Investigation to test potential stereolithography materials for development of an *in vitro* root canal model.

Saif alarab A. Mohmmed[a], Morgana E. Vianna[b], Stephen T. Hilton[c], David R. Boniface[d], Yuan-Ling Ng[e], Jonathan C. Knowles[a]

[a] Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, University College London, London, United Kingdom.

Postal address: 256 Grays Inn Road, London WC1X 8LD

E-mail: Saif.mohmmed.12@ucl.ac.uk

j.knowles@ucl.ac.uk

[b] School of Dentistry, College of Biomedical and Life Sciences, Department of Learning and Scholarship, Cardiff University, Cardiff, United Kingdom.

Postal address: Heath Park Campus, Cardiff CF14 4XY

Email: ViannaM@cardiff.ac.uk

[c] School of Pharmacy, Faculty of Life Sciences, University College London, London, United Kingdom.

Postal address: 406, 29-39 Brunswick Square, London WC1N 1AX

E-mail: s.hilton@ucl.ac.uk

[d] Biostatistics Unit, UCL Eastman Dental institute, University College London, London, UK.

Postal address: 256 Grays Inn Road, London WC1X 8LD

E-mail: d.boniface@ucl.ac.uk

[e] Unit of Endodontology, UCL-Eastman Dental Institute, University College London, London, United Kingdom.

Postal address: 256 Grays Inn Road, London WC1X 8LD

Email: y.ng@ucl.ac.uk

Running title: Assessment of stereolithography materials used to create a root canal model

***Corresponding author:**
Saif alarab Mohmmed

Division of Biomaterials and Tissue Engineering

UCL Eastman Dental Institute

256 Grays Inn' Road

WC1X 8LD

London, UK

Phone: +44 20 3456 1090

E-mail: Saif.mohmmed.12@ucl.ac.uk

Key words: Stereolithography materials; dentine; physico-chemical properties; *Enterococcus faecalis*; biofilm.

Abstract

The aims were to compare the physico-chemical properties (zeta-potential, wettability, surface free energy) of stereolithography materials (STL) (Photopolymer, Accura) to dentine and to evaluate the potential of each material to develop *Enterococcus faecalis* biofilm on their respective surfaces. Eighteen samples of each test material (Photopolymer, Accura, dentine) were employed (total n = 54) and sectioned to 1 mm squares (5mm x 5mm) (n = 15) or ground into a powder to measure zeta-potential (n = 3). The zeta-potential of the powder was measured using the Nano-Zetasizer technique. The contact angle (wettability, surface free energy tests) were measured on nine samples using goniometer. The biofilm attachment onto the substrate was assessed on the samples of each material using microscopy and image processing software. The data were compared using one-way ANOVA with Dunnett *post-hoc* tests at a level of significance $P \leq 0.05$. Both STL materials showed similar physico-chemical properties to dentine. The materials and dentine had negative charge (Accura: -23.7mv, Photopolymer: -18.8mv, dentine: -9.11mv). The wettability test showed that all test materials were hydrophilic with a contact angle of 47.5°, 39.8°, 36.1° for Accura, Photopolymer and dentine respectively, and a surface free energy of 46.6, 57.7, 59.6 mN/m for Accura, Photopolymer and dentine, respectively. The materials and dentine proved suitable for attachment and growth of *E. faecalis* biofilm with no statistical differences ($p > 0.05$). Stereolithography materials show similar physico-chemical properties and growth of *E. faecalis* biofilm to dentine. Therefore, they may be an alternative to *in vitro* tests requiring dentine.

Introduction

It is known that bacteria are the main cause of apical periodontitis (Kakehashi et al., 1965). These bacterial cells are usually attached to surfaces and embedded in an extracellular polymeric matrix known as a biofilm, which makes its elimination difficult (Costerton et al., 1999). It has been shown that certain bacteria (e.g. strains of streptococcus mutans) are able to attach to type I collagen, which is the major organic component (90%) of dentine through the expression of surface adhesions and as such, form biofilms (Switalski et al., 1993). Furthermore, dentinal tubules provide a site for bacterial colonisation and a possible hiding place from the effects of shear forces, such as antimicrobial agents (Peters et al., 2001). Therefore, a major challenge of root canal treatment is the elimination of bacterial biofilm from the root canal system (Nair et al., 2005).

The investigation of biofilm removal from the dentine surface is problematic and often assessed indirectly in *in-vivo* studies by collecting samples from the main canal (Vianna et al., 2006), *ex-vivo* models comprise either histological examination of a part of the root after disinfection procedures (Nair et al., 2005; Vera et al., 2012) or the use of extracted teeth (Schaudinn et al., 2013). Although it might be an ideal environment, it may not be the optimum method to study root canal irrigation as the root canal components (dentine, cementum) are concealed body compartments (Cate-Ten, 1998), making them unavailable for direct visualization. In addition, the use of extracted teeth introduces many variables to the studies (Khalilak et al., 2008), in that sample standardization becomes a challenge with differences in dentinal tubule diameters and unique internal anatomy (Cate-Ten, 1998).

Attempts to mimic the root canal anatomy using converted gypsum to hydroxyapatite (Papageorgopoulou, 2013; Turner et al., 2011) have shown promising anatomical

features, but such materials are unsatisfactory for direct visualisation because they lack transparency. The use of transparent resin blocks has been advocated to study the effects of instrumentation on the shape of the root canals (Khalilak et al., 2008) and has been shown to be useful to study root canal irrigation (Nouioua et al., 2015). These blocks offer the advantage of transparency, but the root canal shapes are standardized circular canals, limiting the options to study the mechanics of fluid in oval canals.

Therefore, it seems justifiable to develop an *in vitro* model that allows the generation of multiple samples with the same anatomical features to investigate the effect of root canal irrigation on bacterial biofilm removal. The first step for an ideal biofilm *in vitro* model is the investigation of materials with regards to bacterial adhesion and growth as it is a crucial factor in the formation of biofilm (Donlan, 2002), ideally this should have properties similar to dentine with regards to bacterial adhesion. Materials that do not fulfill these characteristics affect negatively on microbial growth (Papageorgopoulou, 2013; Thakrar, 2014). Furthermore, it is fundamental that materials be designed with anatomical similarity (e.g. main root canal) as structural mechanics and geometry can influence microbial colonization and growth (Epstein et al., 2011).

