European Journal of Orthodontics 33 (2011) 577–583
doi:10.1093/ejo/cjq126
Advance Access Publication 27 December 2010

Ultrastructure and morphology of biofilms on thermoplastic orthodontic appliances in 'fast' and 'slow' plaque formers

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SUMMARY The aim of this study was to investigate the morphological features and distribution of biofilms on Invisalign orthodontic appliances, in a sample of 'slow' and 'fast' plaque formers using scanning electron microscopy (SEM). Fifty-six Chinese male/female volunteers (aged 19–39 years) were screened for their plaque-forming rate using the plaque percentage index (PPI) coupled with digital photography and computer-based image analysis, after a period of 48 hours of abstinence from oral hygiene procedures. Eleven volunteers (seven males/four females) representing the lowest and highest ends of the plaque formation spectrum were chosen as slow and fast plaque formers, respectively. The subjects wore a full-coverage splint appliance, in which four tiles of Invisalign material were embedded. These tiles were collected at intervals of 1, 3, 6, 12, 24, and 48 hours, as well as 3, 7, and 14 days, immediately fixed in 10 per cent paraformaldehyde in 0.2 M cacodylate buffer solution and prepared for SEM. The surface configuration of the Invisalign appliance was visualized, as well as the chronological pattern of biofilm formation. Significance between fast and slow plaque formers was determined using a Student's *t*-test.

Colonization appeared to centre initially on the raised edges or textured surfaces of the appliance, and initial adhesion was quicker and more abundant in the fast plaque-forming group. In the later stages of biofilm development, both groups showed no discernible differences in biofilm accrual on the surfaces, but the fast group displayed a more complex biofilm structure. More recessed and sheltered areas of the appliance, such as the cusp tips and attachment dimples, harboured more biofilm than the flat surfaces. Hence, it seems that the novel Invisialign orthodontic appliance is a useful tool to investigate the features of biofilm formation in time-course studies.

Introduction

Orthodontic treatment is no longer limited mainly to children and adolescents, with increasing numbers of patients from the older age groups seeking orthodontic treatment. With this increase comes a corresponding interest and the need to develop more aesthetic orthodontic solutions to comply with the increased social and cosmetic demands of adult patients. The concept of using a series of sequential removable flexible orthodontic appliances, first started by Kesling (1945) with the introduction of thermoplastic tooth positioners as a valid mode of orthodontic treatment, has been evolving for several years. That author found that these close fitting positioners were useful for closing small amounts of residual space post fixed banded appliance therapy and hypothesized that with the use of minute movements in sequential study model set-ups, these aligners could be used to achieve significant tooth movement. Other case reports have also noted the use of similar close fitting aligners or positioners for orthodontic tooth movement (Ponitz, 1971; McNamara *et al.*, 1985; Sheridan *et al.*, 1993). The Invisalign orthodontic system introduced in 1997, utilizes computer-assisted design and manufacturing procedures, for the production of sequentially worn accurate 'invisible' clear plastic retainers needed to achieve tooth movement (Vlaskalic and Boyd, 2002). Treatment using these clear appliances typically with 15–20 aligners, each used for a minimum of 2 weeks, may last for up to 24 months (Wong, 2002).

Dental plaque is defined as a complex microbial community found on tooth surfaces, embedded in a matrix of polymer of bacterial and host origin, which harbours the most diverse resident microflora associated with humans (Marsh, 1995). The presence of biofilms can be attributed as the causative factor of many oral pathological conditions, including caries, periodontal disease, and candidiasis (Marsh, 1995; Marsh and Bradshaw, 1995; Costerton *et al.*, 1999). It has been estimated that some 60 per cent of human infections, including dental caries and periodontal disease, are due to microbial biofilms (Costerton *et al.*, 1999). Thus, in order to control the various disease processes that occur in the human oral cavity, an understanding of biofilms, their formation, and control of their development, is vital. It is therefore not surprising that oral biofilms are among the most extensively researched biofilm ecosystems, with many

