***Antibacterial Effect of Titanium Dioxide-Doped Phosphate Glass Microspheres Filled Total-Etch Dental Adhesive on S. mutans Biofilm***

***Running Title: Antibacterial Total-Etch Dental Adhesive***

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**Abstract**

**Purpose:**

to improve the antibacterial action of a two-step total-etch dental adhesive by using titanium dioxide-doped phosphate glass microspheres (GMs) without affecting its penetration ability.

**Materials and Methods:**

Five and 10 wt% of APTES silanized [surface treated with 3-Aminopropyltriethoxysilane (APTES)] and non-silanized GMs have been used as a filler to AdperTM Single Bond 2 Refill. The morphology, chemistry and *ζ -* potential of GMs have been investigated using scanning electron microscopy, Fourier transform infra-red (FTIR) spectroscopy and Zeta-sizer respectively. The chemistry and antibacterial action of filled adhesive have been investigated using FTIR and nitrocellulose filter membranes (NFM) *S. mutans* biofilm model respectively. The number of colony forming units (CFU) per NFM was considered. The contact angle and microtensile bond strength of adhesives to mid-coronal dentin, as a measure of its penetration ability, have been investigated using a Drop Shape Analyzer and microtensile testing machine respectively. AdperTM Single Bond 2 Refill was used as a control.

**Results:**

The size of GMs varied from 60-200 µm. The silanization process was confirmed by reduction in ζ-potential [-7 (±2) mV] and the presence of amide (1500-1600 cm‑1), C-N (1380 cm‑1), Si-O-Si (1096 cm‑1) and Si-O-C (780 cm‑1) peaks. Incorporation of GMs had no adverse effect on monomer conversion. All tested adhesives including the control showed significantly higher antibacterial action (~5-7 log10 reduction in CFU) than the NFM control. All filled adhesives showed significantly higher antibacterial action (~1-2 log10 reduction in CFU) than the control adhesive. The non-silanized GMs filled adhesives showed the highest antibacterial action against *S. mutans* biofilm formation. The presence of silanized GMs did not affect the wetting but increased the microtensile bond strength of the adhesive to dentin.

**Conclusion:**

Glass microsphere modified adhesives could be promising to reduce the possibility of recurrent caries around restorations.

**Key words:** Total-etch adhesive, phosphate glass microspheres, antibacterial, FTIR & microtensile bond strength

**Highlights:**

* AdperTM Single Bond 2 was modified with silanized and non-silanized glass microspheres (GMs).
* GMs has no adverse effect on polymerization or contact angle.
* GMs produced a non-significant increase in microtensile bond strength.
* GMs produced ~1-2 log10 reduction in CFU of *S. mutans*.
* Non-silanized GMs produced the highest reduction in CFU of *S. mutans.*

**Graphical Abstract**



**1. Introduction**

The introduction and advances in the chemistry of bonding agents lead to the evolution of caries management from G. V. Black “extension for prevention” to “minimally invasive cavity preparation design” (1). Therefore, it is no longer necessary to prepare cavities with mechanical retention through unnecessary cutting of tooth structures. Modern dental adhesives are grouped into total- or self-etch. Total-etch (TE) systems rely on the use of a separate etching step using 30-40% phosphoric acid gel to etch both enamel and dentin before the application of a separate primer and bonding agent (three-step TE) or combined primer with bonding agent (two-step TE). Etching dentin removes the smear layer, open the dentinal tubules and exposes the collagen network. Rinsing of enamel and dentin is therefore required after etching with phosphoric acid gel. . Self-etch (SE) adhesives on the other hand, condition both enamel and dentin simaltanoulsy. They depend on the use of acidified primer, generally phosphoric acid ester, which does not require rinising followed by the bonding agent (two-step SE). In some formulations, both acidified primer and bonding agent are combined (one-step SE).. . Reducing the number of steps has been attempted to reduce the application time and technique sensitivity (2, 3).

