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Ida E. Svendsen ^{ab}; Thomas Arnebrant ^a; Liselott Lindh ^b

^a Biomedical Laboratory Science and Technology, Faculty of Health and Society, Malmö University, Malmö, Sweden

^b Prosthetic Dentistry, Faculty of Odontology, Malmö University, Malmö, Sweden

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Validation of mechanically-assisted sodium dodecyl-sulphate elution as a technique to remove pellicle protein components from human enamel

Ida E. Svendsen^{a,b*}, Thomas Arnebrant^a and Liselott Lindh^b

^aBiomedical Laboratory Science and Technology, Faculty of Health and Society, Malmö University, SE-205 06 Malmö, Sweden; ^bProsthetic Dentistry, Faculty of Odontology, Malmö University, SE-205 06 Malmö, Sweden

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The salivary film, denoted the pellicle, formed on oral surfaces is of great importance for oral health and comfort. The present study describes mechanically-assisted sodium dodecyl sulphate (SDS) elution of the *in vivo* pellicle formed on human enamel and visualisation of the desorbed pellicle proteins using two-dimensional gel electrophoresis (2-DE). To verify this removal of the pellicle, a combined mechanical and surfactant procedure was additionally performed on an *in vitro* pellicle formed on human enamel, and the effectiveness was validated by mechanical removal in combination with HCl. As indicated by protein quantitation and one dimensional gel electrophoresis, rubbing with polyamide fibre pellets soaked in a 0.5% SDS solution was optimal for completely removing the adsorbed proteins from the enamel surface, and yet provided separation of the proteins by 2-DE to enable identification in future studies.

Keywords: saliva; gel electrophoresis; surfactant; validation studies; enamel surface

Introduction

The acquired enamel pellicle is known to form instantaneously when oral surfaces are exposed to saliva. This thin organic film is formed by selective adsorption of primarily salivary proteins, where proteins such as proline-rich proteins, statherin, lysozyme, histatins and mucins have been identified (Lendenmann et al. 2000; Hannig and Joiner 2006). The pellicle possesses many important functions such as maintaining tooth integrity by controlling the mineral solution dynamics of the enamel, and having tissue-coating, antimicrobial and lubricating abilities. Since this is of importance for oral health and comfort, there has been a great deal of interest in characterising the composition of salivary pellicles over the years (Lendenmann et al. 2000; Hannig and Joiner 2006; Deimling et al. 2007; Siqueira et al. 2007; Vitorino et al. 2007). For these investigations, different techniques were applied for the removal of pellicles (Carlén et al. 1998; Hahn Berg et al. 2001; Yao et al. 2001; Hannig et al. 2005) where sodium dodecyl sulphate (SDS) has been used frequently as eluent at different concentrations. Carlén et al. (1998) developed a technique for *in vivo* pellicle elution using a 2% SDS solution, which although not validated on human enamel surfaces, was shown to completely desorb surface-bound proteins on modified gold surfaces (Källtorp et al. 2000). Yao et al. (2001) evaluated several

different eluents and showed that, among these, SDS was the most efficient, but still only desorbed approximately 80% of the pellicle proteins from hydroxyapatite (HA) at a concentration of 0.1%. Further, SDS has also been used extensively for elution of *in situ*-formed salivary films where a solution of 0.5% SDS completely removed adsorbed salivary films on hydrophilic silica, whereas some residual material was left on HA surfaces (Hahn Berg et al. 2001). Hannig et al. (2005) investigated the removal of *in situ*-formed pellicles on wet-ground and polished bovine incisors and showed that the pellicle was incompletely removed by polyurethane foam sponges soaked in 2% SDS.