species isolated and interactions responsible documented. The practice of dentistry itself also plays a role in biofilm formation in the oral environment through the introduction of a variety of foreign surfaces, such as dentures and orthodontic appliances, ideal for colonization by oral microbes. It has been demonstrated that biofilms are unique to the environment they inhabit, with changes in growing conditions, such as colonizing surface morphology, surface roughness, and oxygen or substrate availability ultimately dictating the compositional flora, their interactions, as well as the investing matrix of the biofilm (Bowden and Li, 1997; Stodley *et al.*, 1999). Hence, the formation of biofilm on materials and surfaces has been clearly documented in an effort to further understand the impact and implications of the introduction of appliances in the mouth. These include various materials found in orthodontic systems, such as ceramic and stainless steel brackets (Steinberg and Eyal, 2004), titanium mini-anchorage implants (Chin *et al.*, 2007), or nickel–titanium archwires (Eliades *et al.*, 2000).

The formation of plaque on surfaces is also dictated to a certain degree by variations that exist in the individual. Early studies investigating the formation of dental plaque in humans noted population variations in the rate of plaque formation in humans (Theilade *et al.*, 1966; Simonsson *et al.*, 1987), and a previous study by Zee *et al.* (1996) elucidated differences in the composition of the dental plaque in subjects with a 'fast' or 'slow' plaque-forming capacity.

Though the Invisalign appliance has been in use by the orthodontic community for almost 10 years, scant research has been undertaken into the appliance itself. A review reveals mostly case reports of treatment (Vlaskalic and Boyd, 2001; Miller *et al.*, 2002; Chenin *et al.*, 2003) and little data seems to be available on the accretion of bacteria and biofilm on the appliance, as well as the possible impact it has on the oral ecosystem. The aim of this scanning electron microscope (SEM) investigation, therefore, was to highlight the ultrastructural features, characteristics, distribution, and morphology of biofilms that develop on Invisalign orthodontic material, in a sample of patients who represent the extremes of the spectrum of plaque formation over a 48 hour period.

Subjects and method

Ethical approval

Permission to conduct the study was obtained from the Ethics Committee of the Faculty of Dentistry, University of Hong Kong. Informed consent was obtained from all the participants.

Subjects

The subjects of this study comprised undergraduate and postgraduate students at the Faculty of Dentistry, University of Hong Kong. A total of 56 ethnic Chinese male/female volunteers were recruited for the experiment, with an age range of 19–39 years. The participants were screened for active periodontal disease and caries, which none of the subjects exhibited. None were smokers or had been on antibiotic therapy within a 2 week period prior to the experiment. The subjects had to undergo a period without oral hygiene measures, lasting 48 hours. Prophylaxis was performed before the start of the study to ensure a plaque-free upper arch. After 48 hours, the anterior teeth 13–23 were disclosed with disclosing solution D and C Red #28 1.5 per cent w/w (Butler-Gum; Sunstar America Inc., Chicago, Illinois, USA), and four digital photographs were obtained, perpendicular to the buccal surfaces. The photographs were taken at a fixed magnification of 1.5 in manual focus, using a Canon (Canon Inc., Tokyo Japan) 60 mm macro lens on a 300D (magnification factor 1.6). A ring flash model MR-14EX was used with the light intensity set at 0.5 per half manually. The digital photographs were then run though an image analysis system (QWin; Leica Micro Systems GmbH, Wetzlor, Germany), producing percentage plaque coverage over the 48 hour period. The software is able to detect and then calculate the percentage plaque surface coverage. Of the 56 subjects, the five and six subjects with the highest and lowest plaque percentage coverage were designated as slow and fast plaque formers, respectively, as described by Zee *et al.* (1996). Eleven male/female (7:4) subjects were chosen for the final study.

Sample collection

A novel appliance was designed, which allowed collection of samples of biofilm formed on actual Invisalign material without the use of multiple aligners (Figure 1). The appliance comprised a full-coverage upper occlusal splint made from vacuum formed plastic. The appliances were made to be completely passive and produced no tooth movement. Windows were cut out in the premolar area, and four tiles of Invisalign material (measuring 4 × 4 mm) were placed next to the windows and held in place by another piece of vacuum formed plastic and attached with a 0.009 inch orthodontic wire ligature.