For the success of any adhesive restoration, tooth structure to which adhesive is applied also plays a key role. Unlike enamel, bonding to dentin is highly challenging and less predictable due to its complex composition and structure. Unlike enamel, dentin has less inorganic (hydroxyapatite) but more organic content (mainly type I collagen). Dentin is also connected to the pulp; numerous fluid-filled tubules extend from the pulp to dentino-enamel junction. The fluid within the dentinal tubules undergoes a continuous outward pressure from the pulp (25-30 mm Hg) and within each dentinal tubule there is an odontoblastic process extending from the pulp. Each dentinal tubule is surrounded by hypermineralized “peritubular” dentin in contrast to the hypomineralized “intertubular dentin” that lies between the dentinal tubules (4).

Regardless of the high clinical performance of total-etch adhesive (5), one of the common problems associated with its use is the discrepancy between dentin demineralization and monomer penetration depths (6). The depth of dentin demineralization varies from 1.1 ± 0.1 to 8.1 ± 0.6 m when the etching time varies from 5 to 120s (7). Penetration of adhesive monomer into intertubular dentin of superficial layer is responsible for most of bond strength to dentin. Penetration of adhesive monomer however into intertubular dentin deep layers is responsible for higher bond strength to dentin. Another common problem with the total-etch adhesive is the technique sensitivity. Improper air drying technique or dessication of the demineralized dentin after rinsing could lead to collapse of the collagen network. This would prevent the penetration of monomer into deeper areas of demineralized dentin; nanoleakage will be expected (8). Also collapse of collagen network makes whatever has polymerized and adhered to it extremely weak and susceptible to “pull out”.

The bond durability and long-term success of an adhesive restoration depends manily on the quality of seal created between the restoration and tooth structure; this seal is highly essential for reducing bacterial penetration along the tooth-restoration interface (9). The penetration ability of the adhesive will therefore aid in improving the seal along the dentinal walls. The antibacterial action of dental adhesives is also another key element in long-term successful restorations; this action is required to eradicate the destructive effect of residual or invading bacteria on tooth structure (10).

Phosphate-based glasses can be easily prepared with a wide variety of compositions, depending on metal oxides included, to meet the required applications (11). Under suitable conditions, they release ions such as calcium and phosphate (12, 13) required for remineralization or copper (14), silver (15), zinc (16) and titanium (12, 17) known for their therapeutic (antibacterial) effect. Copper-doped phosphate-based glass has been employed as a filler for an experimental phosphate-substituted dental adhesive. To improve the copper ion release and hence their antibacterial action, these glasses were used as irregularly-shaped microparticles and regularly shaped microspheres (13). The incorporation of up to 20 wt% glass filler had no adverse effect on the polymerization process of the experimental adhesive. The incorporation of up to 5 wt% glass filler was found to be significantly effective antibacterial against *S. mutans* and *P. aeruginosa.* The copper ion release was signficnatly increased with the microspheres and this was reflected on their antibacterial action against *P. aeruginosa* when compared with microparticles. On the other hand, the microparticles were more effective than microspheres against *S. mutans*. Regardless of the significant improvement in the antibacterial action, all glass-filled adhesives showed lower antibacterial action than the positive controls. Furthermore, silanaization of the glass fillers was not attempted; accordingly these fillers could weaken the experimental adhesives.

One of the common phosphate glass systems that showed favourable biocompatibility and new bone formation after 5 weeks implantation in rat calvarium is titanium dioxide doped system (17-19). Accordingly, this system could potentially be used for both antibacterial and remineralization action. This study therefore aimed to investigate the effect of incorporation of these glass microspheres on antibacterial action, wettability and microtensile bond strength of a two-step total-etch dental adhesive. Null hypothesis was “addition of glass microspheres into a two-step total-etch adhesive has no significant effect on antibacterial action, wettability and microtensile bond strength to dentin”.