Stereolithography materials, with 3D printing, have been used to create plastic models with simple or complex root canal anatomies (Kfir et al., 2013). The purpose of these models was to facilitate the treatment planning process and provide a trial of treatment approaches for *dens invaginatus* (dental developmental abnormality) (Kfir et al., 2013). The use of 3D printing models to study root canal disinfection has been explored in a preliminary study (Kfir et al., 2013) but the tested stereolithography material; Visijet® EX200 Plastic did not allow bacterial colonization and was not transparent. As such,

materials that are employed in such study should allow bacterial attachment and growth to serve as a useful biofilm model for *in vitro* experiments.

Bacterial adhesion to biotic and abiotic surfaces includes a process of two phases (Derjaguin and Landau, 1941). The initial phase of the bacteria–substrate interaction is determined by the physical and chemical properties (e.g. surface free energy. zeta-potential, hydrophobicity) of the substrate surfaces. This reversible interaction is followed by the second phase of molecular-level nonspecific interactions between the bacterial surface structures and the substrate (Verwey et al., 1999). Surface structures of bacterial cells include fimbrae, pili, and flagella (Tomaras et al., 2003).

It has been demonstrated that wettability and SFE play an important role in a wide range of microbial infections (Doyle, 2000). Microbial wettability is defined by the energy of attraction between a polar or slightly polar cells immersed in an aqueous phase (Van Oss, 1995).

The aim of the present study was to compare the physical and chemical properties (zeta-potential, wettability, and surface free energy) of potential substrate stereolithography (STL) materials (Photopolymer, Accura) to dentine, and to compare the attachment of bacterial biofilm (*E. faecalis*) onto the surface of these substrate materials to dentine.

Materials and Methods

1. Preparation of the samples

1.1 Preparation of dentine samples

A total of eighteen single-rooted, mature apices, and caries-free adult teeth were obtained from the Biobank, UCL Eastman Dental Institute (study reference number 1310). The teeth were stored in sterile water after extraction. Under aseptic conditions, the crown part of each tooth was removed using a rotary diamond wheel (Abrasive

Technology Inc., Westerville, USA), mounted on a straight air motor hand-piece (W&H UK Ltd, St Albans, UK) under water cooling. Pulp tissue in the root canal was removed using a barbed broach (Dentsply Tulsa Dental Specialties, Tulsa, OK, USA). The cementum was ground using a grinding wheel (Struers Ltd, Solihull, UK). Each root dentine was sectioned and 1 mm thick standard squares (5mm x 5mm) were created using a diamond wheel. The method was based on previous study (Sousa et al., 2009) but with changes in the dimensions of samples.

1.2 Preparation of the stereolithography (STL) material samples

Two STL materials were evaluated in this study. Firstly, the acrylic base photopolymer clear™ material (AZoNetwork Ltd., Cheshire, UK), which is composed of a mixture of methacrylic acid esters and photo-initiator. Secondly, the epoxy based Accura® ClearVue™ material (3D Systems, Inc., South Carolina, USA), which is composed of Bisphenol-A epoxy resin. These materials were manufactured by 3D printing technique, and delivered in the form of sheets of different dimensions. A total of eighteen sheets of each STL material were sectioned using a diamond wheel to create 1 mm thick standard squares (5mm x 5mm). All samples were smoothed by using grinder discs for 3 minutes (1200 µm, Struers Ltd, Solihull, UK).

2. Measurements of the zeta-potential of dentine and the stereolithography materials

The zeta-potentials (positive or negative charges) of the dentine and STL materials were determined using the Nano-Zetasizer device (Fletcher and Marshall, 1982; Hsu et al., 2013; Jones et al., 2011), which used the Laser Doppler Micro-electrophoresis technique to measure the charge.

A total of three square samples of each test material, Photopolymer, Accura, and dentine were ground using a Retsch grinding machine (Retsch Gmbh, Hanna,

Germany). This produced powder with particles with maximum size of 5 μm, which was achieved using sieves (Endecotts, London, UK). A total of 10 g of each material powder was mixed with 10 mL Brain Heart Infusion broth (Sigma-Aldrich, St. Louis, Montana, USA), which was vortexed at maximum speed for 30 seconds using a Vortex (IKA, Chiltern Scientific, Leighton, UK). One mL of each mixture was added individually into the cuvette of the Nano-Zetasizer device using 1 mL sterile pipettes (Alpha Laboratories Ltd, Winchester, UK). The software of the device was used to control the measurement of the zeta-potential of each sample. Measurements were taken in triplicate for each sample.

3. Comparison of contact angle and solid surface free energy between dentine and the stereolithography materials

The measurements of contact angle (θ) and surface free energy (*Y*) were achieved by the sessile drop method using a goniometer device equipped with a video camera (KSV instruments, Fairfield, Connecticut, USA) and an image analyser (Fletcher and Marshall, 1982; Hsieh et al., 2007; Jones et al., 2011). Three different liquids that included apolar [diiodomethane (Y=50.8 mN/m)] and polar [glycerol (Y=64 mN/m), water (Y=72.8 mN/m)] were used with each sample. A total of nine square samples of each test material (Dentine, Accura, and Photopolymer) were examined, with three samples per liquid. Each sample was placed on the stage of the goniometer and the contact angle of one drop of the designated liquid was measured. With each liquid droplet, five measurements were made. A manually controlled micrometre syringe was used to push liquid droplets onto the solid surface from above. The video signal of the sessile drop on the solid surface was acquired by use of a CCD camera connected to a digital video processor, which performed the digitization of the image. Attension Theta software (Biolin scientific, Staffordshire, UK) was used to measure the contact angle.

4. Comparison between bacterial biofilm growth and attachment on dentine and biomaterial substrates

4.1 Sterilisation of the samples

A total of six square samples of each test material (Dentine, Photopolymer, Accura) were placed individually into a packaging bags (Sterrad 100S, ASP®, Irvine, CA, USA) and then sterilised using gas plasma with hydrogen peroxide vapour (Sterrad 100S, ASP®, Irvine, CA, USA) for fifty minutes.