Due to contradictory statements in the literature on the pellicle removal efficiency of SDS on different types of surfaces, using various techniques for visualisation of the results, including one-dimensional gel electrophoresis (1-DE) (Carlén et al. 1998; Milleding et al. 2001; Yao et al. 2001; Hannig et al. 2005), immunoblotting (Carlén et al. 1998; Milleding et al. 2001), densitometry (Yao et al. 2001), ellipsometry (Hahn Berg et al. 2001), atomic force microscopy (Milleding et al. 2001) and transmission electron microscopy (Hannig et al. 2005), the aim of the present study was to investigate the effectiveness of SDS in eluting both *in vivo* and *in vitro* pellicles formed on natural human teeth, which had not been subjected to any treatment other than pumicing.

*Corresponding author. Email: Ida.Svendsen@mah.se

Additionally, the choice of SDS concentration would be crucial for the visualisation of the results when using two-dimensional gel electrophoresis (2-DE). Allowing SDS to remain in the sample would decrease sample preparation prior to 2-DE; too high a concentration of a negatively charged surfactant such as SDS, however, would affect the separation in the first dimension (protein separation according to isoelectric point), leading to poor resolution and identification. In addition to the above stated aim it was, therefore, considered relevant to investigate different concentrations of SDS for complete pellicle elution that would additionally yield good separation in 2-DE.

Materials and methods

General

All water used was UHQ (ultra high quality) processed in Elgastat UHQ II (Elga Ltd, High Wycombe, Bucks, England). SDS (L6026, >99% pure) was purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden), and diluted in UHQ to the desired concentrations. All other chemicals used were of at least analytical grade (VWR International, Stockholm, Sweden; Amersham Biosciences, Uppsala, Sweden; Bio-Rad, Sundbyberg, Sweden).

Collection of *in vivo*-formed pellicle

The *in vivo*-formed pellicle was collected from one donor, found to be in good oral health. Informed consent from the donor was given. The collection procedure was initiated 2 h after breakfast, and repeated every day over a 2 week period. The donor refrained from any dietary intake during pellicle formation. In brief, after the teeth had been pumiced with a rubber cup and fine-grade pumice followed by water rinsing, pellicles were allowed to form over a 2 h period. Collection was then performed from the coronal two thirds of all buccal surfaces of the upper and lower jaws, except from restorations, according to Carlén et al. (1998) using polyamide fibre pellets (Quick-sticksTM, Dentonova AB, Huddinge, Sweden) (Milleding et al. 2001) soaked in the respective SDS solution (concentrations investigated: 0.1, 0.25, 0.5 and 2% w/v). Fibre pellets (a total of three) used for the same quadrant were collected in one test tube, and 10 µl of the respective SDS solution were added. The bottom of the test-tube was then perforated, and the SDS solution containing the pellicle eluent was recovered in an outer test-tube after centrifugation for 2 min at 1000 rpm in a table-top centrifuge (Mini centrifuge C-1200, Labnet International Inc, Woodbridge, NJ, USA). All pellicle samples of the same SDS concentration were pooled, and finally

stored at -20°C until subjected to 2-DE. This study was approved by the Committee for Research Ethics at Lund University (Approval No: LU 518-02).

Saliva collection and *in vitro* validation of pellicle collection

Human whole saliva (HWS) was collected as described by Dawes (1974) from the same donor as the *in vivo* formed pellicle.

A total of 15 extracted human teeth were pumiced as described above (collection of *in vivo*-formed pellicle), after which they were rinsed in water and blown dry with filtered air. Each pumiced tooth was then rubbed with 1 Durapore membrane filter (PVDF, type HVLP, 45 µm pore size, 13 mm diameter; Millipore, Bedford, MA, USA) (Yao et al. 2001) soaked in 0.32 M HCl followed by 1 PVDF membrane soaked in water. This acid/water treatment was repeated twice, according to Bennick et al. (1983). This first HCl-water treatment will be referred to as HCl-1 in the following text. The teeth were then rinsed in excessive amounts of water and blown dry with filtered air. All 15 teeth were then placed (crown facing down) in a shallow Petri dish filled with freshly collected HWS and an *in vitro* pellicle was allowed to form for 2 h at room temperature. This temperature was chosen based on previous studies (Carlén et al. 1998). No contact between the roots of the extracted teeth and the saliva was allowed. After 2 h the teeth were rinsed in large amounts of water, and blown dry. Fibre pellets, soaked in 0.5% SDS were then used to collect the pellicle, as described above. Afterwards the teeth were again rinsed with water and dried as described, and finally the teeth were treated with HCl and water in the same manner as described earlier to validate if the pellicle had been totally removed by the SDS solution. This second HCl-water sample is referred to as HCl-2 in the following text. Elution from the PVDF membranes was carried out according to Yao et al. (2001). Elution from the PVDF fibre pellets was performed as described above for the *in vivo*-formed pellicle. All samples were stored at -20°C until subjected to 1-DE.

