria into a dense stain to provide darkness around the specimen, thus illuminating the sample utilizing a negative contrast [15]. NS-EM involves coating the specimen with a thin stain layer of cationic or anionic heavy-metal salts. NS-EM can produce high-contrast images with these coated heavy-metal stains [4], since the heavy-metal stains more strongly scatter electrons than do the lighter atoms within the proteins themselves [16–19].

Preparation of the sample for NS-EM can be easily adapted in any laboratory. Heavy metal stains allow for higher electron dose tolerance,

Abbreviations: EM, electron microscopy; NS, negative staining; OpNS, optimized negative staining; cryo-EM, cryo-electron microscopy; IPET, individual-particle electron tomography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; UF, uranyl formate; PTA, phosphotungstic acid; SNR, signal-to-noise ratio; CVD, cardiovascular disease

* Corresponding author. Tel.: +1 510 495 2375.
E-mail address: gren@lbl.gov (G. Ren).

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improved contrast, and also function as a fixative for the specimen. NS-EM also delivers images that contain high SNR, and an intermediate (near a nanometer) resolution [3] for reconstruction of three-dimensional (3D) models [20]. However, certain effects of the heavy-metal stain produce undesirable outcomes due to interactions with the specimen, such as aggregation, molecular dissociation, and artifacts of stacking [4,21,22].

Since the early 1970s, the NS method has been used to examine the structure and morphology of lipoproteins [15,23–30]. Lipoproteins are composed of lipids and apolipoproteins (apo), and are known to be structurally dynamic and flexible in nature [8,29–34]. The structures of lipoproteins are also sensitive to the buffer pH, salt concentration and chemical reagents. An obvious artifact of lipoprotein structure observed for decades is how the lipoprotein particles regularly present stacking rouleau in NS images [35–40]. This is significant in that rouleau formation is absent from serum analysis, native-gel, small-angle scattering, and cryo-EM studies [12,22,29,30,36,39,41–44]. Considering that rouleau formation displayed in NS images could lead to inaccurate interpretations of lipoprotein structure and function, a thorough investigation or comparison of the NS protocols used to examine the lipoprotein structure is necessary.

Here, we have reviewed the literature by focusing on the structure and morphology of lipoprotein-related NS sample preparation parameters. The comparisons were performed from three parameters: operational procedures, salt concentrations, and the staining reagents themselves. These comparisons show that the optimized NS (OpNS) protocol eliminates the artifact of rouleau in lipoproteins and delivers structural features that are most similar in size and shape to that from cryo-EM images. OpNS features make it a suitable protocol to study lipoprotein structure and morphology. Moreover, OpNS displays itself as a reliable and powerful protocol revealing the mechanics and structures of small and asymmetric proteins, such as 53 kDa cholesteryl ester transfer protein (CETP) [45], and an individual 160 kDa IgG antibody (with ~1 nm high-resolution 3D reconstruction) [11].

2. Rouleau artifact in lipoproteins under negative-staining

With certain popular and conventional NS protocols [15,46], lipoprotein particles regularly present stacking into rouleau formation [35–40]. For example, apoE4-contained high-density lipoprotein (HDL) (Fig. 1A), apoA-I-contained discoidal nascent HDL (Fig. 1B), and even apoB-contained human plasma LDL (Fig. 1C) form rouleau by the conventional NS method [29,30]. The rouleau formation seems unrelated to comprising apolipoprotein types or particle concentrations, although it may relate to lipid component type (cationic or ionic). However, rouleau formation is absent in the results from nondenaturing polyacrylamide gradient gel electrophoresis, mass spectrometry [47,48], X-ray crystallography [49,50], small-angle X-ray diffraction data [43], or cryo-EM studies [29,47,51–53]. Thus, rouleau formation is generally believed to be an artifact of the conventional NS-EM protocol [29,30]. Unfortunately, the observation of rouleau formation by conventional NS protocol (because of the challenges in imaging the lipoproteins in their near native-state by cryo-EM) has for decades been an indicator of successful synthesis of HDL particles.