4.2. Preparation of microbial strain

A Gram-positive bacterial strain, *Enterococcus faecalis* (ATCC 19433) was grown onto the square samples of the dentine and STL materials. The strain was supplied in the form of frozen stock of Brain-Heart Infusion (BHI) broth (Sigma-Aldrich, St. Louis, Montana, USA) and 30% glycerol stored at -70 °C. Prior to the experiments, *E. faecalis* identity was confirmed using specific primers and conventional PCR (Sedgley et al., 2006). The strain was thawed to 37 °C over 10 minutes and vortexed for 30 seconds (Siqueira et al., 2002). After thawing, one hundred microliter aliquots of the bacterial strain was pipetted and plated onto a BHI agar plate with 5% defibrinated horse blood (E&O Laboratories Ltd, Scotland, UK) and incubated at 37 °C in the 5% CO_2 incubator for 24 hours. Bacterial morphology and catalase were confirmed before generation of biofilms. For this, two colonies of the strain were separately harvested using a sterile inoculating loop (VWR, Leicester, UK), and subjected to catalase test using 3% H_2O_2 (Sigma-Aldrich Ltd, Dorset, UK) and Gram staining test (BD Ltd., Oxford, UK).

4.3 Generation and staining of E. faecalis biofilm on the stereolithography material surfaces.

A concentration of 10^8 CFU/mL of *E. faecalis* cells was used as standard inoculum. For that, six colonies were harvested from the agar plate, placed into 20 mL of BHI

broth, and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. BHI containing *E. faecalis* was adjusted to 0.5 absorbance at wavelength of 600 nm measured using a spectrophotometer (NanoDrop™ Spectrophotometer ND-100, Wilmington, USA) (Al Shahrani et al., 2014). Inoculum concentration was confirmed in parallel to the experiment using six ten-fold serial dilutions, and dilutions plated on BHI agar plate (Sigma-Aldrich, St. Louis, Montana, USA) with 5% defibrinated horse blood (E&O Laboratories, Scotland, UK) and incubated at 37 °C in the 5% CO_2 incubator (LEEC, Nottingham, UK) for 24 hours. The colony forming units per milliliter (CFUs/mL) of 1.1 × 10^8 CFU/mL was determined.

Each sample was incubated with 1 mL of *E. faecalis* inoculum, which was delivered into a sterile 7 mL plastic bijou bottle (Sarstedt), containing the samples, using a sterile syringe (BD Plastipak™, Franklin Lakes, NJ, USA) and a 21-gauge needle (BD Microlance™, Franklin Lakes, NJ, USA). The samples were then incubated at 37 °C in the 5% CO_2 incubator (LEEC) for 10 days. Every two days, half of the inoculum that surrounded the sample was discarded using a syringe and a 30G needle and replaced with fresh BHI broth using a sterile syringe and needle (De-Deus et al., 2007).

After incubation, all samples with biofilms were removed from the plastic bottle and the biofilm on the surface of three samples of each material was observed using scanning electron microscopy (SEM) (FEI XL30 FEG SEM, FEI, Eindhoven, Netherlands). For this, the sample was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 ˚C overnight. Then, they were dehydrated in a graded series of alcohol (50, 70, 90, and 100%), placed in hexamethyldisilazane for 5 minutes and air-dried. Samples were mounted onto aluminium pin stubs, and sputter coated with gold/palladium (Polaron E5000, QUORUM Technology, UK) before examination using SEM. The other three samples were placed onto a slide and rinsed with 1 mL sterile

distilled water (Roebuck, London, UK) for 1 minute using a sterile 10 mL syringe (Plastipak, Franklin Lakes, New Jersey, USA) to remove loosely attached cells. Using a micropipette, 1 µL of CV stain (Merck, Darmstadt, Germany) was applied to the biofilm and left for 1 minute for staining. Each sample was subsequently washed with 3 mL of sterile distilled water for 1 minute to remove excess stain (Izano et al., 2007).

4.4 Assessment of bacterial growth and attachment

To quantify the surface coverage by biofilm, each sample was placed on the stage of an optical microscope coupled to a recording CCD camera (BX51, Olympus Optical Co., Ltd., Tokyo, Japan), and viewed using an objective lens (×20 magnification) (Cerca et al., 2005). For standardisation of measurement, a template was created using AutoCAD® software (Autodesk, Inc., San Rafael, CA, USA). The template was printed on transparency printer paper of the same size as the sample (5 mm × 5 mm) to provide a grid of 25 squares each of 1 mm^2. The template was placed over the sample and five squares of one mm^2 were imaged, the first square was located in the centre of the template and the other four at each corner of the center square. Surface area coverage with bacterial biofilm onto the surface of the five squares of each sample was quantified, using Image-pro plus 4.5 (MediaCybernetics®, Silver Spring, USA).

The method used to assess the attached biofilm was based on Cerca *et al.* study (Cerca et al., 2005). Each sample was grasped in the horizontal plane using tweezers, and immersed slowly for 10 seconds in 100 mL distilled water in a sterilised 100 mL glass tube (Sarstedt Ltd, Nümbrecht, Germany). The immersion cycle was repeated three times. The sample was then dried for 3 minutes at room temperature. The sample surface with bacterial biofilm was imaged and the difference in percentages

surface area of substrate coverage with bacteria biofilm attached to the samples before and after water immersion was quantified.

5. Statistical analysis

All data were analysed using SPSS (BM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). For evaluating differences in physico-chemical properties, means and standard deviations were calculated and descriptive analysis was used. The mean values of percentage surface area of dentine versus Photopolymer, Accura) coverage with *E. faecalis* biofilm before immersion experiments were compared using one-way ANOVA with Dunnett *post-hoc* test. The same test was used for the comparison of the mean difference in percentage surface area coverage with biofilm before and after immersion between dentine and STL material surfaces. All tests were performed at a level of significance $p \leq 0.05$ with a confidence level of 95%.

Results

1. Measurement of the zeta-potential

Mean value and standard deviation of the zeta-potential of the dentine and STL materials (Accura, Photopolymer) are presented in Table 1.

Both STL materials had the same negative charge as root dentine. However, the concentration of anionic electrolytes of dentine [-9.11 (±4.1)] mv) was less than Photopolymer [-18.8 (±3.5)] mv) and Accura [-23.7 (±6.9)] mv).