Protein quantitation

The protein concentrations of the samples were determined according to the instructions of Bio-Rad RC DC Protein assay (Bio-Rad Laboratories, Syndbyberg, Sweden). The absorbance was measured at 750 nm in a spectrophotometer (Hitachi model U-2000, Hitachi Ltd. Tokyo, Japan). Bovine serum albumin (Bio-Rad no 500-0007) was used as the standard. The quantitation was performed at least twice for each protein sample with a variation <5%.

Two-dimensional gel electrophoresis (2-DE)

The different collections of *in vivo* pellicles using different concentrations of SDS were subjected to 2-DE for determination of the most suitable SDS concentration for optimal separation. There were no attempts to remove SDS from the pellicle samples. 2-DE was carried out as previously described (Svendsen et al. 2004) using 7 cm Immobiline DryStrip linear immobilised pH gradient gel strip (pH 3–10, Amersham Biosciences, Uppsala, Sweden) for the first dimension (separation according to charge) and a linear gradient (10–14%) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was used for the second dimension (separation according to size). Thirty five microliters of each of the pellicle samples were diluted 1:3 in rehydration buffer and then subjected to 2-DE. Duplicates were made of each sample.

One-dimensional gel electrophoresis (1-DE)

12% Ready Gel[®] Precast SDS-PAGE gels (Bio-Rad Laboratories, Sundbyberg, Sweden) were used to visualise potential proteins in the *in vitro* validation trial (HCl-1, HCl-2 and the *in vitro* SDS eluted pellicle). The maximum volume (approximately 10 μ l) of HCl-1, SDS pellicle and HCl-2 was loaded on the SDS-PAGE. SDS-PAGE Molecular weight standard Broad Range (6–200 kDa) (Bio-Rad, Sundbyberg, Sweden) was used as the standard. Gels were run at a constant 120 V for approximately 2 h.

Also, SDS-PAGE was used to extract any possible proteins that had not been eluted from the fibre pellets used for the *in vitro* pellicle collection. This extraction was performed according to Siqueira et al. (2007) which had shown a 97% recovery of proteins from collection strips. Briefly, two fibre pellets together with sample buffer (approximately 20 μ l), were placed in one well of the SDS-PAGE. Also, clean fibre pellets were subjected to SDS-PAGE which verified that no proteinaceous contaminants were present. For all 1-DE the same composition of sample buffer was used: 0.125 M Tris-HCl, 4% SDS, 25% glycerol, 10% 2-mercaptoethanol and 0.01% bromophenol blue.

Staining procedures

The gels were stained according to the “Brilliant Blue G – Colloidal concentrate” staining protocol for proteins (Coomassie Brilliant Blue (CBB), Sigma, St Louis, MO, USA, No. B-2025), followed by the “Silver Staining Protocol for Proteins” (No. 71-7177-00, Amersham Bioscience, Uppsala, Sweden). Combining CBB and silver staining is known to enhance the staining sensitivity of proteins (Irie et al. 1982).