The subjects wore the appliance for a period of 2 weeks. They were encouraged to brush their teeth and the appliance as normal with toothpaste during the time that they wore the appliance but were told to refrain from soaking it in any cleaning solution. They were also asked to refrain from the use of antibacterial mouthwash during the trial period. The subjects wore the appliance full time (24 hours day), removing it only to eat and perform oral hygiene. At designated intervals of 1, 3, 6, 12, 24, and 48 hours and 3, 7, and 14 days, the appliance was briefly removed to harvest the tiles.

Specimen preparation for SEM

Specimens for SEM were prepared as described previously (Seneviratne *et al.*, 2008). Immediately after removal from

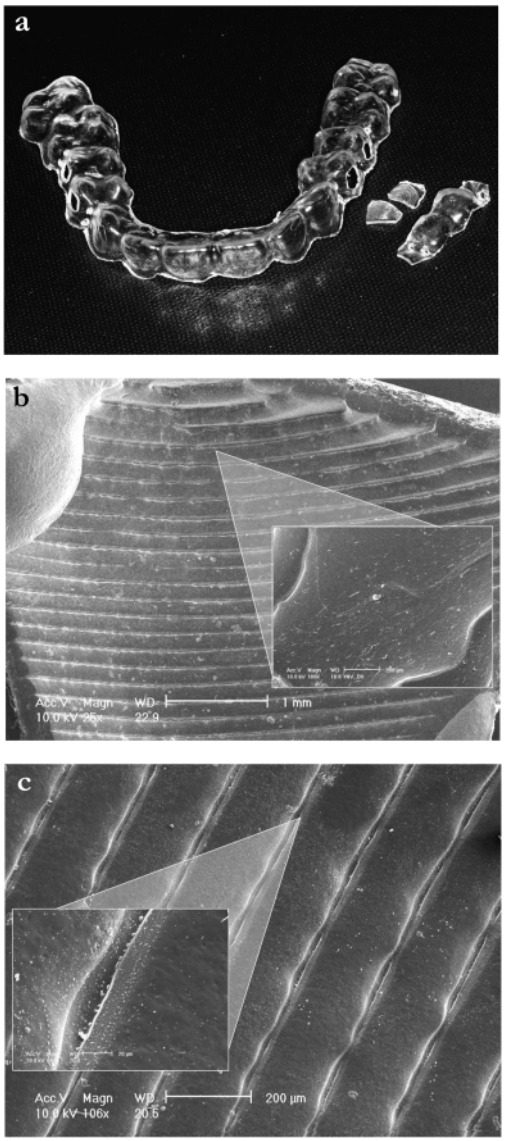

Figure 1 (a) Novel appliance used for collection of biofilm samples *in vivo*. (b) Inner surface of the Invisalign appliance, with corrugated three-dimensional surface. Inset: surface of an unworn Invisalign appliance, scratch marks, and microabrasions present on smooth surface. (c) Magnification of 'peaks' on Invisalign surface, with undercut area highlighted in magnified inset .

the appliance, the harvested tiles were immersed in a fixative of 10 per cent paraformaldehyde in 0.2 M cacodylate buffer solution within 2 minutes of removal from the mouth and refrigerated. The tiles were prepared by serial dehydration in ascending concentrations of acetone, desiccated by critical point drying, mounted on aluminium stubs, and sputter coated with elemental gold (approximate thickness 15 nm, Fine Coat Ion Sputter JFC1100; Jeol, Tokyo, Japan). These were then examined using SEM (Philips XL 30 CP, Eindhoven, Netherlands) at 10 kV, with magnifications ranging from ×30 to ×4000. Only the tooth facing/inner surfaces of the appliance was examined.

Method of biofilm/plaque assessment

Morphology of the appliance material was examined with SEM, first at lower magnifications, for features and configurations of the material that may not be visible to the naked eye. Higher magnifications were used to determine the distribution of biofilm formation on inner surfaces, as well as to elucidate the basic morphological characteristics of bacteria colonizing the appliance material, categorized as cocci, rods, and filaments.

Statistical analysis

Statistical significance between fast and slow biofilms formers was performed with a Student's *t*-test using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, Illinois, USA).

