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Cryo scanning electron microscopy of *Plasmodium falciparum*-infected erythrocytes

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Plasmodium falciparum invades erythrocytes as an essential part of their life cycle. While living inside erythrocytes, the parasite remodels the cell's intracellular organization as well as its outer surface. Late trophozoite-stage parasites and schizonts introduce numerous small protrusions on the erythrocyte surface, called knobs. Current methods for studying these knobs include atomic force microscopy and electron microscopy. Standard electron microscopy methods rely on chemical fixation and dehydration modifying cell size. Here, a novel method is presented using rapid freezing and scanning electron microscopy under cryogenic conditions allowing for high resolution and magnification of erythrocytes. This novel technique can be used for precise estimates of knob density and for studies on cytoadhesion.

Key words: *Plasmodium falciparum*; scanning electron microscopy; knobs; plunge freezing.

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After having resided in a hepatocyte, *Plasmodium falciparum* liver merozoites invade erythrocytes initiating the asexual erythrocytic life cycle. While maturing, the parasite remodels the erythrocyte surface introducing multiple nanoscale surface protrusions termed knobs (1). Detailed studies using scanning electron microscopy (SEM) have shown that knob density, size, and distribution changes during parasite maturation. From trophozoite to schizont stage, the knobs become more numerous and smaller in size (1).

Plasmodium falciparum benefits from producing the knobs. The knobs bear antigens that aid in parasite sequestration (2) and loss of the knobs results in impaired cytoadhesion (3). It should be noted that knobs are not essential for binding as *Plasmodium* species such as *Plasmodium vivax* and *Plasmodium chabaudi* without knobs bind to tissues (4–6).

The knobs are small, having a diameter of approximately 100 nm (1). Thus, high magnification is required to visualize and quantify the structures. Traditionally, this field has mainly been explored by SEM, and also by using transmission electron microscopy, protein-dense protrusions are seen (2, 7, 8). Recently, it was described that a parasite-produced spiral and knob-associated histidine-rich protein make up the knob skeleton (8). Atomic force microscopy (AFM) has also been used to successfully study *falciparum*-induced knobs (9–13). The results obtained with AFM are comparable to those obtained with SEM. Yet, with AFM, the height of the knobs could be determined and more structural details on knob organization were revealed (9, 12).

In studies using conventional SEM and AFM the cells are fixed and also dehydrated in order to perform SEM; processes that change cell size and appearance (10, 11, 14). Cryo-immobilization can also be employed overcoming the need of chemical fixation. The process relies on very high cooling rates that prevent the formation of ice crystals that are deleterious to cellular structure but instead produce vitreous ("glassy") ice. The vitrification leaves the cells in a native and hydrated state during imaging (14). By using high pressure freezing, cryo-immobilization can preserve specimens up to

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200-μm thick (15). Plunge freezing can be used for surfaces and vitrifies samples up to 1 μm in thickness (15).

Here, a novel method based on cryo-immobilization and SEM imaging (cryoSEM) for high-magnification assessment of *P. falciparum*-infected erythrocytes and their interactions with adherent cells is described.

MATERIALS AND METHODS

Parasite culture and synchronization

Plasmodium falciparum (strain FCR3) was cultured essentially as described previously (16). In short, the parasites were grown in culture flasks at 37 °C at 4% hematocrit in HEPES-buffered RPMI-1640 medium (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 5 mg/mL Albumax II (Life Technologies, Thermo Fischer Scientific, Waltham, MA, USA), 0.02 mg/mL hypoxanthine (Sigma-Aldrich, St. Louis, MO, USA), 0.05 mg/mL gentamicin (Gibco, Life Technologies), and 0.18 mg/mL L-glutamine (Sigma-Aldrich) in an atmosphere of 2% O_2, 5.5% CO_2, and 92.5% N_2. Subculture with the addition of blood group O erythrocytes was done throughout the study. Human blood was obtained with verbal, informed consent from healthy volunteers, a procedure that is permitted without ethics approval from the Ethics Committee in the Capital Region of Denmark. Prior to experiments, parasites were synchronized to late stage by gelatin flotation (16). Parasite stage and parasitemia was determined by microscopy of Giemsa-stained thin blood smears. Parasitemia after gelatin flotation was 50–60%.

Chinese hamster ovary (CHO) cells and cultivation

CHO cells stably expressing the human receptor CD36 (CRL-2092, American Type Culture Collection (ATCC), Manassas, VA, USA) and wild type (CCL-61, ATCC) were grown in HEPES-buffered RPMI-1640 (Biological Industries) supplemented with 10% fetal bovine serum (Life Technologies) and 0.05 mg/mL gentamicin (Life Technologies) and in 5% CO_2.

Selection of parasites

Selection for CD36 binding was carried out as previously described by initially using wild-type CHO cells without CD36 for negative selection and then CD36-expressing CHO cells for positive selection (16, 17).