Results

Choice of appropriate SDS concentration for use in 2-DE

In Figure 1, the results using 2-DE of *in vivo* collected pellicles removed by 0.25% (Figure 1A) and 0.5% (Figure 1B) and 2% SDS (Figure 1C) solutions are shown. A larger number of proteins were visible on the 2-DE using 0.5% SDS compared with the 2-DE using 0.25% SDS. A concentration of 0.1% SDS revealed an even lower number of proteins visible in 2-DE (data not shown). Protein quantitation also showed that pellicles removed by 0.1% SDS and 0.25% SDS had a lower amount of proteins compared with 0.5% SDS (Table 1). The 2% SDS solution interfered with the isoelectric focusing gel electrophoresis (Figure 1C), therefore, 0.5% SDS was chosen for the *in vitro* pellicle collections.

Figure 1. CBB and silver stained 2-DE of pellicles collected *in vivo* using different SDS concentrations. A = 0.25% SDS, B = 0.5% SDS, C = 2% SDS. The isoelectric points of the proteins were separated in the horizontal direction (from pH 3 (left) to pH 10 (right)), and the sizes of the proteins were separated in the vertical direction with the size of the standard given in kDa on the right.

Table 1. Protein concentrations of the validation samples, as well as the *in vitro* and *in vivo* pellicles.

Sample	Average protein concentration ($\mu\text{g ml}^{-1}$)
<i>In vivo</i> pellicle (collected with 0.5% SDS solution)	150
<i>In vivo</i> pellicle (collected with 0.25% SDS solution)	130
<i>In vivo</i> pellicle (collected with 0.1% SDS solution)	110
HCl-1 (before <i>in vitro</i> pellicle formation)	50 ^a
<i>In vitro</i> pellicle (collected with 0.5% SDS solution)	150
HCl-2 (after <i>in vitro</i> pellicle collection)	n.d. ^a

^aSample concentrated 20 times.

n.d. = no proteins detected.

Because 0.5% SDS was shown to completely remove the pellicle, the 2% SDS pellicle was not included in the protein quantitation analysis. $n \geq 2$ and variation $< 5\%$.

Validation of complete pellicle removal using 0.5% SDS

Figure 2 shows 1-DE of HCl-1 (before pellicle formation), *in vitro* pellicle collected with 0.5% SDS, and HCl-2 (after pellicle collection). As can be seen in Figure 2A, HCl-1 showed no protein bands, indicating that no protein components were present on the clean surfaces in any detectable amounts. Protein quantitation of the sample showed no signs of any proteins present. However, after concentrating the sample 20 times (using a Lyovac GT 2 (Leobold Heraeus GMBH, Köln, Germany)) the HCl-1 sample contained $50 \mu\text{g protein ml}^{-1}$ (Table 1).

The 1-DE of SDS eluted *in vitro* pellicle (Figure 2B) showed a few protein bands in the resolved molecular weight range (6–200 kDa).

1-DE of HCl-2 illustrated no detectable protein bands, indicating that the pellicle was completely removed by 0.5% SDS (Figure 2C). Also, the protein quantitation analysis indicated that no proteins were present in this sample (Table 1). For further assurance, this sample was also concentrated 20 times in the same manner as HCl-1, after which the protein concentration was investigated again. The results still showed no indication of proteins being present in the sample.

The fibre pellets used for the *in vitro* pellicle collection was also subjected to 1-DE to determine whether all proteins had been eluted from the fibre material. The results are illustrated in Figure 3. It was evident that not all proteins had been eluted from the fibre pellets.

Figure 2. CBB and silver stained SDS-PAGE of (A) HCl-1, (B) *in vitro* pellicle eluted with 0.5% SDS, and (C) HCl-2. The sizes of the proteins were separated in the vertical direction with the size of the standard given in kDa on the right.

Figure 3. CBB and silver stained SDS-PAGE of the fibre pellets used for the collection of *in vitro* pellicle. The sizes of the proteins were separated in the vertical direction with the size of the standard given in kDa on the right.