Results

Plaque percentage coverage

The plaque percentage index (PPI) of the 56 volunteers after a period of 48 hours of abstinence from oral hygiene procedures ranged from 8.0 to 67.2 per cent. For the slow plaque formers, the mean PPI was 13.1 per cent (range 11.3–15.2 per cent); for the six fast plaque formers, the mean PPI was 63.7 per cent (range 61.1–67.2 per cent; $P < 0.01$). The mean PPI for the remaining unselected volunteers ranged from 17.7 to 56.3 per cent.

Morphology of the Invisalign appliance material

SEM of the Invisalign material revealed a corrugated furrowed appearance, which was attributed to the mode of manufacture of the appliance, using stereomodels (Figure 1b). The average distance between the corrugated 'peaks' was approximately 0.17 mm. At a magnification of ×845, the individual peaks of the material appeared to possess a degree of undercut, which provided even more minute niches to encourage bacterial colonization (Figure 1c). The average width of the undercut areas was 20 m, and from the SEM photograph, these were the focus of pellicle development as well as initial bacterial colonization.

Morphological features 1–48 hours

Slow plaque formers. Ten tiles from the Invisialign appliance of five subjects were collected for SEM analysis. Although there were individual variations, slow plaque formers showed a distinctly different sequence of events compared with fast biofilm formers. In general, after 1 hour, globular deposits were evident on the surface of the tiles gathered from the slow plaque formers (Figure 2a). The deposits were distributed across the entire surface but seemed to be concentrated around the raised edges of the furrowed surface. From 0 to 6 hours, surface colonization was sparse with few bacteria colonizing the surfaces.

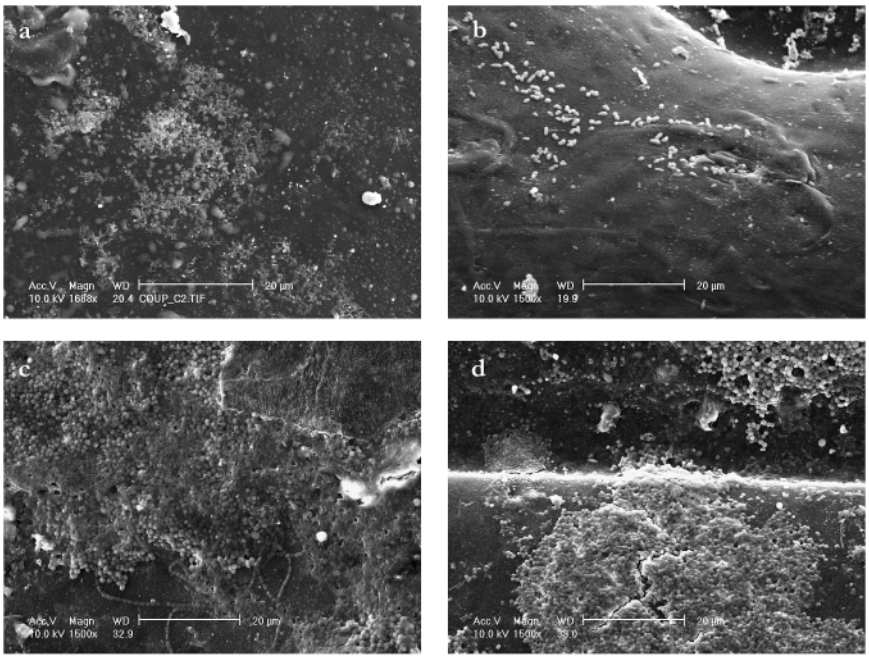

Figure 2 Scanning electron microscopic images of biofilm development over a period of 48 hours. (a) 1 hour tile, globular and amorphous pellicle deposits formed on surface. (b) 6 hour tile, initial colonizing species adhering to appliance surface, composed predominantly of cocci species. (c) 24 hour tile, large mass of coccoid species dispersed in thick extracellular matrix. Cocci in chain configuration present. (d) 48 hour tile, large masses of coccoid species interlaced with extracellular polymeric substance. Evidence of filamentous and rod species.

At 6–12 hours, colonization of the surface by organisms became more evident, with the majority of the bacteria belonging to the coccal species (Figure 2b). At 24–48 hours, the tiles exhibited a more florid bacterial biofilm, with evidence of filamentous and rod forms, as well as coccal forms, in cluster and chain formations (Figure 2c–2d). At this stage, the organisms were encased in a coat of extracellular polymeric substance (EPS). There were, however, individual variations in the morphology and quantity of the biofilms that developed on the tiles, between subjects, especially on the 48 hours tiles.