2. Measurement of the contact angle (wettability and surface free energy measurements)

Substrates physico-chemical characteristics presented by the contact angle and surface free energy parameters were obtained using the three liquids tested and are given in Table 2.

Measurement of contact angle showed that all test materials (Accura, Photopolymer, and Dentine) were hydrophilic (θ < 90). Dentine had the lowest water contact angle (more hydrophilic) [θ = 36.1° (±2.8°)]. Similarly, photopolymer material had hydrophilic properties [θ = 39.8° (±3.1°)], which was closer to dentine in comparison to the Accura material [θ = 47.5° (±0.3°)].

On the other hand, the surface free energy showed a variation consistent with the size of the standard deviations. The highest SFE was associated with dentine [59.6(±0.9) mN/m] followed by Photopolymer [57.7(±1.7) mN/m], while the lowest SFE was associated with Accura [46.6(±1.7) mN/m].

3. Assessment of biofilm growth and attachment

Representative SEM images of the biofilm onto the surface of the dentine and the STL materials are presented in Figure 1. The images indicate that the biofilms grew on the surface of dentine and the STL materials.

Interestingly, the surface area coverage with bacterial biofilm grown on the dentine surface was observed to be more abundant than on the two stereolithography surfaces as shown in Figure 2. The distribution of the biofilm on the surface of dentine and the STL materials is obviously different.

The mean values of percentage surface area coverage with *E. faecalis* biofilm attached to dentine *versus* the STL materials before 3 cycles of immersion in water are presented in Figure 3.

The mean value of percentage surface area coverage with biofilm was the highest on the dentine [68.7%, (±4.96)], while the lowest on Photopolymer substrate [64.2%, (±7.55)]; however, there was no statistically significant difference between these means (p = 1.00).

The 3 cycles of immersion in water of all experimental groups had a minimal effect on the removal of attached biofilm from the surface of the substrates. The mean difference of biofilm percentages before and after immersion in water is shown in Table 3.

It revealed that there was no statistically significant difference between biofilm grown on dentine surface and that on STL materials (Dentine vs Photopolymer p = 0.63, Dentine *vs* Accura p = 0.99).

Discussion

The present study aimed to compare the physico-chemical properties (zeta-potential, wettability, surface free energy) of stereolithography materials (Photopolymer, Accura) to dentine and to evaluate the substrate potential to develop *Enterococcus faecalis* biofilm.

Previous investigations reported that the wettability, surface free energy, and zeta-potential could influence bacterial adhesion to solid substrates (Cerca et al., 2005; Marshall et al., 1971). The best method to determine bacterial wettability and SFE is by contact angle measurements (Doyle, 2000). The effect of SFE of substrate on bacterial adhesion has been critically discussed in the literature. Some authors report that materials with low SFE result in less bacterial adherence (Bürgers et al., 2009; Liu and Zhao, 2005); whilst others report, that bacterial adhesion decreased with increasing surface energy of substrates (Absolom et al., 1983; McEldowney and Fletcher, 1986). However, the mechanism of bacterial adhesion is complex and depends on several factors including the physical and chemical properties of substrates and of the bacterial cell (Derjaguin and Landau, 1941). In general, the initial bacterial adhesion can be illustrated by Derjaguin, Landau, Vervey, and Overbeek (DLVO) theory (Derjaguin and Landau, 1941; Verwey et al., 1999) of calculating the

interaction energy between cells and substrate as a function of separation distance (Doyle, 2000). Adhesion can be mediated by non-specific interactions, with long-range characteristics, including van der Waals forces, electrostatic forces, and acid-base interaction forces (Van Oss, 1995). As soon as microorganisms reach a surface, they are either attracted to, or repelled by it, depending on the sum of the different non-specific interactions (Fonseca et al., 2001). Hydrophobic interactions and surface free energy (SFE) are usually the strongest of all long-range forces (Teixeira and Oliveira, 1999). In the present *in vitro* study, the two STL materials (Photopolymer and Accura) showed similar physico-chemical properties to that of dentine, and showed they were suitable for growth and attachment of single species biofilm (*E. faecalis*).

The selection of both substrates, Photopolymer and Accura, was related to their excellence in terms of optical transparency, which will enable direct and real-time imaging of biofilm removal by antibacterial agents (e.g. NaOCl).

The Gram-positive facultative *E. faecalis* type strain was selected to generate the biofilms because these species have been associated with secondary root canal infections (Endo et al., 2014). It has been reported that *E. faecalis* has a hydrophilic and negatively charged cell wall (George and Kishen, 2007) and exhibits an inherent resistance to antimicrobial agents, as well as possessing the capacity to adapt to changes in environmental conditions (Laplace et al., 1997). Furthermore, *E. faecalis* is able to develop a biofilm under different growth conditions, including aerobic, anaerobic, nutrient-rich and nutrient-deprived environments (George et al., 2005). However, single species biofilm may be considered a limitation of the present study and future investigations using multi-species biofilms, including Gram-negative species may be valuable to be explored in the future.

The initial inoculum concentration was in accordance with study (Sena et al., 2006), which was around 10^8 CFU/mL. In addition, this concentration represents cell concentrations (of total bacteria) found in infected root canal systems determined by culture (Zavistoski et al., 1980).

A total of ten days of *E. faecalis* biofilm growth was chosen for this study as it has been shown to produce standardised biofilm models for testing the efficacy of antimicrobial agents (Sena et al., 2006). Furthermore, this species have been used to evaluate efficacy of irrigation solutions (Al Shahrani *(Al Shahrani et al., 2014; Sena et al., 2006)*. The results of the present study have shown that microbial colonization was consistent on dentine and STL material surfaces.

In order to assess biofilm attachment, staining with crystal violet and washing with water, as this is a simple and standardized method to verify bacterial adhesion to surfaces (Adetunji and Isola, 2011; Stepanović et al., 2004), three cycle of sample immersion was selected as it had previously been adopted in another study (Cerca et al., 2005).

Optical microscopy and image processing software were used to image biofilm grown onto dentine and substrates surfaces. This type of microscopy has previously been used to assess oral biofilms growth (Wang et al., 2014), and attachment to substrates (Cerca et al., 2005). One major advantage of this technique is that it allows a direct visualisation of the samples, without need for fixation, dehydration or the disturbing of biofilm structures. Nevertheless, one limitation associated with the microscopy images was the presence of images surrounded by "halos" around the outlines of details. These are optical artifacts, which may obscure the boundaries of details. This may be

related to thickness of samples, which can interfere with light illumination (Wilson and Bacic, 2012).