Discussion

The present investigation aimed to elucidate the possibility of removing the *in vivo* formed pellicle by means of using SDS as a chemical reagent in combination with mechanical rubbing of the tooth enamel surfaces. Previous *in situ* studies on the removal of salivary films from HA showed incomplete removal when the salivary film was subjected to rinsing with a 0.5% SDS solution (Hahn Berg et al. 2001). The results presented here show that 0.5% SDS was sufficient to completely remove the pellicle, and it appears that mechanical rubbing with fibre pellets had a large influence on the removal of adsorbed proteins. Surface properties are also of importance in this context. Hannig et al. (2005) showed results indicating that the most important factor in pellicle harvesting was mechanical removal; similar fractions were desorbed from *in situ*-formed pellicles using polyurethane foam

sponges soaked in 2% SDS and water, respectively. However, results from the study reported here, as well as previous reports using mechanical pellicle collection in combination with desorption agents (Carlén et al. 1998; Yao et al. 2001), showed that the elution agent and its concentration are important.

Different mechanisms have been proposed for the removal of adsorbed proteins by SDS (Wahlgren and Arnebrant 1991). In this particular case, when dealing with enamel surfaces that have been reported to be negatively charged (Arends and Jongebloed 1977; Arends 1979), SDS is expected to form complexes with adsorbed proteins, which then are electrostatically repelled from the surface. The concentration interval of SDS was chosen based on previous pellicle collection studies (Carlén et al. 1998; Hahn Berg et al. 2001; Yao et al. 2001; Hannig et al. 2005) and on the fact that the critical micelle concentration (cmc) of SDS in water is known to be 8.3 mM (0.25% w/v). The results indicate that there is a critical concentration at approximately $2 \times \text{cmc}$ (0.5% SDS) that completely desorbs the pellicle. Previous studies have shown that complete SDS-induced desorption of lysozyme takes place at a concentration close to the cmc (Fröberg et al. 1999), which also was shown to be true for salivary films adsorbed on hydrophilic silica (Hahn Berg et al. 2001). However, differences in surface properties, adsorbed film composition, protein adsorption time and the desorption procedure affect the threshold concentration of SDS at which complete film removal is observed (Rapoza and Horbett 1990).

For the 2-DE visualisation, the large difference in protein separation between 0.5% and 2% SDS indicates the existence of a crucial concentration of SDS. Previous studies (Ames and Nikaido 1976; Gorg et al. 2000) have shown that exceeding a concentration of 0.25% of SDS in the rehydration-loading solution could interfere with isoelectric focusing. It also has been suggested that this was due to insufficient replacement of SDS by the non-ionic/zwitterionic detergent present in the rehydration buffer (Gorg et al. 2000). For the pellicle collection using 0.5% SDS, the final concentration in the rehydration buffer was 0.17% SDS; for the collection with 2% SDS, the resultant concentration was 0.67%. As 0.5% SDS was sufficient for complete pellicle harvesting while still not interfering with 2-DE, several time-consuming steps of sample purification prior to 2-DE (eg dialysis) could be eliminated.

The validation of the pellicle removal was performed *in vitro* on intact human teeth, which had been extracted as part of orthodontic treatment. These teeth had not been subjected to any other treatment than pumicing before the validation test so that the surfaces

would resemble the *in vivo* situation as closely as possible. Previous studies of desorbing agents for the removal of pellicles formed *in situ* used bovine incisors that had been subjected to wet-grinding and polishing (Hannig et al. 2005). Hannig and co-workers showed that polyurethane foam sponges soaked in 2% SDS did not completely desorb the pellicle using a method similar to the one used in the investigation reported here. Although this previous study increased awareness of differences between pellicle collection techniques, the authors of the current study believe it is of importance to investigate SDS desorbability on actual human enamel teeth. Studies have shown that, even though there are several similarities between bovine and human enamel, they still differ in many aspects, eg in surface free energy (Brown et al. 1992). Surface free energy is an important factor when investigating adsorption from saliva and proteins therein, as numerous studies have shown (Arnebrant 2003). Furthermore, differences in surface treatment procedures (such as wet grinding) are also of relevance for pellicle formation and composition as surface treatment may modify surface properties. Therefore, it is of value to do these types of studies on human enamel, which has only been subjected to pumicing as in the clinical situation. Furthermore, compositional differences between the polyurethane foam sponges used by Hannig et al. (2005) and the polyamide fibre pellets used in the present investigation also may contribute to the differences in the results obtained.

