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GOLD COATING OF RESPIRATORY CILIA FOR SCANNING ELECTRON MICROSCOPY

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Abstract

The optimal thickness of gold coating of cilia for scanning electron microscopy was studied using respiratory mucosa obtained from pigs. We tested 8 different coating times, from 10 seconds to 4 minutes, which resulted in gold layer thicknesses varying from 16 $\pm$ 1 nm to 100 $\pm$ 3 nm. The thickness of the gold layer with a coating time of 60 seconds and voltage of 2.5 kV was 43 $\pm$ 5 nm. This thickness of gold layer gave good image quality without causing any electric charging. With thinner gold films, the amount of electric charging increased. When the coating time was longer, the gold layer was thicker but image quality did not improve. The thicknesses of the gold layers were measured using transmission electron microscopy (TEM).

Key Words: Respiratory cilia, gold coating, scanning electron microscopy, ciliary ultrastructure.

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Introduction

Non-conductive specimens examined by scanning electron microscopy (SEM) must be coated with a thin film of conductive material. The coating layer is primarily used to eliminate or reduce the electric charge which builds up rapidly in a non-conducting specimen when it is scanned by a beam of high energy electrons (Jazbi and Sayegh, 1978; Echlin, 1981). Sputter coating is used routinely for SEM studies (Bell *et al.*, 1987). However, very few investigations have been performed regarding the suitable thickness of the gold coating layer for SEM of respiratory mucosa and cilia. From a clinical standpoint, the correlation of ciliary ultrastructure and respiratory diseases, such as immotile cilia syndrome, is of current interest (Afzelius, 1981; Herzon, 1981; Rautiainen *et al.*, 1990).

In the present study, the correlation between the image quality and the thickness of the coating layer was examined using eight different coating times with gold in a diode sputter-coating system. The aim was to determine the optimal thickness of the gold layer which would provide good image quality and contrast without any charging effects.

Materials and Methods

Eighteen specimens of respiratory mucosa from two healthy pigs were used in this study. Specimens were cut from the tracheae, above the bifurcation and randomly divided into 9 different groups (each group containing two specimens). Eight different coating times were used to study the structures and the thicknesses of coating layers: 10 seconds, 30 seconds, 1 minute, 1 minute 30 seconds, 2 minutes, 2 minutes 30 seconds, 3 minutes, and 4 minutes. In addition, two samples, which had no metal coating, were also examined.

To avoid mechanical artefacts, the specimens were taken from the trachea immediately after the animal had been killed by electric shock. The material was obtained from a local slaughterhouse. The specimens were first washed in 0.9% NaCl and then fixed in 1% glutaraldehyde and 4% formalin cocktail fixative with phosphate