For standardisation purposes and to reduce chances of bias, the same areas of all samples were examined; five fields of view were selected in central area of the sample. Although the areas measured may not represent the whole surface area of the sample, literature suggests that measurements from a regular array of points is more accurate than random assessment (Loebl, 1985).

Image analysis software (Image-Pro Plus) has been used to analyse the images from optical microscopy. This software has also been adopted in other studies for quantification of surface area coverage with simulant biofilms (Huang et al., 2008; McGill et al., 2008; Thakrar, 2014; Turner et al., 2011). One criticism that can be made in relation to all image-analysis techniques is that the areas measured are, to some extent, subjectively measured by the examiner. In order to reduce this limitation, a semi-automatic approach to measuring the biofilms was applied and imaging software was used to manually draw the biofilm outlines in the biofilms prior washing and the same template was used to obtain and calculate the biofilm area after washing, without further interference of the operator. This semi-automatic method provided operator-independent quantitative results.

In order to obtain an alternative STL model to dentine that allows microbial growth, it is important that these materials exhibit comparable properties to dentine. The physicochemical properties of the STL materials was shown to be similar to that of dentine. The negatively charged property of the STL material may be explained by the aqueous environments applied during measurement that result in more cations that can be solvated in comparison to anions on the surface (Shaw et al. 1988). The

hydrophilic property of the STL materials could be related to the hydrophilic hydroxyl groups in the molecules of resin materials (Wang et al., 2010). This is consistent with Fourier transform infrared spectroscopy (FTIR) readings where the two STL tested materials showed distinct peaks (≈ 3340 cm^{-1}) for hydrophilic hydroxyl groups (data not shown). According to DLVO theory, the negative charges of the bacterial cell (*E. faecalis*) and the substrates (dentine, photopolymer, and Accura) hinder bacterial adhesion due to charge-charge repulsions (Doyle, 2000). However, the findings of the present study showed that biofilm was able to attach and grow on the STL material and dentine. It could be also due to the formation of a conditioning layer over the model's surface by the bacteria itself using proteins within the BHI broth (Lehner et al., 2005). This layer may reverse the charge of the substrates surfaces to positively charge and promote the adhesion of planktonic microbial cells to the solid surface (Donlan, 2002). A second possible reason for the abovementioned bacterial attachment may be related to the hydrophilic properties of the test materials, which serve to overcome the repulsive force that exists between the negatively charged surfaces of both bacterial cells and test materials (Donlan, 2002). This finding is consistent with other studies that had shown more bacterial attachment on hydrophilic solid surfaces (Absolom et al., 1983; Almaguer-Flores et al., 2012). Nevertheless, other studies have failed to identify a correlation between surface hydrophobicity and the attachment of bacteria to a solid surface (Espersen et al., 1994).

Although the distribution of biofilm on the dentine and STL materials was different, the results showed no significant difference between the surface area coverage with biofilm onto the surface of STL substrates and that of dentine. Some factors could explain these results: (a) the level of charges could play a role in the bacterial adhesion, as dentine presented lower negative charges which could reduce the

repulsion charges; (b) the roughness of dentine favoured biofilm attachment and subsequent growth, (c) the presence of type I collagen in the dentine could provide extra substrate for bacterial growth (Kishen et al., 2008). Therefore, the type of substrate can influence the distribution of the grown biofilm.

The results of the physico-chemical properties measured and bacterial adhesion demonstrate that stereolithography materials are an exciting option for the development of a novel biofilm model to be used for *in vitro* experiments with the advantages of direct visualisation and the development of a biofilm somewhat the same as in the natural environment. This is particularly important to study the outcomes of the interaction between an irrigant (and/or irrigant method) and bacterial biofilm within the root canal system. Also, the mechanics of fluids within the root canal could be investigated in real time.

Although the agreements between the tests were good and the aims were indeed achieved, the sample size may be considered as a limitation, considering that forty-five samples were used with n = 3 per group was a relatively small number. As a robust calculation of the optimal sample size is important for the minimization of the risk of type I or II errors (Schuurs et al., 1993), a larger sample size may be able to indicate larger differences so as to indicate either Photopolymer or Accura as the most suitable material to create the *in vitro* biofilm models, whereas the present study only revealed that both materials presented similarly relative to dentine.

Overall Photopolymer or Accura proved to be potential materials to create an *in vitro* biofilm model to study irrigation. However, the adhesion mechanism to the STL substrate remains to be explored in future investigations, as well as the adhesion and growth of multi-species biofilms.

Conclusion

Within the limitations of the present study, the physical and chemical properties of stereolithography materials, Photopolymer, and Accura, were shown to be comparable to those of dentine. Furthermore, they allowed the attachment and growth of *E. faecalis* biofilm onto their surface to a similar extent to that of dentine. Within the limitations of this *in vitro* study, the tested stereolithography materials demonstrated good potential for use in *in vitro* tests that require microbial colonization with the advantage of transparency when compared to dentine. This could be applied to the study of root canal disinfection strategies using artificially infected models, in order to evaluate the fluid dynamics of biofilm removal during root canal irrigation.

Acknowledgements

The authors declare that there is no conflict of interest. The authors acknowledge that this study is based on ideas and concepts originated, developed and under investigation by Professor Kishor Gulabivala, strands of which have been presented at conferences and as Masters Dissertations. This work was partially supported by the Iraqi Ministry of Higher Education through a PhD Scholarship (# 1031). We would like to thank valuable guidance though technical aspects of this project received from Haitham Hussain, Graham Palmer and Nicola Mordan.

References

Absolom DR, Lamberti FV, Policova Z, Zingg W, van Oss CJ, Neumann A. 1983. Surface thermodynamics of bacterial adhesion. Appl Environ Microbiol 46(1): 90-97.

Adetunji VO, Isola TO. 2011. Crystal violet binding assay for assessment of biofilm formation by Listeria monocytogenes and Listeria spp on wood, steel and glass surfaces. Global Veterinaria 6(1): 6-10.