**2. Materials & Methods**

**2.1. Glass Microspheres**

**2.1.1. Preparation and Silanization**

Calcium phosphate glass - 40P2O5·16CaO· 24MgO·17.5NaO·2.5TiO2 mol % - was prepared using the P2O5, CaHPO4, MgHPO4, NaH2PO4, and TiO2 (Sigma-Aldrich, U.K.) as precursors –Table (1). These precursors were weighed and dried in a 100 mL volume Pt/5% Au crucible at 350 °C for 30 min in an air furnace and then melted and held at 1280°C for 2 h. The molten glass was then poured onto a steel plate and allowed to cool down to room temperature. The cooled glasses were then ground into microparticles using a Retsch PM100 milling machine. To control the size of powder particles, the ground powder was passed through a series of sieves with different pore sizes ranging from 60 to 125 μm. Solid glass microspheres were then produced using the sieved glass particles via ﬂame spheroidization process utilising a thermal spray gun (Metallisarion, U.K.) according to the manufacturing process described elsewhere (13, 20).

Glass microspheres were treated with 3-Aminopropyltriethoxysilane (APTES, 99%, Sigma-Aldrich, UK) 1 h (21) by adding 1 g of glass microspheres to 20 mL of 0.2 M APTES solution (in 90% ethanol 10% DI water) followed by a gentle agitation by hand for 1 minute. The microspheres were then allowed to soak in the APTES solution for 1 h at room temperature. The treated samples were then washed three times with the same solvent (i.e., 90% ethanol 10% deionized water) to remove any unbound APTES and then dried in an oven at 50 ºC for 4 h.

**2.1.2. Characterization**

***2.1.2.1. Scanning Electron Microscopy***

The morphology of glass microspheres was studied using scanning electron microscopy (SEM, JEOL JSM6480LV, USA) after sputter coating with gold at an accelerating voltage of 20 kV.

***2.1.2.2. ζ - Potential***

Zeta*(ζ)* potential measurements were conducted using a Zeta-sizer (Malvern Zeta-sizer Nano ZSP®, UK). Dilute suspension (0.1 wt%) of samples in deionized water were placed in the capillary electrode cell and the ζ-potentials were measured as an average of 5 measurements from 100 scans each.

***2.1.2.3. Fourier Transform Infra-Red Spectroscopy (FTIR)***

The chemical groups of the silane treated glass microspheres were identified using a FTIR spectroscopy (Tensor-27, Bruker, Germany) equipped with a standard attenuated total reflectance (ATR) cell (Pike Technology, UK). The microspheres were scanned in transmittance mode over the wavenumber range from 2000 to 550 cm-1 and at a resolution of 1 cm−1.

**2.2. Glass Microspheres Filled Adhesives**

**2.2.1. Preparation**

A two-step total etch dental adhesive (AdperTM Single Bond 2 Refill, 3M ESPE, USA) was modified with 5 and 10 wt% glass microspheres (silanized and non-silanized).Unmodified adhesive was used as a control – Table 1. The glass microspheres were weighted and simply added to the adhesive bottle at the correct wt%. For proper dispersion of the glass microspheres within the adhesive, the mix was initially mechanically stirred using vortex mixer (ZX3 Advanced Vortex Mixer, VELP Scientifica SrL, Italy) for 5 min. Additionally prior to each application, the filled adhesives was mechanically stirred for 2 min.

***Table 1: Materials and codes of different adhesives used in this study.***

|  |  |  |  |
| --- | --- | --- | --- |
| **Titanium dioxide doped phosphate glass microspheres** | | | |
| **Precursors Used/Chemical Formula** | **Oxides Required /Chemical Formula** | | **Oxides Mole %** |
| Phosphorous pentoxide/ P2O5 | Phosphorous pentoxide /P2O5 | | 40 |
| Calcium hydrogen phosphate/ CaHPO4 | Calcium oxide/CaO | | 16 |
| Magnesium hydrogen phosphate/MgHPO4 | Magnesium oxide/MgO | | 24 |
| Sodium dihydrogen phosphate/NaH2PO4 | Sodium oxide/Na2O | | 17.5 |
| Titanium dioxide/TiO2 | Titanium dioxide/TiO2 | | 2.5 |
| **Glass microspheres filled adhesives** | | | |
| **Description** | | **Codes** | |
| AdperTM Single Bond 2 Refill Adhesive | | Adh | |
| 5 wt% non-silanized glass microspheres-filled adhesive | | 5% nSiGMs-Adh | |
| 10 wt% non-silanized glass microspheres-filled adhesive | | 10% nSiGMs-Adh | |
| 5 wt% silanized glass microspheres-filled adhesive | | 5% SiGMs-Adh | |
| 10 wt% silanized glass microspheres-filled adhesive | | 10% SiGMs-Adh | |