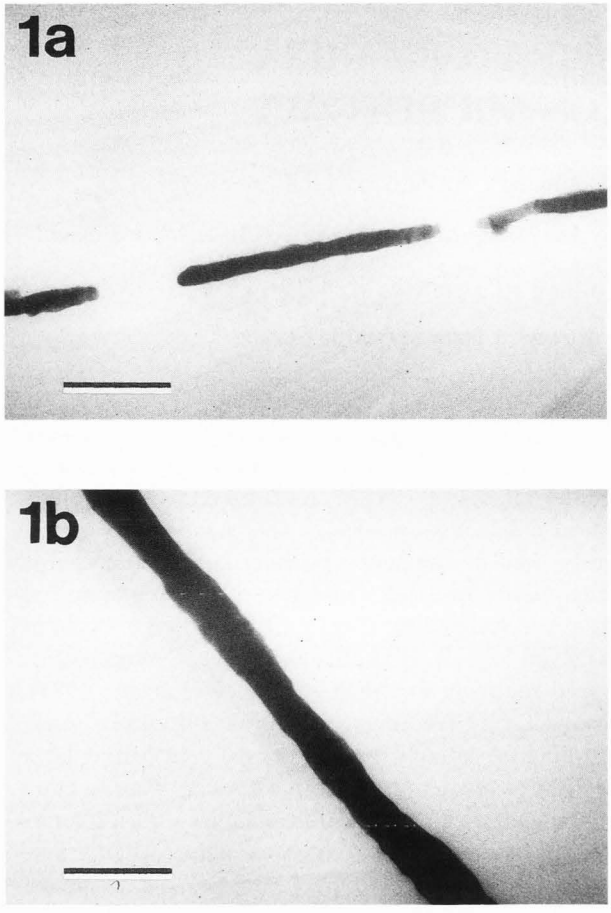

Figure 1. The thicknesses of gold layers and corresponding coating times studied by TEM. (**a**) 16 ± 1 nm, 10 seconds; (**b**) 43 ± 5 nm, 1 minute; and (**c**) 100 ± 3 nm, 4 minutes. Bars = 100 nm.

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buffer (1300 mOsmol/kg, pH 7.0) (McDowell and Trump 1976; Rautiainen *et al.*, 1987). The specimens were then handled according to modified thiocarbohydrazide procedure (OTOTO-method) as described by Malick *et al.* (1975), dehydrated in graded ethanol series (50%, 60%, 70%, 80%, 90%, 94%, absolute ethanol) at 10 minutes for each step. They were then dried with CO_2 in a critical point dryer (Balzers, Liechtenstein). Specimens were mounted by conductive carbon adhesive (Hydro-Collage, Balzers) on aluminum stubs.

Specimens were coated with gold in a sputter coater (Polaron II E51002). The distance of the gold source from the specimen was 40 mm, and the voltage used for coating was 2.5 kV. Coating was carried out in an argon atmosphere with a current of 20 mA; the pressure in the chamber was 5 Pa.

To measure the thickness of these eight different gold layers, small epoxy (Ladd LX 112) sticks were

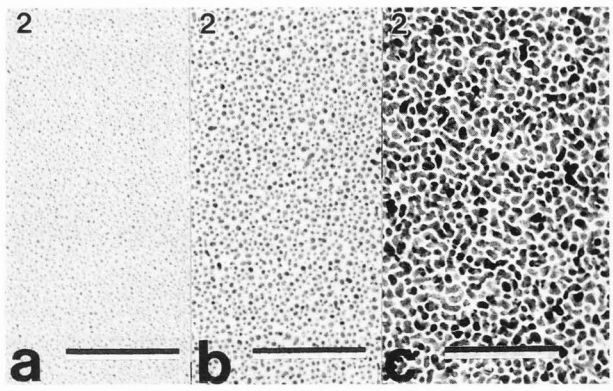

Figure 2. The surface structure and corresponding thicknesses of gold layers obtained with different coating times and studied by TEM. (**a**) Ten second coating time, 16 ± 1 nm thick, the size of gold grains is 1.3 ± 0.2 nm. (**b**) One minute coating time, 43 ± 5 nm thick, the size of gold grains is 2.7 ± 0.5 nm. (**c**) Four minute coating time, 100 ± 3 nm thick, the size of the gold grains is 5.1 ± 0.7 nm. Bars = 100 nm.

