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Supramolecular pellicle precursors

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Saliva contacting with solid surfaces in the oral cavity forms a coat termed the pellicle. However, its formation is not fully understood. Although indications for the existence of supramolecular pellicle precursors have been reported, the possible relationship between them and pellicle formation is unclear. This study investigates the ability of supramolecular precursors to form the pellicle via interaction with a solid surface. Fixed and unfixed salivary globes were spread onto a microscopic grid and examined by transmission electron microscopy. Biochemical pretreatment of saliva revealed that neither disulphide links nor transglutaminase-mediated crosslinking are responsible for maintaining the salivary globes, i.e. supramolecular pellicle precursors. However, the detergent, sodium dodecyl sulphate, caused dissociation of the salivary globes, indicating their micellar nature. Saliva contacting a formvar film for 10 s did not form a complete surface coating, but single supramolecular pellicle precursors were observed attached to the surface. After extension of the contact time to 60 s, a surface layer was formed by clustering and fusion of the supramolecular pellicle precursors. The supramolecular pellicle precursors are unstable and attain a thermodynamically more favourable state by adhesion to a solid surface. As a result, a layer of fused precursors covering the solid surface is formed – the salivary pellicle.

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The acquired salivary pellicle is an organic coating that forms on the enamel surface from salivary components (predominantly proline-rich proteins, PRPs) (1) and provides protection of the enamel against acidic challenges (2). However, the exact mechanisms of the initial stages of pellicle formation have not been completely elucidated. Two mechanisms appear plausible: the adsorption of dissolved salivary components; or attachment of supramolecular pellicle precursors to the enamel surface and their subsequent clustering and fusion. The observation of salivary colloidal globes (globular protein aggregates, micelle-like structures) using cryo-transmission electron microscopy (3) and their attachment to carbon surfaces (4, 5), the almost identical amino acid composition (6, 7) of acidified salivary sediment and pellicle, as well as the globular appearance of the pellicle surface (8–11), all suggest that the salivary globes (micelle-like structures) are very probably supramolecular pellicle precursors.

However, apart from the morphological similarity, no other confirmation exists that the salivary globe-like structures observed in the salivary pellicle, and the salivary globes detected on carbon coats of microscopic grids, are the same structures. There is insufficient empirical evidence of an ability of salivary globes to form the pellicle layer. Thus, the importance of salivary globes in the *in vivo* formation of pellicles has remained uncertain. In addition, no information has been available regarding the arrangement of components inside the supramolecular pellicle precursors. Moreover, the type of bonds responsible for the arrangement and maintenance of the supramolecular pellicle precursors has not been elucidated. Non-covalent forces (aggregation as a consequence of the amphiphilic character of the PRPs) have been hypothesized (6), and crosslinking with disulphide links has been suggested in the salivary cysteine-containing phosphoproteins (12), with cysteine found in 2-h-old enamel pellicle (13–15). Crosslinking of acidic PRPs via transglutaminase has also been suggested in the formation of the salivary-mucosal pellicle (16) and in the pellicle precursor proteins (17, 18).

The aims of the present work were, first, to provide clear evidence that the salivary globes are indeed the supramolecular pellicle precursors which are able to form the pellicle via fusion and, second, to investigate the shape and dimensions of the precursors, as well as the type of the bonds responsible for their maintenance.

Material and methods

Scanning electron microscopy

Saliva was collected from four donors without using stimulation. All saliva donors were male, healthy and non-smokers. The native saliva used in all experiments was not older than 5 min. Two-hundred microlitres of saliva from each donor was passed through a 5-μm filter (Minisart NML; Sartorius, Göttingen, Germany) and a 0.2-μm filter (Cellulosenitrat Membranfilter; Sartorius), followed by 5 ml of saline. The saliva filtrates and the controls (saline filtrates) were then processed for scanning electron microscopy (SEM), by fixing with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at room temperature, then

postfixed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h. The postfixed filters were dehydrated in an ascending series of ethyl alcohols, critical-point-dried in a drying equipment CDP 030 (BAL-TEC, Balzers, Liechtenstein) and subsequently sputtered with gold (*c.* 10 nm). The specimens were examined in an environmental scanning electron microscope, ESEM XL30 (FEI, Philips, Eindhoven, the Netherlands), operating at 20 kV.