**2.2.2. Characterization**

***2.2.2.1. Fourier Transform Infra-Red Spectroscopy (FTIR)***

The chemical groups of each adhesive were identified before and after light curing using a FTIR spectroscopy. Before polymerization, a 100 µl of each adhesive was placed on ATR cell and scanned. For polymerized adhesives, a 100 µl of each adhesive was placed between two celluloid sheets, light cured for 10s using a light curing gun [light-emitting diode (LED) curing unit; 3M ESPE, Elipar, Seefeld, Germanydelivering 1200 mW/cm2, at 430–480 nm] and then scanned in transmittance mode as described above.

***2.2.2.2. Antibiofilm Analyses***

Stock culture of *S. mutans* NCTC 10449 strain was grown on brain heart infusion agar (BHI) before biofilm experiment. Biofilms of *S. mutans* were grown on nitrocellulose filter membranes (NFM; 47 mm diameter, 0.45 μm pore size; Invitrogen Ltd, Paisley, UK) which were treated with different adhesives given in Table 1. The antiadhesive nature and inhibition of biofilm formation of different adhesives was compared with NFM negative control.

Firstly, 100 µl of each adhesive was placed on NFM that was placed onto the centre of each BHI plate and light cured for 10s. A 100 µl of *S. mutans* suspended in phosphate buffer solution (PBS) (approx.108 cells/ml) was spread evenly across the surface, using a sterile spreader, for 1 min. The resulting BHI plates were incubated at 37°C in aerobic cabinet for 72 h. After incubation, the NFM from the plates were transferred to respective sterile containers with 20 ml PBS to disrupt the biofilm. The resulting cell suspensions were then serially diluted in PBS and cultured on BHI plates to enumerate viable bacteria in terms of total colony-forming units (CFU, which were log10 transformed) present per NFM. The results were compared with negative control that had biofilm growth on NFM. All experiments were conducted in triplicate.

***2.2.2.3. Contact Angle Measurements***

Extracted human molars, collected from Oral Surgery Clinic, King Abdulaziz University Dental Hospital, were used after obtaining an ethical approval (no. 102-11-17) and informed consents from the patients. Teeth were attached to rectangular cold cure acrylic (Davis Schottlander & Davis Ltd, UK) molds, used as holders, using a sticky wax (Kerr Corporation, USA) to facilitate the removal of occlusal enamel, superficial dentin and whole roots using diamond metal bond blades (102 x 31 x 12.7 mm) mounted on TechCut 4TM precision low speed saw (TechCut 4TM, Allied, USA). The cutting was carried out under 500 rotation per minture (RPM) and water coolent. Fifteen dentin discs of approximately 1mm thickness, prepared from the mid-coronal dentin to ensure consistency between samples in term of dentinal tubule size and density, were prepared. Then they were finished at the top surface with #240, 400, and 600 grit silicon carbide papers (Struers, USA) under water coolent using automatic polishing machine (Struers, USA) to creat a standardized smear layer and to provide flat and smooth dentin surfaces. The dentin discs were etched for 10s with 37% phosphoric acid gel to remove the smear layer and plug and to demineralize the dentin. The etched dentin samples were rinsed with water for 10s, gently air-dried. During the air-drying, the distance between the the sample and water-air syring was kept constant. Dentin samples were then divided into three groups according to the adhesive used (Adh, 5% SiGMs-Adh and 10% SiGMs-Adh). A drop of each adhesive was applied on the surface of each dentin disc and the contact angle measurement was taken after 5s using a Drop Shape Analyzer (DSA100, Krüss).