Fast plaque formers. The early morphological features of the biofilm formed on the fast Invisalign tiles were comparable with their counterparts in the slow group, beginning with deposition of a pellicular material along the material surface. The initial colonizers were, as before, coccoid in nature, but at 6 hours, more bacteria colonizing the surface were evident. Colonization centred predominantly around the corrugated portions and in the vicinity of cracks or grooves in the Invisalign material. After 24 hours, in a fast plaque former, the base of the cusp indentation was heavily colonized. After 48 hours, the fast plaque-forming group produced more biofilm, which covered a larger area of the tile surface. These biofilms appeared to be more abundant, larger, and thicker in nature, and generally comprised a single morphotype, mostly cocci. They were invariably found encased in a thick covering of EPS. As with samples collected from the slow group, large variations were found in the amount and dispersal of biofilms.

Morphological features 48 hours–14 days

All samples collected during this period (2–14 days) displayed more extensive and fully developed biofilms compared with samples from earlier time points. All samples displayed bacteria encased in a thick matrix of EPS. At time intervals after 7 days, SEM analysis revealed irregular and smooth crystalline structures, which appeared to be calcifications of biofilm material or the formation of microcrystalline structures (Figure 3a–3c).

Discussion

For the present study, the collection of biofilm samples, which were as intact and undisturbed as possible, was paramount in allowing visualization of the morphological development of the biofilms. The selected location for the sample tiles, on the buccal surface of the upper premolars, was chosen to allow easy placement and harvesting, as well as being relatively unobtrusive to maximize subject compliance and comfort. In various investigations that required the collection of *in vivo* biofilm, several methods have been used for whole/intact plaque, including cementation of enamel blocks to tooth surfaces (Zee *et al.*, 1997) or the use of various

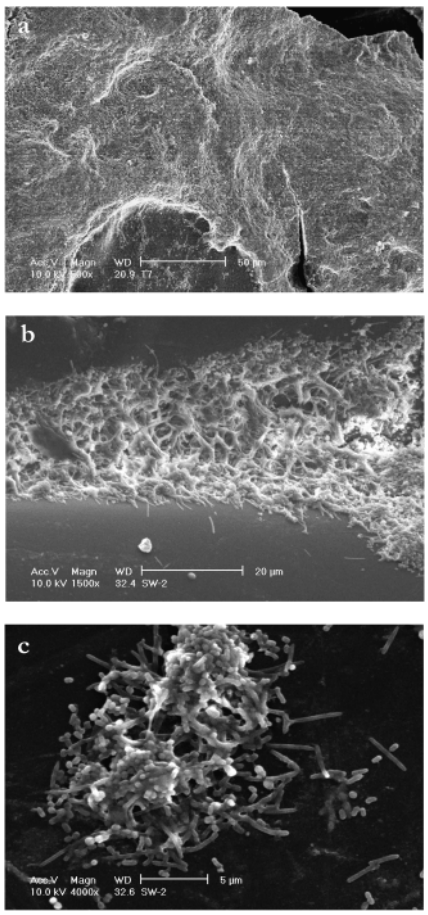

Figure 3 Scanning electron photo micrographs of tiles collected from 'fast' plaque formers at day 7 and 14. (a) 7 day tile, spreading sheet-like morphology of coccoid biofilm encased in thick extracellular matrix. (b) 14 day tiles, showing mass of biofilm comprised of coccoid, rod, and filamentous species. Extracellular polymeric substance and large interstitial voids visible. (c) 14 day tile showing bacterial colony of coccoid and rod species, arranged in three-dimenstional configuration.

removable appliances housing enamel or glass blocks, which can be subsequently harvested (Palmer *et al.*, 2001; Auschill *et al.*, 2005). Previous investigators have demonstrated that the buccal surface of the upper premolars is representative of most other areas of the oral cavity, in terms of plaque development as well as bacterial colonization and viability (Arweiler *et al.*, 2004; Auschill *et al.*, 2004).