Transmission electron microscopy

One millilitre of native saliva was fixed with 1 ml of 2% glutaraldehyde (pH 6.5 with 0.2 M sodium cacodylate) for 5 min at room temperature and then passed through a 5-μm, and subsequently through a 0.2-μm, filter (Minisart NML; Sartorius). Formvar-coated copper grids were placed on a drop of the fixed filtered saliva for 10 s or 1 min, blotted with filter paper and then placed on two drops of bidistilled water and blotted. The grids were negatively stained by use of 1% uranyl acetate in bidistilled water.

Other formvar-coated copper grids were placed on a droplet of native saliva for 10 s or 1 min, blotted and subsequently placed on two drops of bidistilled water, blotted and negatively stained as described above.

Enzymatic cleavage – One drop of native saliva was mixed with one drop of neuraminidase type III from *Vibrio cholerae* (Sigma-Aldrich Chemie, Steinheim, Germany), at a final concentration of 0.0044 U ml^{-1}, buffered to pH 5.0 at 37°C. After 2 h, a formvar-coated grid was placed on the mixed droplet for 1 min, blotted, subsequently placed onto two drops of bidistilled water, blotted and negatively stained. One drop of native saliva was mixed with one drop of trypsin type I from bovine pancreas (Sigma-Aldrich Chemie), at a final concentration of 195 U ml^{-1}, buffered to pH 7.6 at 25°C. After 2 h, negatively stained specimens were prepared as described above.

Analysis for disulphide links, crosslinking of acidic PRPs via transglutaminase, and non-covalent bonds – Formvar-coated grids were placed on droplets of 2-h-old saliva for 1 min, and negatively stained specimens were prepared as described above. These specimens served as a control. In order to block the transglutaminase activity, 0.5 ml of native saliva was supplemented with the same quantity of 2% EDTA, pH 8.0, for 2 h (16–18). Formvar-coated grids were placed on a drop of this saliva for 1 min, and negatively stained specimens were prepared as described above.

Five-hundred microlitres of native saliva was supplemented with the same quantity of 2% *N*-acetyl-L-cysteine, pH 8.0, for 2 h to disrupt the disulphide links. Formvar-coated grids were placed on a drop of this saliva for 1 min, and negatively stained specimens were prepared as described above.

In addition, 0.5 ml of native saliva was supplemented, after 2 h, with the same quantity of 2% sodium dodecyl sulphate (SDS), pH 8.0, for 30 min. Formvar-coated grids were placed on a drop of this saliva for 1 min, and negatively stained specimens were prepared as described above.

Transmission electron microscopy (TEM) was performed with an LEO EM 910 (LEO Elektronenmikroskopie, Oberkochen, Germany) operating at 80 kV.

Results

SEM analysis

Large numbers of desquamated epithelial cells, covered with micrometer-long mucin-like fibres, were observed on the 5-μm Minisart filters (Fig. 1A). Salivary globes, with a diameter of nearly 50 nm, and long fibres similar to those observed on the 5-μm filters, were found on the 0.2-μm filters. The salivary globes adsorbed to the filter surface and, as a result, the filter appeared warty (Fig. 1B). By contrast, the control filter samples (i.e. washed with only saline) appeared smooth (Fig. 1C). In some samples, in addition to the warty filter surface, large numbers of salivary globes were observed in clumps on the filter surface, and some fibres with salivary globes attached were also evident (Fig. 1D).

TEM analysis

The mucin-like fibres from non-filtered fixed saliva attached to the formvar film and stained positively with uranyl acetate (Fig. 2A). As the staining was very intense and the quantity of fibres very high, supramolecular pellicle precursors could not be distinguished. After filtration of the fixed saliva to remove the main mass of fibres, the supramolecular precursors stained with uranyl acetate were clearly visible (Fig. 2B). Their diameter was $\approx$ 30 nm. By contrast, the diameters of supramolecular aggregations that attached during the 10-s grid exposure to native whole saliva, varied from 20–60 nm (Fig. 2C). The fibres from native saliva did not attach to the formvar film. The dimensions of the supramolecular aggregations attached during the 1-min grid exposure to native whole saliva varied even more considerably (Fig. 2D). The supramolecular aggregations completely covered the formvar surface, forming partly overlapping clumps. Clumps and piled formations indicate a granulated surface layer originating from fused supramolecular aggregations.

A formvar-coated grid, exposed for 1 min to a droplet of 2-h-old native saliva, showed few supramolecular aggregates (Fig. 3A). The enzymatic cleavage did not significantly affect the supramolecular aggregations (Fig. 3B,C). The micrographs from the formvar-coated grids exposed to saliva supplemented with 2% EDTA, pH 8.0, or with 2% *N*-acetyl-L-cysteine, were similar to those from the 2-h-old native saliva (data not shown). By contrast, the 30-min treatment with 2% SDS caused complete dissociation of the supramolecular aggregations (Fig. 3D).