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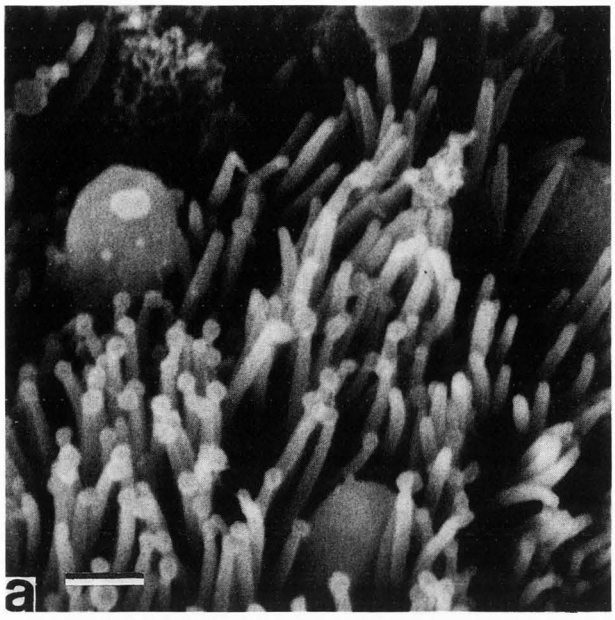
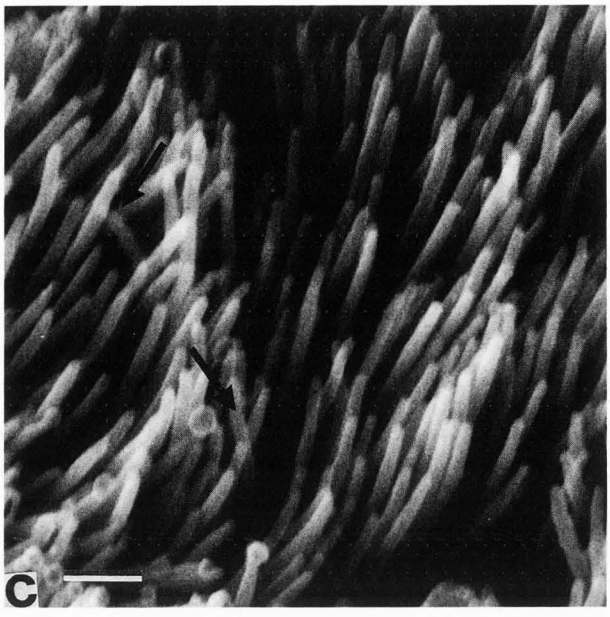
coated at the same time as the specimens. The sticks were then embedded in epoxy resin, and after polymerisation, ultrathin cross-sections were cut. The structures of the gold layers were studied from copper grids covered with formvar film; these were coated at the same time as the specimens. The structures and the thicknesses of the gold layers were studied in a JEOL JEM 1200 EX (Peabody, MA, USA) transmission electron microscope (TEM) operated at an acceleration voltage of 80 kV. Specimens were photographed at primary magnifications of 25000x. The thicknesses of gold layers and the size of the grains were measured from micrographs using a measuring magnifier (Polaron 7x). The results were expressed as mean values of ten measurements.

Specimens of respiratory mucosa were examined in a scanning electron microscope (JEOL JSM-35) operated at an accelerating voltage of 15 kV. From ciliated areas of the mucosa, 10 randomly selected areas were photographed at the primary magnifications of 600x and 6000x. The final magnifications of the micrographs were 800x and 8000x.

Results and Discussion

The thicknesses of the gold layers ranged from 16 ± 1 nm (Fig. 1a; 10 second coating time) to 100 ± 3 nm (4 minute coating time). The structure of the metal coating film in TEM was continuous when the gold layer was 43 ± 5 nm (1 minute coating time) or thicker. The structures of thinner films were no longer homogenous. The metal grains were larger when the gold layer was





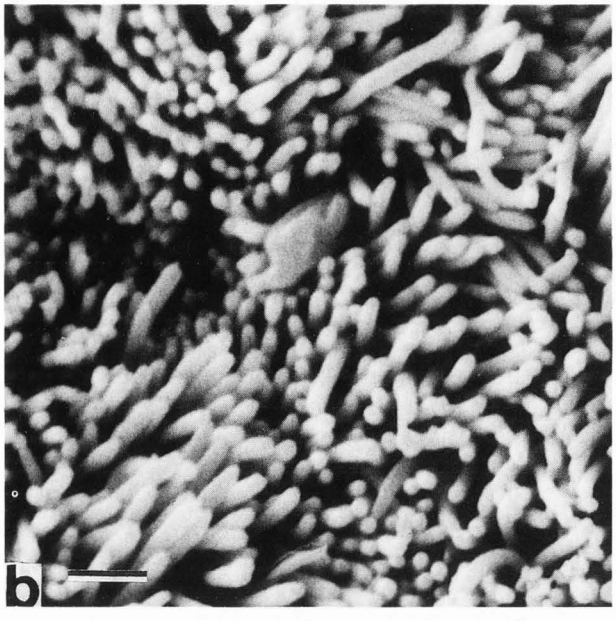


Figure 3. (**a**) Pig respiratory mucosa coated with a 43 ± 5 nm thick gold film. Image quality is good, and there was no electric charging. (**b**) Respiratory mucosa coated with a 100 ± 3 nm thick gold film. Cilia are significantly thicker than with a 43 nm layer. (**c**) Respiratory mucosa without gold coating. Cilia are transparent and the cell membranes are unclear (arrows). Bars = 1 μm.