Al Shahrani M, DiVito E, Hughes CV, Nathanson D, Huang GT-J. 2014. Enhanced Removal of Enterococcus faecalis Biofilms in the Root Canal Using Sodium

Hypochlorite Plus Photon-Induced Photoacoustic Streaming: An In Vitro Study. Photomed Laser Surg 32(5): 260-266.
Almaguer-Flores A, Olivares-Navarrete R, Wieland M, Ximénez-Fyvie L, Schwartz Z, Boyan B. 2012. Influence of topography and hydrophilicity on initial oral biofilm formation on microstructured titanium surfaces in vitro. Clin Oral Implants Res 23(3): 301-307.
Bürgers R, Schneider-Brachert W, Rosentritt M, Handel G, Hahnel S. 2009. Candida albicans adhesion to composite resin materials. Clin Oral Investig 13(3): 293-299.
Cate-Ten A. 1998. Oral histology: development, structure, and function. Saint Louis, Missouri: Mosby.
Cerca N, Pier GB, Vilanova M, Oliveira R, Azeredo J. 2005. Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of Staphylococcus epidermidis. Res Microbiol 156(4): 506-514.
Costerton JW, Stewart PS, Greenberg E. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284(5418): 1318-1322.
De-Deus G, Brandão M, Fidel R, Fidel S. 2007. The sealing ability of GuttaFlow™ in oval-shaped canals: an ex vivo study using a polymicrobial leakage model. Int Endod J 40(10): 794-799.
Derjaguin B, Landau L. 1941. The theory of stability of highly charged lyophobic sols and coalescence of highly charged particles in electrolyte solutions. Acta Physicochim. URSS 14: 633-652.
Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis 8(9): 881-890.
Doyle RJ. 2000. Contribution of the hydrophobic effect to microbial infection. Microbes and infection 2(4): 391-400.
Endo MS, Signoretti FGC, Marinho ACS, Martinho FC, de Almeida Gomes BPF. 2014. PCR identification of endodontic pathogens and DNA quantification in samples from teeth with posttreatment apical periodontitis. Clin Lab Res Dent 20(4): 197-208.
Epstein A, Hochbaum A, Kim P, Aizenberg J. 2011. Control of bacterial biofilm growth on surfaces by nanostructural mechanics and geometry. Nanotechnology 22(49): 494007.
Espersen F, Wurr M, Corneliussen L, HøG A-L, Rosdahl VT, Frimodt-Møller N, SkinhøJ P. 1994. Attachment of staphylococci to different plastic tubes in vitro. J Med Microbiol 40(1): 37-42.
Fletcher M, Marshall K. 1982. Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. Appl Environ Microbiol 44(1): 184-192.
Fonseca A, Granja P, Nogueira J, Oliveira D, Barbosa M. 2001. Staphylococcus epidermidis RP62A adhesion to chemically modified cellulose derivatives. J Mater Sci Mater Med 12(6): 543-548.
George S, Kishen A. 2007. Effect of Tissue Fluids on Hydrophobicity and Adherence of Enterococcus faecalis to Dentin. J Endod 33(12): 1421-1425.
George S, Kishen A, Song P. 2005. The Role of Environmental Changes on Monospecies Biofilm Formation on Root Canal Wall by Enterococcus faecalis. J Endod 31(12): 867-872.
Hsieh Y, Gau C, Kung Wu S, Shen E, Hsu P, Fu E. 2007. Dynamic recording of irrigating fluid distribution in root canals using thermal image analysis. Int Endod J 40(1): 11-17.

Hsu LC, Fang J, Borca-Tasciuc DA, Worobo RW, Moraru CI. 2013. Effect of micro- and nanoscale topography on the adhesion of bacterial cells to solid surfaces. Appl Environ Microbiol 79(8): 2703-2712.

Huang TY, Gulabivala K, Ng YL. 2008. A bio-molecular film ex-vivo model to evaluate the influence of canal dimensions and irrigation variables on the efficacy of irrigation. Int Endod J41(1): 60-71.

Izano EA, Wang H, Ragunath C, Ramasubbu N, Kaplan JB. 2007. Detachment and killing of Aggregatibacter actinomycetemcomitans biofilms by dispersin B and SDS. J Dent Res 86(7): 618-622.

Jones M, JP, NM, JvdM, KG, Y-LN. 2011. Comparison of substrates for the fabrication of an artificial tooth-model to test endodontic disinfection protocols. Paper presented at the wladimir adlivakine european sosciety of endodontology research prize. Abstract retrieved from http://www. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2591.2011.01914.x/epdf. Abstract: Int Endod J. p 1189.

Kakehashi S, Stanley H, Fitzgerald R. 1965. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Patho 20(3): 340-349.

Kfir A, Telishevsky-Strauss Y, Leitner A, Metzger Z. 2013. The diagnosis and conservative treatment of a complex type 3 dens invaginatus using cone beam computed tomography (CBCT) and 3D plastic models. Int Endod J46(3): 275-288.

Khalilak Z, Fallahdoost A, Dadresanfar B, Rezvani G. 2008. Comparison of extracted teeth and simulated resin blocks on apical canal transportation. Iran Endod J 3(4): 109.

Kishen A, Sum C-P, Mathew S, Lim C-T. 2008. Influence of Irrigation Regimens on the Adherence of Enterococcus faecalis to Root Canal Dentin. J Endod 34(7): 850-854.

Laplace J-M, Thuault M, Hartke A, Boutibonnes P, Auffray Y. 1997. Sodium hypochlorite stress in Enterococcus faecalis: influence of antecedent growth conditions and induced proteins. Curr Microbiol 34(5): 284-289.

Lehner A, Riedel K, Eberl L, Breeuwer P, Diep B, Stephan R. 2005. Biofilm formation, extracellular polysaccharide production, and cell-to-cell signaling in various Enterobacter sakazakii strains: aspects promoting environmental persistence. J Fo Prot 68(11): 2287-2294.

Liu Y, Zhao Q. 2005. Influence of surface energy of modified surfaces on bacterial adhesion. Biophys Chem 117(1): 39-45.

Loebl J. 1985. Image analysis: principles and practice: Oxford University Press, USA.

Marshall K, STOUT R, Mitchell R. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J Gen Microbiol 68(3): 337-348.

McEldowney S, Fletcher M. 1986. Variability of the influence of physicochemical factors affecting bacterial adhesion to polystyrene substrata. Appl Environ Microbiol 52(3): 460-465.