Studies have indicated differences in composition between *in vivo* and *in vitro*-formed pellicles (Carlén et al. 1998; Yao et al. 2001). *In vitro* pellicles were shown to contain more intact salivary proteins compared with *in vivo* pellicles (Yao et al. 2001), where proteolysis was found to have a larger influence (Jensen et al. 1992). However, Mayhall (1970) performed amino acid analysis of *in vivo* and *in vitro* pellicles on human enamel that showed a high degree of similarity. Further, many proteins identified in *in vivo* pellicles also have been identified *in vitro* (Carlén et al. 1998; Lendenmann et al. 2000; Yao et al. 2001) and, therefore, use of an *in vitro* model for the validation reported here was justified. The protein bands observed in 1-DE (Figures 2B and 3) were in good agreement with previous results performed at physiological temperatures (Yao et al. 2001; Hannig et al. 2005). This indicates a minor influence of the temperature at which the *in vitro* pellicle was formed (room temperature vs. physiological temperature). As the present study focused on the validation of pellicle removal, no attempts were made to identify the proteins.

HCl has been used in previous studies for the removal of so called "old pellicle", and the collection of pellicles by decalcification (Mayhall, 1970; Bennick

et al. 1983). These studies showed that the HCl-extracts had an amino acid profile very similar to that of enamel proteins, clearly indicating that the whole pellicle had been removed. The $50 \mu\text{g ml}^{-1}$ protein found in the concentrated HCl-1 sample could indicate a partial dissolution of the protein matrix of the enamel. In addition, previous studies have found that pumicing did not completely remove the "old pellicle" (Yao et al. 2001) and, therefore, this also may be an explanation for the protein content in HCl-1. Because HCl is used for decalcification, it can be stated that the surface of the enamel after HCl-treatment is completely free of protein components. Although HCl may have caused minor effects on the enamel surface, this treatment was considered vital for the validation test, as it would not be possible to validate the removal of the pellicle if the surface from the beginning was not completely free from protein components. Characterisation of the wettability of the surfaces before and after elution should give additional indications of a complete removal of protein. However, as the surfaces were pre-etched by acid before treatment, comparison with clinical values of contact angles may not be completely straightforward.

The technique used for elution of HCl-1 and HCl-2 from the PVDF membranes has previously been described by Yao et al. (2001). They showed that $83\% \pm 5\%$ of adsorbed proteins could be desorbed. In their test, no elution agent was present, and it seems likely that the presence of SDS would result in increased elutable fractions from the membranes, although it is possible that protein components in HCl-2 may still be adsorbed to the membranes. However, as the 1-DE and protein quantitation showed complete absence of proteins in this sample, it seems unlikely that even a minor amount of proteins would be present.

1-D and protein quantitation may seem insufficient as techniques for accurate determination of minor concentrations of protein components. These techniques have been used in previous studies for the validation of different elution tests (Källtorp et al. 2000; Yao et al. 2001), giving excellent results both in quality and quantity. Therefore, the results obtained by combining these techniques were considered reliable.

Conclusions

In the present study, different concentrations of SDS were used in combination with mechanical rubbing of the surfaces for complete desorption of pellicle formed *in vivo* on human enamel. The results showed that a solution containing 0.5% SDS was optimal for complete removal of the pellicle. Further, this study showed that there was no need to remove SDS from

the pellicle sample prior to 2-DE, thereby considerably decreasing the time needed for the experimental procedures.

Although optimisation of the elution of pellicle material from the polyamide fibre pellets used for the collection is needed, the protocol used here for pellicle harvesting from clinically relevant human enamel may serve as a guide for improved pellicle collections in the future, thus enabling identification of pellicle components.

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