Discussion

The observation of salivary supramolecular globes attached to a solid surface (the carbon coat of a microscope grid) (4–7) does not explain their origination. As adsorption of dissolved salivary molecules onto solid surfaces could result initially in such aggregations, it is necessary to preserve the pre-existing structures of saliva.

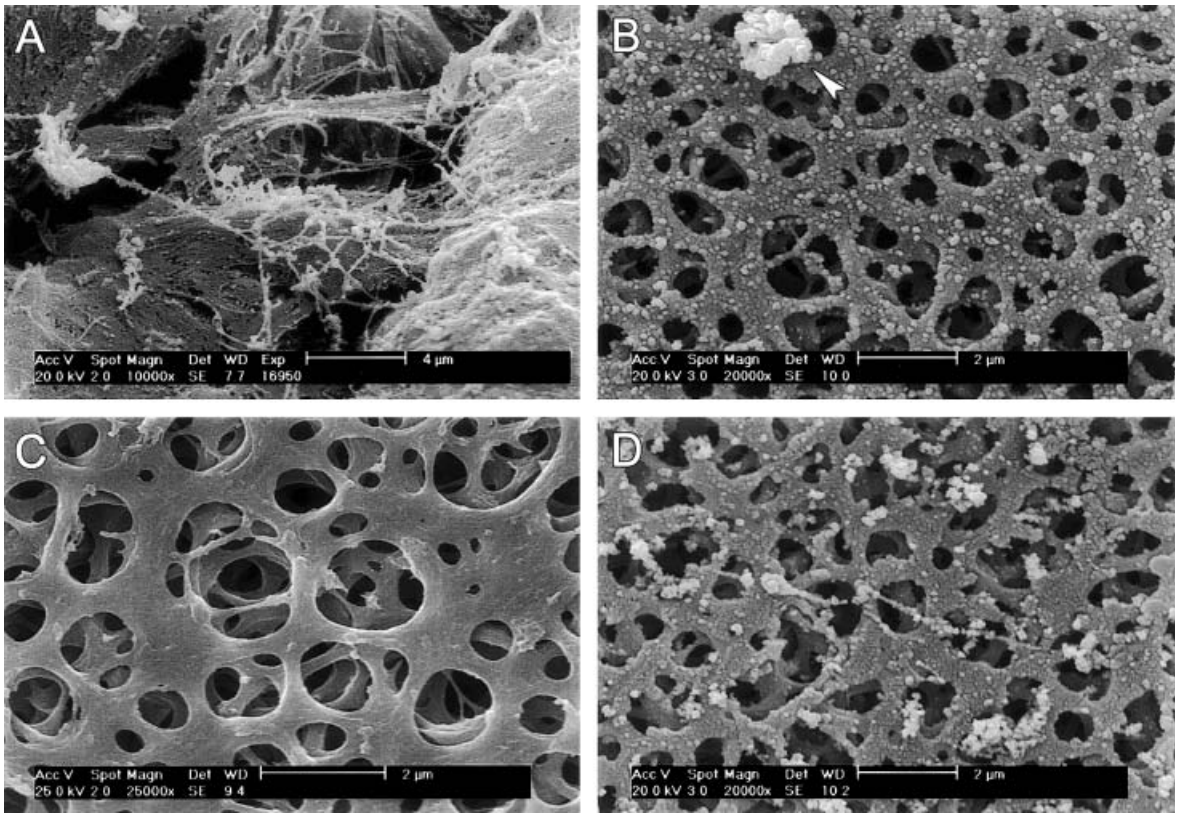

Fig. 1. Scanning electron microscopy. (A) A micrograph of a 5-μm filter with epithelial cells covered by long mucin-like fibres forming a network. (B) A micrograph of a 0.2-μm filter. Large numbers of salivary globes are attached to the filter surface, causing its warty appearance. The arrow indicates a cluster of salivary globes. (C) Control sample: a 0.2-μm filter washed with saline. (D) A micrograph of a 0.2-μm filter. A large number of salivary globes were grouped in clusters on the filter surface. The centre of the micrograph shows two mucin-like fibres covered with salivary globes.