McGill S, Gulabivala K, Mordan N, Ng YL. 2008. The efficacy of dynamic irrigation using a commercially available system (RinsEndo®) determined by removal of a collagen 'bio-molecular film'from an ex vivo model. Int Endod J41(7): 602-608.

Nair P, Henry S, Cano V, Vera J. 2005. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit"

endodontic treatment. Oral Surg Oral Med Oral Patho, Oral Radiology, and Endodontology 99(2): 231-252.
Nouioua F, Slimani A, Levallois B, Camps J, Tassery H, Cuisinier F, Bukiet F. 2015. A preliminary study of a new endodontic irrigation system: Clean Jet Endo. Odontostomatol Trop 38(149): 13-22.
Papageorgopoulou L. 2013. Pilot studies to develop an artificial tooth model with standardized apical anatomies for the evaluation of the efficacy of root canal irrigation. (Unpublished master's thesis).Eastman Dental Institute, UCL. London, UK.
Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ. 2001. Viable Bacteria in Root Dentinal Tubules of Teeth with Apical Periodontitis. J Endod 27(2): 76-81.
Schaudinn C, Jaramillo D, Freire MO, Sedghizadeh PP, Nguyen A, Webster P, Costerton JW, Jiang C. 2013. Evaluation of a nonthermal plasma needle to eliminate ex vivo biofilms in root canals of extracted human teeth. Int Endod J46(10): 930-937.
Schuurs A, WU MK, Wesselink P, Duivenvoorden H. 1993. Endodontic leakage studies reconsidered. Part II. Statistical aspects. Int Endod J26(1): 44-52.
Sedgley C, Buck G, Appelbe O. 2006. Prevalence of Enterococcus faecalis at Multiple Oral Sites in Endodontic Patients Using Culture and PCR. J Endod 32(2): 104-109.
Sena N, Gomes B, Vianna M, Berber V, Zaia A, Ferraz C, Souza-Filho F. 2006. In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms. Int Endod J39(11): 878-885.
Siqueira JF, Rôças IN, Lopes HP. 2002. Patterns of microbial colonization in primary root canal infections. Oral Surg Oral Med Oral Patho, Oral Radiology, and Endodontology 93(2): 174-178.
Sousa C, Teixeira P, Oliveira R. 2009. Influence of surface properties on the adhesion of Staphylococcus epidermidis to acrylic and silicone. Int j biom 7: 1-9.
Stepanović S, Ćirković I, Ranin L. 2004. Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. Lett Appl Microbiol 38(5): 428-432.
Switalski L, Butcher W, Caufield P, Lantz M. 1993. Collagen mediates adhesion of Streptococcus mutans to human dentin. Infect Immun 61(10): 4119-4125.
Teixeira P, Oliveira R. 1999. Influence of surface characteristics on the adhesion of Alcaligenes denitrificans to polymeric substrates. J adh sci tech 13(11): 1287-1294.
Thakrar M. 2014. Development of an in vitro artificial infection model with standardised apical root canal anatomy. (Unpublished master's thesis).Eastman Dental Institute, UCL. London, UK.
Tomaras AP, Dorsey CW, Edelmann RE, Actis LA. 2003. Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. Microbiology 149(12): 3473-3484.
Turner D, NM, JvdM, KG, Y-LN. 2011. Investigation of artificial tooth models for assessing the efficacy of root canal irrigation protocols using sodium hypochlorite. Paper presented at the wladimir adlivakine european sosciety of endodontology research prize. Abstract retrieved from http://www. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2591.2011.01914.x/epdf. Abstract: Int Endod J. p 1189.

Van Oss C. 1995. Hydrophobicity of biosurfaces—origin, quantitative determination and interaction energies. Coll Surf B: Bioint 5(3): 91-110.
Vera J, Siqueira Jr JF, Ricucci D, Loghin S, Fernández N, Flores B, Cruz AG. 2012. One- versus Two-visit Endodontic Treatment of Teeth with Apical Periodontitis: A Histobacteriologic Study. J Endod 38(8): 1040-1052.
Verwey EJW, Overbeek JTG, Overbeek JTG. 1999. Theory of the stability of lyophobic colloids: Courier Corporation.
Vianna ME, Horz HP, Gomes BPFA, Conrads G. 2006. In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. Int Endod J 39(6): 484-492.
Wang X, Huang J, Huang K. 2010. Surface chemical modification on hyper-cross-linked resin by hydrophilic carbonyl and hydroxyl groups to be employed as a polymeric adsorbent for adsorption of p-aminobenzoic acid from aqueous solution. Chemical Engineering Journal 162(1): 158-163.
Wang Y, da Silva Domingues JF, Subbiahdoss G, van der Mei HC, Busscher HJ, Libera M. 2014. Conditions of lateral surface confinement that promote tissue-cell integration and inhibit biofilm growth. Biomaterials 35(21): 5446-5452.
Wilson SM, Bacic A. 2012. Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. Nat. Protocols 7(9): 1716-1727.
Zavistoski J, Dzink J, Onderdonk A, Bartlett J. 1980. Quantitative bacteriology of endodontic infections. Oral Surg Oral Med Oral Patho 49(2): 171-174.

Table 1: Mean values (n = 3) of the zeta-potential of the dentine and stereolithography materials (Photopolymer, Accura).

Type of measurement	Sample Name	Zeta Potential (mV) (±SD)
zeta-potential	Dentine	-9.11(±4.1)
zeta-potential	Accura	-23.7(±6.9)
zeta-potential	Photopolymer	-18.8(±3.5)

SD = Standard deviation

Table 2: Mean values of the contact angle (θ) and surface free energy of the test materials (n = 3) at the liquid/solid interfaces.

Substrate	Contact angle θ (±SD)			Surface free energy (mN/m) (±SD)		
	Water	Glycerol	Diiodomethane	Y total	Y^d	y^p
Dentine	36.1(±2.8)	42.3(±3.4)	31.7 (±2.9)	59.6(±0.9)	44.5	15.1
Accura	47.5(±0.3)	68.8 (±2.2)	46.7 (±1.5)	46.6(±1.7)	27.9	18.8
Photopolymer	39.8 (±3.1)	47.7 (±3.4)	33.6 (±2.0)	57.7(±1.7)	36.9	20.8

Y total = surface free energies with their dispersive (Y^d) and polar (Y^p) components, SD = Standard deviation.