Morphology and ultrastructure of the biofilms

SEM was employed in the present study to visualize the general morphology and the detailed structural or ultrastructural features of biofilm formed on the tiles. The use of SEM to determine morphology and ultrastructure of bacteria and biofilms has been utilized in several other studies (Lie, 1979; Nyvad and Fejerskov, 1987; Zee *et al.*, 1997; Monsenego, 2000; Sukontapatipark *et al.*, 2001). SEM represents a rapid and convenient means of assessing the pattern of colonization as well as screening samples for major bacterial morphotypes (Samarananyake, 1996). Hence, SEM was used to investigate the detailed surface structure and configuration of the material, which may lead to an understanding of the patterns of biofilm formation on Invisalign appliances.

There are however some drawbacks inherently associated with SEM as the identification of morphological features of the object is largely based on visual morphology (Sukontapatipark *et al.*, 2001). The preparation of specimens for SEM to remove the water content also introduces the possibility of deformation, shrinkage, and the inclusion of artefacts, all of which may distort the biofilm from its original state (Little *et al.*, 1991; Surman *et al.*, 1996). The bacterial extracellular matrix is particularly susceptible to distortion by dehydration due to its aqueous content and may condense to as little as 1 per cent of its original volume (Fischer *et al.*, 1988). Thus, care was taken in interpretation of the photomicrographs. No software has to date been developed to quantitatively analyse the morphological features obtained through SEM images, which is another drawback of this technology. Other advanced imaging modalities, which were not used, could be considered by future investigators to overcome the latter drawbacks. These include environmental SEM, an analogue of SEM, which utilizes a hydrated specimen chamber, eliminating the need for dehydration and coating of specimens to reduce electron absorption, which should reduce sample distortion (Slayter and Slayter, 1992; Surman *et al.*, 1996). Confocal laser scanning microscopy also allows inspection of aqueous live biofilm samples with relatively less distortion (Wood *et al.*, 2000). The use of quantitative image analysis also allows for the determination of live/dead cell counts and their distribution within the biofilm (Arweiler *et al.*, 2004). These along with concurrent investigations into the microbiological identification of bacterial species involved in biofilm formation warrant further study.

Structure of the Invisalign appliance

SEM images showed that the corrugated structure of the appliance material corresponded to the stereolithographic fabrication of the study models on which the aligners were fabricated (Wong, 2002). When visualized at high magnification, the surfaces were not completely smooth but exhibited microabrasions and irregularities, which may contribute to bacterial adhesion. The overall surface configuration, with its furrowed corrugated façade would appear to make the appliance more conducive to bacterial and biofilm accumulation. This appears to be the first description of the topographic features of an Invisalign appliance in the indexed literature.

Formation of biofilm on the appliance

Biofilm formation tended to centre around the textured areas of the appliance and on the flat non-textured surfaces, where the biofilm coverage was limited. Though there are

numerous other factors that play a role in influencing the initial attachment of colonizing species to a specific surface, including material reactivity, surface-free energy, and hydrophobicity, the most important factor has been shown to be related to surface roughness and configuration (Teughels *et al.*, 2006). More irregular surfaces provide protected niches in which bacteria are sheltered from shearing or dislodging forces that are common in the oral cavity. Increases in surface area also encourage bacterial adhesion by increasing the physical surface area for adhesion by a factor of 2–3, and rougher surfaces have been found to be more difficult to clean and thus promote regrowth by surviving organisms, as opposed to complete surface recolonization (Teughels *et al.*, 2006).

Fast plaque formers appear to accrue bacterial biofilms at a greater rate within the initial 48 hour period compared with subjects in the slow group. The initial colonization appears to begin at around 6–12 hours in both groups, prior to which globular or diffuse precipitates are visible on the polyurethane surface. These deposits seem to correspond to the deposition of salivary or pellicle glycoproteins, an important first step in the cascade of bacterial colonization. Ultrastructural SEM studies of denture surfaces have shown the formation of an electronically dense pellicle 2–6 m thick, different from the normal dental pellicle (Monsenego, 2000). However, it should be borne in mind that the composition of this conditioning film is influenced by the properties of the underlying substrate and the selective adsorption of environmental macromolecules, which can dictate the composition of the biofilm, which forms on the appliance (Lee *et al.*, 1974; Rykke and Sönju, 1991; Teughels *et al.*, 2006). Thus, it may be surmised that the composition of the salivary proteins adsorbed onto the surface of Invisaligners is compositionally different from that on enamel surfaces, leading to differing flora in the subsequent biofilm.