thicker. With 10 second coating time, the size of the gold grain was 1.3 ± 0.2 nm; with 1 minute coating time, it was 2.7 ± 0.5 nm; and with 4 minute coating time, it was 5.1 ± 0.7 nm (Fig. 2).

The best result was achieved with a 43 nm thick gold film which was obtained with a coating time of 60 seconds (Fig. 1b). This resulted in good image quality without any electric charging (Fig. 3a). Specimens coated with a gold layer thicker than 43 nm had no electric charge. However, the image quality did not improve, and the diameter of cilia increased significantly when the coating time was longer (Fig. 3b). With a 90 nm gold layer, the mean diameter of cilia was measured at 280 ± 10 nm, but when there was only a 43 nm layer, it was 240 ± 10 nm. When there was no coating at all, the mean diameter of cilia was 230 ± 20 nm. The measurements were made from the micrographs about 1 μm from the tip of ten cilia. In percentage terms, the diameter of cilia increased from the uncoated sample value by 4% with a 43 nm gold layer and by 22% with a 100 nm layer (Fig. 3).

The resolution was significantly worse when the coating film was thinner than 43 nm because electric charging made it difficult to see cilia and their fine details, such as cilia tips. The samples, which had no metal coating, also had an electric charge which made electron microscopic examination of their cilia difficult. In these specimens, cilia appear "transparent," and the cell membrane and its outlines are unclear (Fig. 3c).

In recent years, the ultrastructure of cilia has been studied intensively. However, most of these studies are based on TEM observations (Satir, 1961; Rautiainen *et al.*, 1987, 1991). A major advantage of SEM is the possibility of obtaining three-dimensional images of large

cilia populations. In our experience, SEM is very useful in studying ciliary structure and should be used more frequently (Toskala *et al.*, 1994).

The OTOTO-procedure on its own without any metal coating has been used for SEM studies of organ tissues. The advantage of this technique is that it should eliminate charging without having metals which can obscure the specimens (Malick *et al.*, 1975; Murphy, 1978). Nonetheless, in the present study, while the preparation of a specimen by the OTOTO-method alone decreased electric charging, it did not eliminate it completely, and a metal coating was required to obtain a better image quality.

In conclusion, gold coating of specimens is advantageous in SEM of respiratory cilia compared to the OTOTO-method alone. A thickness of 43 nm resulted in a homogenous gold layer, and with the sputter coater used in this study, this was obtained within one minute. This allowed the examination of specimen with a good image resolution and good contrast, but without any electric charging.

Platinum, which has finer grains than pure gold, is used as a coating for high resolution SEM. However, platinum is very expensive, and for conventional SEM, which still is used in many laboratories, gold is the routine coating material when low resolution will suffice.

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Discussion with Reviewers

G.M. Roomans: Did you compare OTOTO-prepared, coated specimens to conventionally prepared (no OTOTO), coated specimens?

Authors: Yes we did, but our experience is that OTOTO improves image quality; however, it alone is not sufficient, and therefore, also needs coating. Specimens without OTOTO preparation also need a thicker gold layer.

M. Lindroth: Are there osmotic artefacts that look like blebbing at the tips of the cilia?

Authors: Blebbings can be caused by osmotic changes, but that is still only a hypothetical explanation because the origin of blebbings is not clear.

M. Lindroth: How can measurements of grain size be of importance? After all, there is a high degree of decoration artefacts due to the properties of gold; i.e., the grain size varies a lot.

Authors: Even though the size of the gold grains varied, clearly, the size was larger at longer coating times. This explains why cilia were thicker in SEM micrographs with longer coating times.