Binding of infected erythrocytes to surfaces and immobilized cells

Prior to performing SEM, infected erythrocytes were immobilized either by using poly-L-lysine (MW 70–140 kDa, Sigma-Aldrich) coating of glass cover slips or by binding to CHO-CD36 cells. In 24-well plates, a poly-L-lysine-coated coverslip was placed in a well and infected erythrocytes ($2*10^6$/well) were added and left to incubate for 30 min. When co-incubated with CHO cells, CHO cells initially were seeded on fibronectin (20 μg/mL, Sigma-Aldrich) and then left to grow until confluency was reached. Non-adherent erythrocytes were removed by washing with phosphate-buffered saline containing 5% fetal bovine serum (Life Technologies).

CryoSEM

After removing excess cells, the cover slip was removed from the well and placed on an OCT-covered (Sakura Finetek, Alphen aan den Rijn, The Netherlands) metal stub. The stub was rapidly plunge-frozen in slushed liquid nitrogen under vacuum (−220 °C) directly in the special transfer container attached to the microscope (Quanta FEG 3d, FEI, Hillsboro, OR, USA). Under vacuum, the temperature was increased to −130 °C. Sublimation was carried out at −95 °C for 120 min. Images were taken during the sublimation process. When sublimation was complete, the samples were platinum (Pt)-coated (~15 nm) and imaged at −95 °C at 2 kV.

This procedure repeatedly resulted in removal of excess ice allowing for imaging of infected erythrocytes.

Image analysis

Erythrocyte diameter was measured by using the open source software FiJi (18, 19). Distinction of infected vs uninfected was based on the presence on knobs. Knob size was measured using FiJi. Measured knob size was 80–100 nm as previously described (1, 12).

RESULTS

CryoSEM imaging results in high-quality images

Sublimation is critical for high-quality cryoSEM images. During freeze plunging, extracellular ice is deposited masking cells of interest (20). Sublimation at −95 °C for 120 min removed extracellular deposits (Fig. 1). However, after sufficient sublimation, imaging of immobilized cells was possible enabling both imaging of cytoadhesion (Fig. 2A) and knobs on infected erythrocytes (Fig. 2B, C). Sublimations at −65 °C was also attempted but resulted in destruction of cells (data not shown). The lack of fixation renders the cells relatively sensitive. Thus, the upper limit of magnification is approximately 24 000×; at that magnification, cells are destroyed at prolonged exposure to electrons (Fig. 2D).

CryoSEM represents cells at their native size

Due to dehydration, conventionally processed cells for SEM result in considerable shrinkage. The diameter of uninfected and infected erythrocytes was measured. The diameter of uninfected cells was 7.7 μm ± 410 nm (mean ± standard deviation) and 6.8 μm ± 502 nm for knob-positive cells.

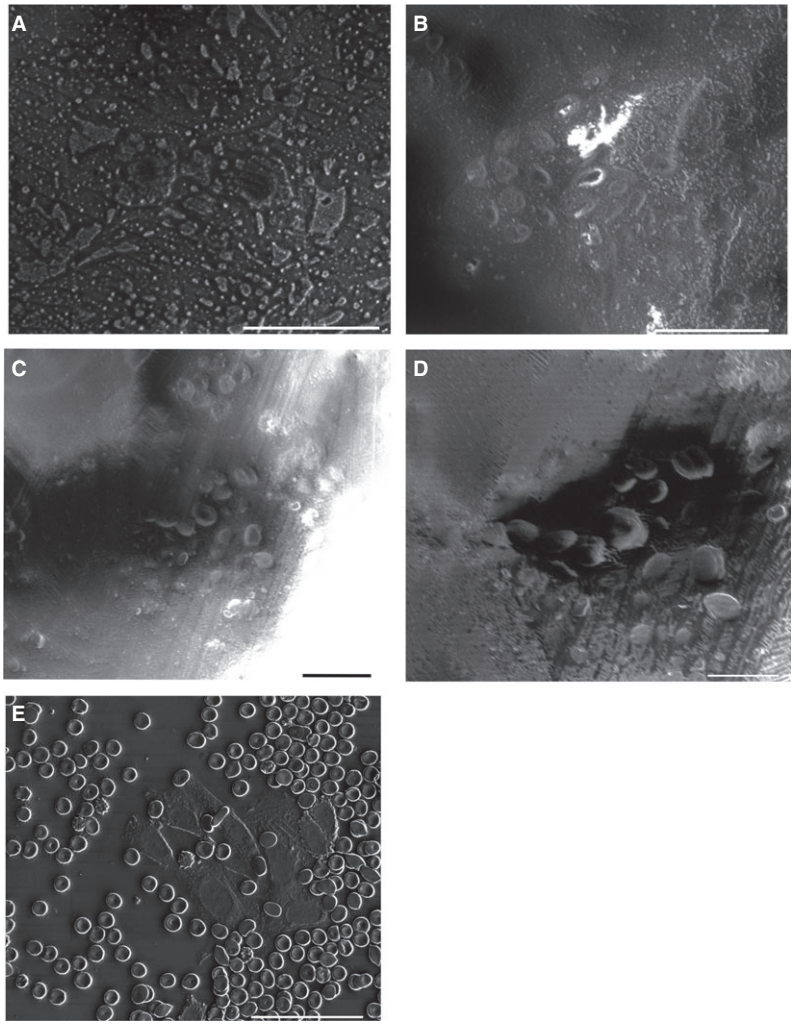

Fig. 1. Sublimation slowly removes extracellular ice. Time series showing removal of ice at time = 0 (A), time = 30 min (B), time = 60 min (C), time = 90 min (D), and time = 120 min (E). Images A–D were taken without Pt coating resulting in charging of surface. E was taken after sufficient sublimation and Pt coating. Scale bars equal 100 μm: B, C; 50 μm: A, D, E.