Saliva fixation enables the preservation of morphological features of supramolecular aggregations, thus enabling differentiation between supramolecular structures pre-existent within saliva and structures originating a short time after the saliva has been in contact with a solid surface. Thus, the observation of supramolecular globular aggregations from fixed saliva provides evidence for their pre-existence (3). In addition, as native supramolecular aggregations deform and flatten as a result of attachment to a solid surface (19), fixation of salivary globes before spreading on a grid enables a more exact estimation of their size.

The almost identical amino acid content of acidified salivary sediment and pellicle (6, 7) strongly suggests that the salivary globes are the pellicle precursors. The existence of small quantities of sialic acid residues on the surface of the pellicle has been previously reported (20, 21). However, according to our findings, their removal did not considerably affect the structure of the salivary globes.

Spontaneous micellation (self-assembly) occurs predominantly as a consequence of repulsion of the hydrophobic parts of the molecules building up the micelle by hydrophobic interactions with water molecules. As a result, the hydrophilic parts are orientated towards the micellar surface and the hydrophobic ones are hidden in the micellar core. PRPs are very appropriate for micellar formation, as they exhibit a marked amphiphilic nature (20% hydrophilic and 80% hydrophobic amino acid residues) (22, 23). Addition of detergents, e.g. SDS, causes dissociation of most self-assemblies (24–26). Dissociation of the salivary globes by SDS strongly suggests their supramolecular structure. As they are responsible for the pellicle formation, they can be denoted as supramolecular pellicle precursors. The resistance of supramolecular precursors to trypsin is very probably a consequence of the inaccessibility of trypsin to residues 31, 75, 92 and 107 of PRPs, where tryptic digestion takes place (23). The typical micellar structure is characterized by closely packed protein molecules with hydrophobic amino acid residues immersed in the micellar core. All the above-mentioned residues are hydrophobic; consequently they are confined to the supramolecular precursor core and are therefore inaccessible to trypsin. Acquisition of tryptic resistance, as a consequence of a protein self-assembling, has also been reported (27, 28). The proteolytic resistance of the supramolecular pellicle precursors may be a protective mechanism to withstand proteolytic challenges by bacteria. Thus, neither disulphide links nor crosslinking of acid PRPs via transglutaminase are responsible for the maintenance of supramolecular pellicle precursors. Instead, non-covalent bonds, particularly hydrophobic forces (29), are important. The molecules forming the supramolecular pellicle precursors may be regarded as possessing