***2.2.2.4. Microtensile Bond Strength (MPa)***

Teeth were attached to rectangular cold cure acrylic (Davis Schottlander & Davis Ltd, UK) molds, used as holders, using a sticky wax (Kerr Corporation, USA). The occlusal enamel, superficial dentin and 2/3 of the roots were removed using diamond metal bond blades (102 x 31 x 12.7 mm) mounted on TechCut 4TM precision low speed saw (TechCut 4TM, Allied, USA). The cutting was carried out under 500 rotation per minture (RPM) and water coolent. Flat dentin surface was prepared using #240, 400, and 600 grit silicon carbide papers with water coolent. Samples were etched, rinsed with water, gently air-dried and divided into three groups according to the adhesive used (Adh, 5% SiGMs-Adh and 10% SiGMs-Adh) as described above. Two coats of the adhesive was applied over 15 using microbrush, gently air dried for 5s and light cured for 10s using a light curing gun [light-emitting diode (LED) curing unit; 3M ESPE, Elipar, Seefeld, Germanydelivering 1200 mW/cm2, at 430–480 nm] according to the manufacturer’s instructions. During the air-drying and light curing, the distance between the the sample and water-air syring as well as the LED was kept constant. Then the universal restorative resin composite Filtek Z350 XT (3M, USA) was used to build-up a composite of approximately 6 mm height in increments of 2 mm thickness each. The surface of each increment was pressed flat and smooth by using a glass slab and through which the composite was light cured for 20s before applying the next increment. A single operator carried out all the bonding procedures. The bonded samples were then sectioned parallel to the long axis of the tooth using diamond metal bond blades (102 x 31 x 12.7 mm) mounted on TechCut 4TM precision low speed saw (TechCut 4TM, Allied, USA). After cutting, each slice was then attached again to acrylic molds using a sticky wax and sectioned into quadrangular beams of approximately 1 mm thickness and 1 mm width . A diagrammatic representation of sample preparation is shown in Figure1. Prior to testing, the dimension of each beam was measured using a digital calliper (Vernier Caliper Electronic Digital LCD Screen Micrometer) and then attached to the jig of a microtensile testing machine (Mecmesin, UK) using a cyanoacrylate adhesive (Weicaon Contact Cyanoacrylate Adhesive). The bars were then loaded under a 0.5 mm/min untile failure and the microtensile bond strength of each adhesive to dentin (n=10) was measured in MPa.



***Figure 1: Steps of samples preparation for microtensile bond strength measurements.***

**2.3. Statistical Analysis**

The *t*-test was then used to compare the means between each two groups at 0.5% significance level using GraphPad software (San Diego, Calif., USA).

**3. Results**

**3.1. Glass Microspheres**

**3.1.1. Scanning Electron Microscopy**

Glass microspheres were produced via the flame spheroidization process (22). Figure 2a shows the yield of the solid microspheres with the size ranges from 60 to 200 µm. All the particles were spherical shaped. However, the size range of microspheres was bigger compared to the starting glass particles.

**3.1.2. *ζ* – Potential**

The ζ-potential value of the phosphate-based glass microspheres was -30 (±3) mV, as presented in Figure 2b. However, the silanized microspheres showed the ζ-potential value reduced to -7 (±2) mV.

**3. 1.3. Fourier Transform Infra-Red Spectroscopy (FTIR)**

Figure 2c shows the FTIR spectra of silanized and non-silanized glass microspheres. The silanized glass microspheres showed amide group associated peaks at 1500-1600 cm‑1. The bands at 1380, 1096 and 780 cm‑1 were due to C-N, Si-O-Si and Si-O-C groups from 3-Aminopropyltriethoxysilane, respectively.



**Figure 2: SEM (a), *ζ - Potential* (b) and FTIR (c) of glass microspheres.**

**3.2. Glass Microspheres Filled Adhesives**

**3.2.1. Fourier Transform Infra-Red Spectroscopy (FTIR)**

AdperTM Single Bond 2 Refill adhesive was also characterized via FTIR spectroscopy before and after LED light curing to identify any changes in their functional groups (Figure 3a). According to the manufacturer product specification data sheet (23), the AdperTM adhesive contains BisGMA, HEMA, dimethacrylates, ethanol, water and a novel photo initiator. In addition, the adhesive incorporates 10 wt% of spherical silica particles (diameter ~ 5nm).