DISCUSSION

A novel method aiding in characterizing malaria-induced modifications to the erythrocyte surface is presented. Without the use of chemical fixation and dehydration, parasite-induced modifications of the erythrocyte surfaces can be studied in detail. Importantly, by using cryoSEM, cells do not shrink. Previous studies of knob density have relied on either conventional SEM and/or atomic force microscopy. The former has the advantage of being fast in terms of scanning the cells, whereas the latter has the advantage of imaging hydrated cells. Thus, with this method knob density can be determined with high precision without overestimating the density due to erythrocyte shrinkage (11). CryoSEM has recently been described for freeze fractured *P. falciparum* schizonts but this protocol avoids the freeze fracture and further enables studies on cytoadhesion (8).

It is not known how fixation affects the precision of knob density determination. While maturing, the parasite remodels the intracellular spectrin network causing increased membrane rigidity due to increased number of vertical links (21). It could be speculated that dehydration may not shrink the infected erythrocytes to the same extent when comparing knob regions with non-knob regions. When considering the newly published studies on knob density being affected by both cultivation length and expression phenotype, precise determination of knob density may be of greater importance than previously expected (10, 11).

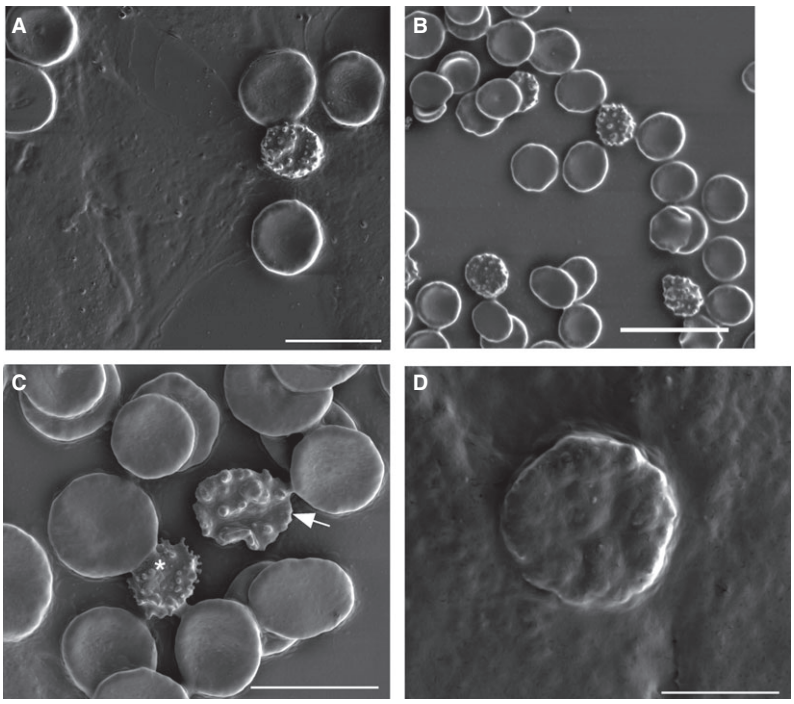

Fig. 2. CryoSEM imaging makes it possible to study surface topology of hydrated, cryo-preserved specimens at high magnification. (A) Immobilized CHO cells with adherent erythrocytes; one with apparent knobs. (B) and (C) Immobilized erythrocytes enabling the study of knob density. Echinocytes are also seen occasionally. In (C), one cell is knob-positive (arrow) and one is crenated (marked with asterisk). (D) High magnification of cellular interaction. At approximately 25 000× magnification beam damage was evident as cracks in cell surface. Scale bars equal: 5 μm: D, 10 μm: A and C, 20 μm: B.

The method described enables detailed analyses of changes in cell surface topology. There is a maximum in terms of magnification as the cells are not fixed and may be harmed by the electrons. As previously described, imaging cells at more than 25 000× magnification induces beam damage (20). However, the combined used of both platinum and carbon coating can be employed to perform imaging at higher magnifications if needed (20).

CryoSEM imaging suggests that erythrocyte diameter is decreased in knob-positive cells. The size is in agreement with a previous publication using AFM (11). Previous reports using volumetric methods have shown contrasting results (22, 23). Small changes in volume may occur yet relative surface area is decreased in schizonts meaning that they will have a round shape compared with uninfected erythrocytes. In terms of changes in diameter that would lead to a small reduction similar to that observed with cryoSEM.

In conclusion, the described method facilitates detailed studies of surface topology of hydrated, malaria-infected erythrocytes including knobs and intercellular interactions.

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