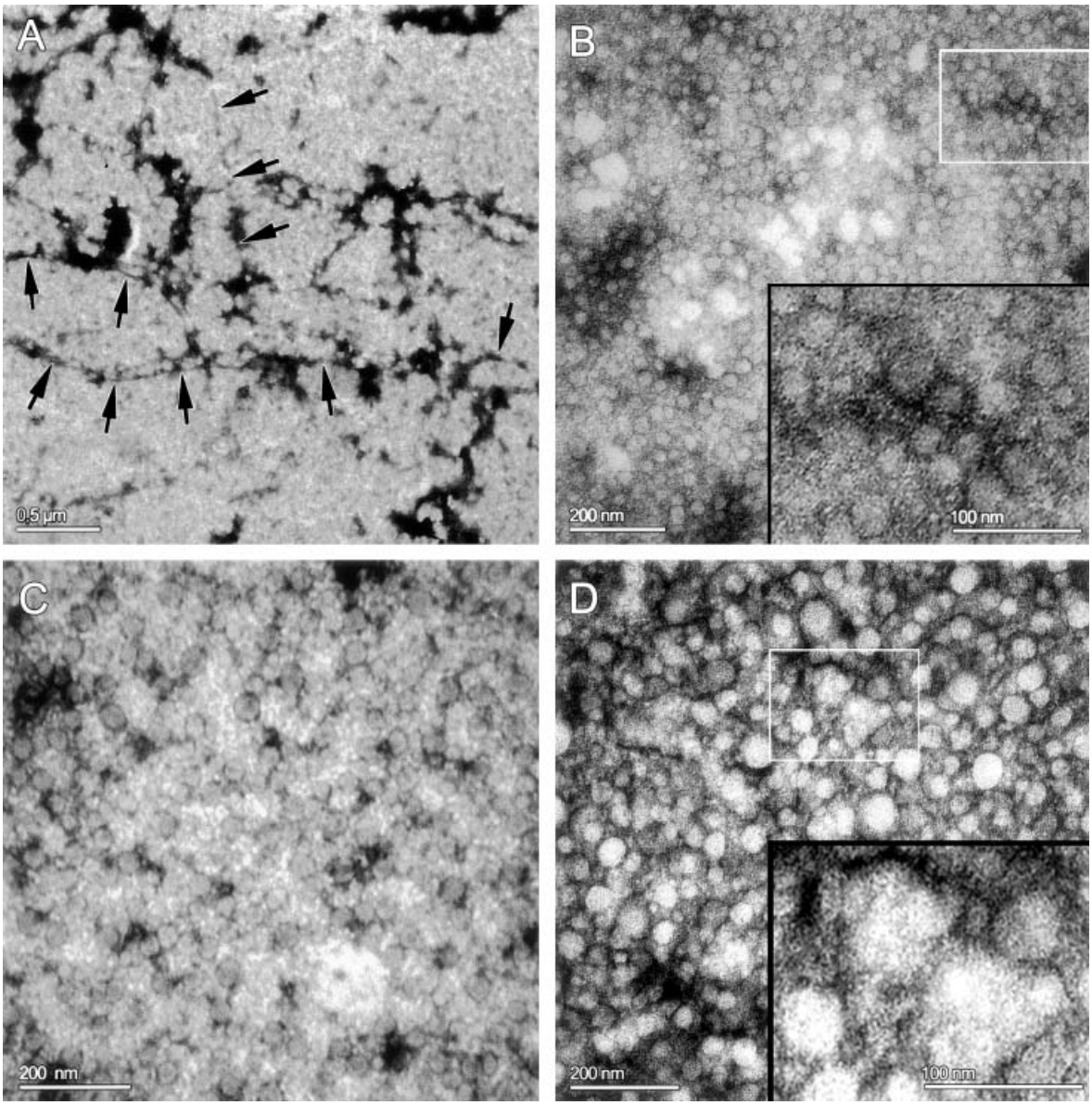

Fig. 2. Transmission electron microscopy. (A) A micrograph of fixed saliva. The arrows indicate fibres attached to the formvar film and stained positively with uranyl acetate. The fibres staining positively completely mask the negative staining. (B) A micrograph of salivary globes spread on the formvar film after a 10-s exposure to fixed saliva. The spherical shape of the salivary globes is clearly distinguishable. Inset: a detail. (C) A micrograph of a formvar film exposed for 10 s to native saliva. The attached native salivary globes are larger than the fixed ones. (D) A micrograph of a formvar film exposed for 60 s to native saliva. The presence of giant salivary globes and clumping of attached globes strongly suggest that the fusion of the salivary globes is caused by their attachment to the solid surface. Inset: a detail with fused globes.

lipid-like properties only in the sense of being amphiphilic and therefore tending to be self-assembling.

The exposure of formvar-coated grids for 60 s to native saliva resulted in attachment of large quantities of the supramolecular pellicle precursors to the formvar surface. Also, the presence of giant precursors, and clumping of attached precursors, strongly suggest that precursor fusion is caused by attachment to the formvar surface. Thus, the salivary pellicle is a result of the clustering and fusion of supramolecular precursors. Salivary pellicle forms in the oral cavity within 30 s and also has a granulated surface (11, 30, 31). These observations advocate that the pellicle formation does not originate via adsorption of single protein molecules to solid surfaces, but is a result of adsorption and subsequent clustering and fusion of supramolecular pellicle precursors. The protein molecules of the supramolecular pellicle precursors attain an adsorbed state after contact with solid surfaces. This state is less energetic and thermodynamically favourable (32), hence it elicits the formation of an adsorbed layer via clustering and fusion of the attached micellar aggregations.