Recently, Ren et al. have faced these same challenges in imaging lipoproteins under near physiological buffer conditions by cryo-EM [8,11,12,29,44,54]. We investigated the NS protocols by using the images obtained from cryo-electron microscopy (cryo-EM) of apoE4 HDL as a standard control for comparison, and reported an optimized negative-staining (OpNS) protocol (Fig. 2) [29]. OpNS eliminates the rouleau artifact of apoE4 HDL, and provides images that are highly similar (difference <5%) in size and shape to that from cryo-EM (Fig. 2A), but with higher contrast [29]. A validation of OpNS protocol has been conducted by examining the elimination of rouleau artifact in various lipoprotein samples [30].

To further illuminate the different NS protocol effects in the structure and conformation of lipoproteins, we cataloged the effects of NS protocols from the three NS parameters, i.e. NS sample preparation procedures, salt concentrations, and staining reagents.

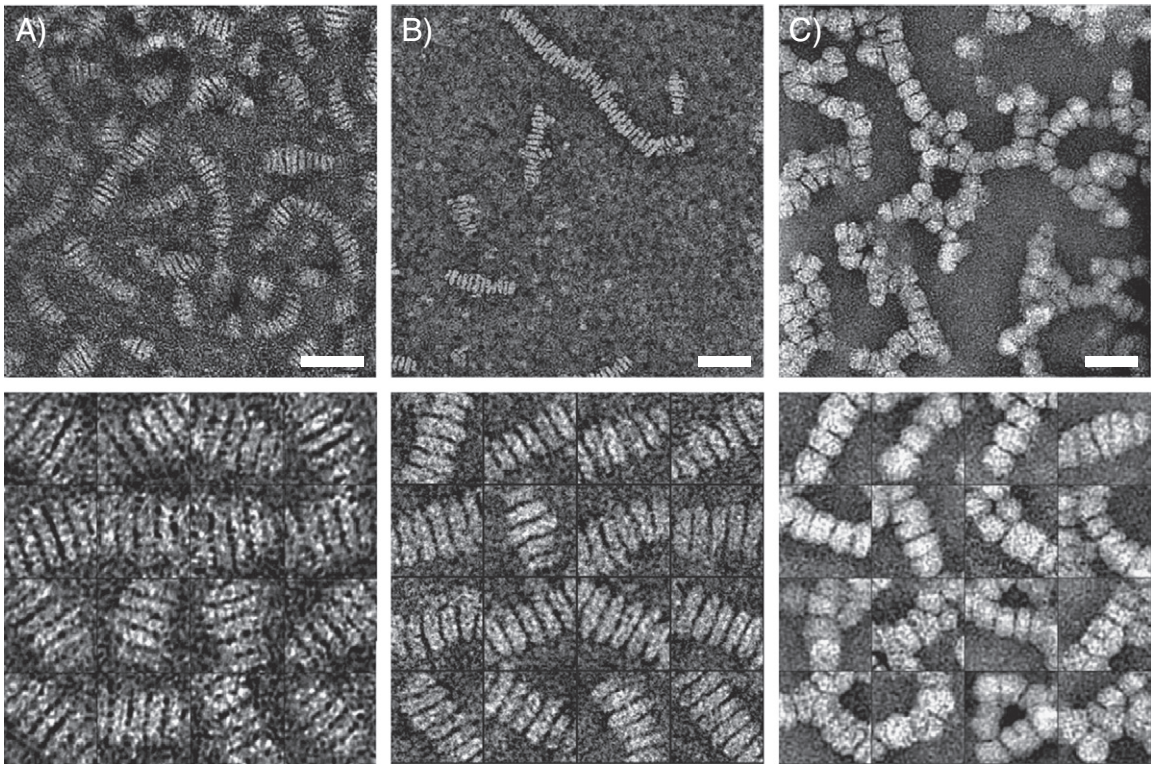

Fig. 1. Electron micrographs presented with rouleau of different lipoproteins by NS with parameters of PTA, and the mix procedure. (A) apoE4-contained rHDL. (B) 9.6-nm nascent rHDL. (C) LDL. Micrographs (up panel) and selected particles (down panel) are shown. Bars: 50 nm. Particle window size: A and B, 30 nm; C, 60 nm. This research was originally published in the Journal of Lipid Research as reference [29,30] @ the American Society for Biochemistry and Molecular Biology.