Table 3: One-Way ANOVA to compare the effect of water immersion on *E. faecalis* biofilm between dentine and stereolithography material (n = 3 per group).

Stereolithography material (reference category)	Mean difference (±SD)	95% Confidence interval for coefficient	*P* value
Photopolymer (Dentine)	0.3(±0.7)	1.2, -0.5	0.7
Accura (Dentine)	0.1(±0.7)	0.9, -0.8	0.9

SD= Standard deviation

Figure 1: SEM images (x2000, x10000 magnification) illustrate that the *E. faecalis* biofilm grown onto the surface of the (a) dentine, (b) Photopolymer, or (c) Accura sample surface after ten-day incubation.

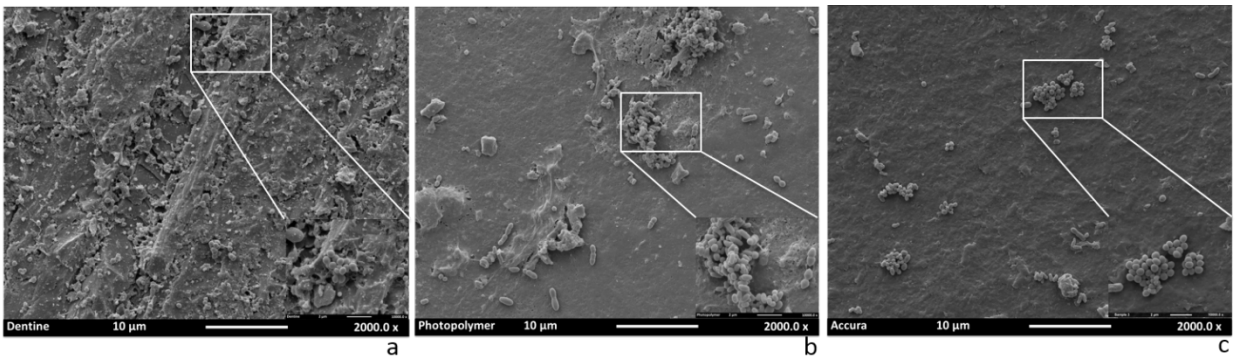
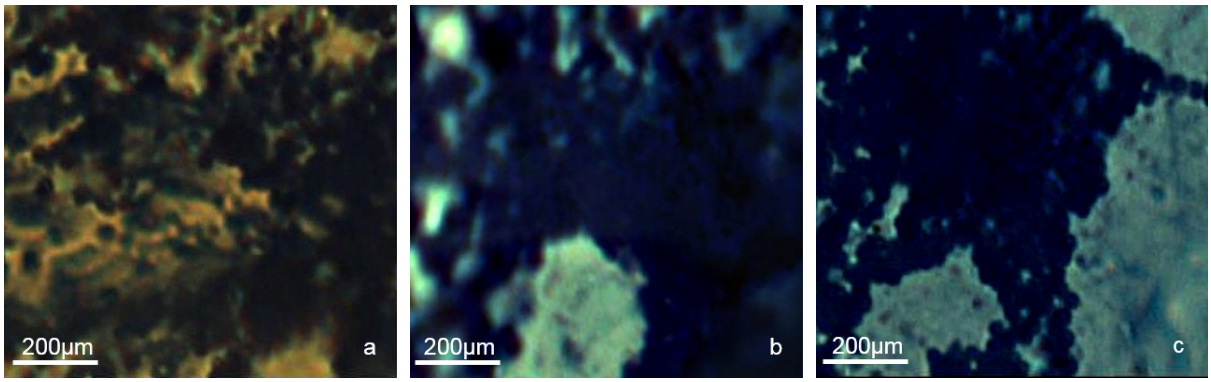


Figure 2: Microscopy images (×20 magnification) of crystal violet stained *E. faecalis* biofilm on one of the (a) dentine, (b) Photopolymer, or (c) Accura sample surface after ten-day incubation.



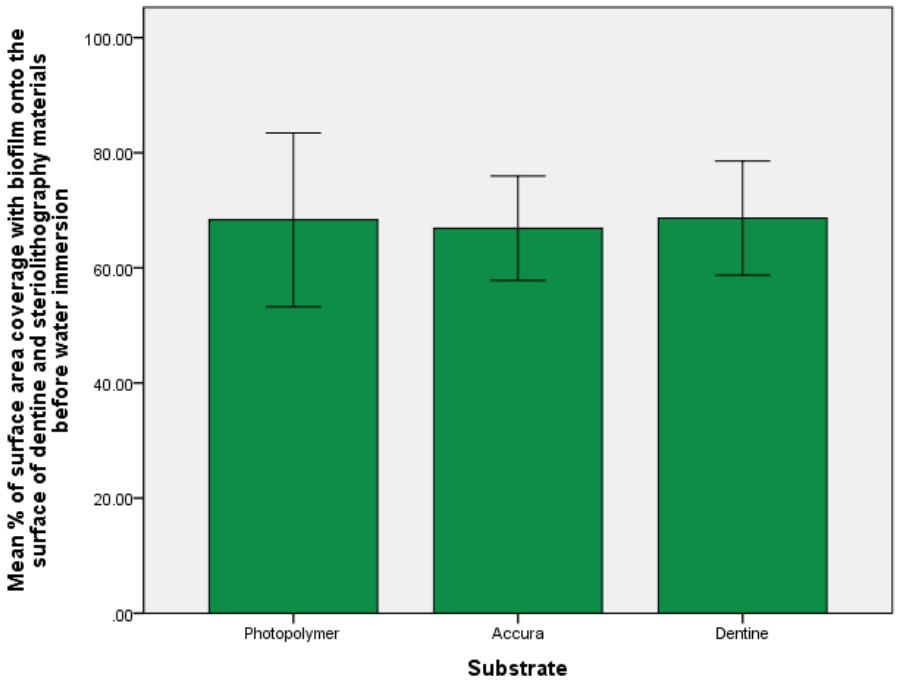


Figure 3: Mean and standard deviation values of percentage area of surface coverage with biofilm, stratified by substrate material (dentine, Photopolymer, Accura).