Initial pioneer species and the bacterial morphotypes of early biofilm were predominantly cocci or rod species, as has been demonstrated in a number of studies and on various surfaces (Lie, 1979; Nyvad and Fejerskov, 1987; Zee *et al.*, 1997; Sukontapatipark *et al.*, 2001). Zee *et al.* (1997) reported a similar trend in rapid and slow plaque formers, with cocci and some rods representing the majority of the bacterial species in early (up to 7 day) dental plaque. They also found differences in the proportions of gram-positive bacteria between the slow and fast groups over the initial period. In the latter stages of plaque development, from day 3 to 14, there appeared to be no major differences in the biofilms in either group in the present study, which is in agreement with the findings of Zee *et al.* (1997), yet the fast formers appeared to produce a more voluminous and complex intermicrobial matrix. However, these observations are qualitative in nature as no software was used to quantify the volume of biofilms between the two groups. On occasions, macroscopic indentations on the surface of the aligner were noted, which corresponded to 'attachments' fabricated on the tooth surface to aid tooth movement. These indentations, due to the recessed nature, provided an additional area of colonization that seem to encourage a greater degree of biofilm formation. The inner surface of the dental cusp tips also provided an ideal niche, which encouraged bacterial adhesion and biofilm formation, due to the sheltered locale as well as the difficulty with access for cleaning.

The predominant early flora in other intraoral appliances such as fitting surfaces of dentures have also been found to be predominantly gram positive and mostly cocci in nature (Monsenego, 2000). Sukontapatipark *et al.* (2001) in a time-dependent SEM study on dental plaque adjacent to orthodontic brackets observed typical morphological features of cleaned enamel surface, bonding resin, metal, and elastomeric surfaces of the appliance. The early stage of plaque formation was seen in the first week of the experiment. The enamel surface cervical to the bracket showed only a discrete layer of cocci whereas surfaces with excess composite, brackets, and ligatures exhibited a more coherent biofilm consisting of cocci and short rods embedded in an extracellular matrix. By the second week, the biofilm included more cocci, rods, and filamentous organisms giving a corncob appearance. Complex plaque structures could be seen on the composite surface within 2–3 weeks whereas the enamel surface showed only an early stage of plaque formation. The bacterial composition of plaque on stainless steel ligatures and elastomeric rings in orthodontic patients has been investigated (Brêtas *et al.*, 2005). It was found that there was no difference in microorganism retention between stainless steel ligatures and elastomeric rings. However, no detailed description of the SEM images was presented. Further studies are required to determine the quantity and nature of the dental plaque biofilm on various orthodontic appliances and to compare these with plaque biofilms formed on tooth surfaces.

A recent study revealed that patients who undergo Invisalign therapy are no more susceptible to periodontal disease than their fixed appliance counterparts and no statistically significant increase was found in probing depths during active treatment (Miethke and Vogt, 2005). The oral hygiene of patients with Invisalign appliances was also found to improve during the trial, attributed possibly to the generally older age of the patient pool, better motivation, and the removable nature of the appliance. In the present investigation, a normal oral and appliance hygiene regimen was instituted among the subjects, but a significant amount of bacteria and biofilm were found on the appliance surface. These surviving colonies may form the reservoirs of planktonic bacteria, which can be liberated into the oral environment to colonize other sites and may encourage faster re-establishment of biofilm on cleaned surfaces (Bowden and Li, 1997). Although in healthy individuals, this may not be a significant concern, this may have adverse consequences in patients with more compromised oral conditions.

Conclusions

The ultrastructure and morphology of dental plaque biofilm formed on the novel Invisalign orthodontic appliance used in the present study simulate the typical features of dental plaque biofilms seen with other intraoral appliances, with initial colonization by coccal and rod species. Therefore, the novel appliance could be utilized as a tool to study the nature of biofilm formation and its effect.

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