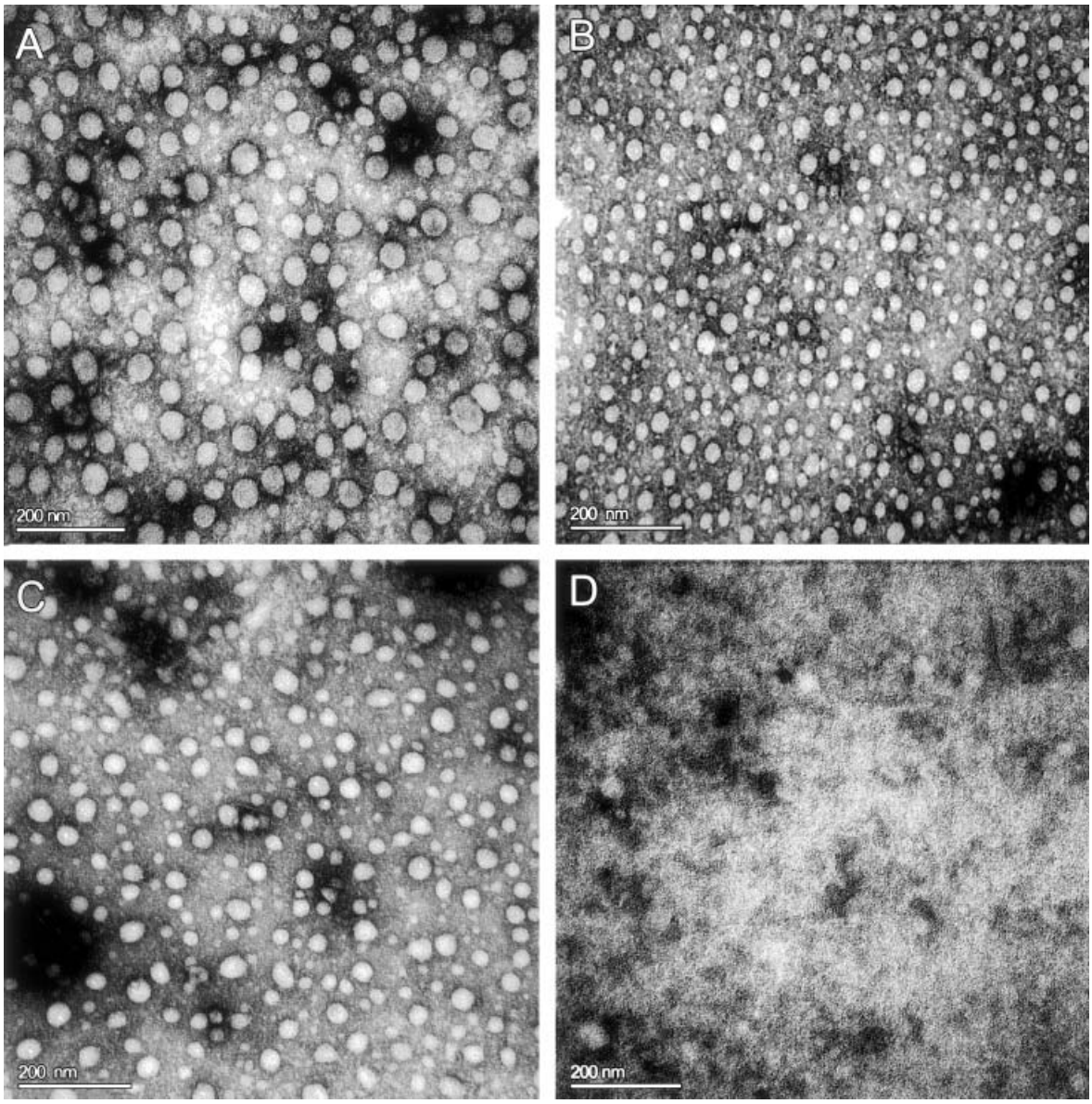

Fig. 3. Transmission electron microscopy. (A) A micrograph of a formvar film exposed for 60 s to 2-h-old saliva. Note the reduced number of salivary globes as compared to Fig. 2D. (B) A micrograph of a formvar film exposed for 60 s to sialidase-treated 2-h-old saliva. No significant differences compared to Fig. 3A are detectable. (C) A micrograph of a formvar film exposed for 60 s to trypsin-treated 2-h-old saliva. No significant differences are visible in comparison to Fig. 3A. (D) A micrograph of a formvar film exposed for 60 s to 2-h-old saliva treated with 2% sodium dodecyl sulphate (SDS) for 30 min. No salivary globes are evident.

The observed long fibres, which in the native state did not attach to formvar, graphite (31), Si wafer (31) or mica (31), but stuck onto the filter surface and, after fixation, to formvar, and also stained positively with uranyl acetate, are very probably mucins. The ability of the major salivary mucins MG1 and MG2 to form fibres and gel-like structures has been well documented (33, 34).

The supramolecular pellicle precursor clustering observed in some SEM micrographs is very probably a result of the preparation procedures. A similar clustering of precursors (also termed subunits) onto carbon film has been previously demonstrated (3,4). Cluster-like distribution of nanoparticles is often observed and is usually artificial (35). It is mainly a result of the spreading and drying of the particles onto a solid surface, e.g. carbon film.

Supramolecular pellicle precursors of micellar nature attach to, and spread out on, solid surfaces during their contact with them. The attached supramolecular precursors fuse when they are in contact with each other and, as a result, a layer coating the solid surface originates – the salivary pellicle.

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