The assignments of the bands for the methacrylate-based mixed monomers (BisGMA, HEMA and dimethacrylates) were at 2974 cm-1 (-CH2 stretching), 1710 cm-1 (C=O stretching), 1638 cm-1 (aliphatic C=C stretching), 1456 cm-1 (-CH3-CH2 group), 1167 cm-1 (C–O stretching), and 810 cm-1 (C=C twist) (24). The broad band at 3000–3700 cm-1 (O–H stretching) was associated with the -OH stretching group of water. The peak at around 1100 cm-1 was attributed to the Si-O-Si group of the silica nanoparticles. After LED light curing, the disappearance of some of the major peaks in the fingerprint region (700-1250 cm-1) suggesting the crosslinking of the monomers into a complex polymer system. However, with the incorporation of varying amount (5 and 10 wt%) of silane treated glass microspheres into the adhesive, there was no noticeable change in their FTIR spectra (Figure 3b), as the microspheres were assumed to be coated by the adhesive resin during the LED light curing.



***Figure 3: FTIR spectra of a) AdperTM Single Bond 2 Refill adhesive before and after LED light cure and b) silanized glass microspheres modified AdperTM Single Bond 2 Refill adhesives after LED light cure in comparison with unfilled adhesive.***

**3.2.2. Antibiofilm Analyses**

The effect of fillers on biofilm growth of *S. mutans* was investigated using NFM biofilm model. Biofilm growth was measured in terms of colony forming units (CFU) per NFM. All samples, including the control adhesive, showed statistically significant (p<0.0001 for all formulations) growth inhibition (~5 log10 CFU reduction) of *S. mutans* compared with NFM control (Figure 4). Furthermore, all microspheres filled adhesives showed significantly (p<0.0001, p = 0.0045, p<0.0001, p = 0.0003 for 5% nSiGMS-Adh, 10% nSiGMS-Adh, 5% SiGMS–Adh and 10% SiGMS-Adh respectively) higher reduction in CFU when compared with the positive control adhesive (AdaperTM Single Bond 2 Refill). Both 5% and 10% non-silanized microspheres filled adhesives were the most active agents against *S. mutans* biofilm formation in this model; ~6-7 log10 CFU reduction was observed. An increase in concentration of silane treated microspheres filled adhesive from 5 to 10 wt% resulted in a significant (p = 0.008) decrease in antibiofilm action.



***Figure 4: Effect of* *glass microspheres modified AdperTM Single Bond 2 Refill adhesives on S. mutans biofilm formation, in terms of colony forming units. AdperTM Single Bond 2 Refill adhesive was used as positive control while NFM nitrocellulose filter membrane was used as negative control. \* Significant at 0.05.***

***3.2.3. Contact Angle Measurements***

As shown in Figure 5, addition of glass microspheres into adhesive did not produce any significant (p = 0.078 and 0.617 for 5% SiGMS-Adh and 10% SiGMS-Adh respectively) change in contact angle measurements.

***3.2.4. Microtensile Bond Strength (MPa)***

As shown in Figure 5, a linear increase in immediate microtensile bond strength was observed with increasing the microspheres filler content. This increase however was not statistically significant ( p = 0.2819 and 0.0589 for 5% SiGMS-Adh and 10% SiGMS-Adh respectively).



***Figure 5: Contact angle (°) and microtensile bond strength (MPa) of silanized glass microspheres modified AdperTM Single Bond 2 Refill adhesives in comparison with unmodified adhesive as a control.***

**4. Discussion**

The development of dental materials with antibacterial action is highly required to reduce the recurrence of caries. Having dental adhesives with antibacterial properties is a viable option to suppress the bacterial colonization around restorations and prevent the replacement of restorations due to recurrence of caries. Some commercially available dental adhesives are modified by the inclusion of antibacterial agents such as glutraldehyde (eg, Gluma 2 Bond) (25), chlorhexidine (eg, Peak Universal Bond) (26) and methacryloyloxydodecylpyridinium bromide (MDPB) (eg, Clearfile SE Protect) (27). Regardless of the antibacterial action of these adhesives, none of them showed remineralization potential (ie, ability to remineralize dentin in deep areas where the adhesives might fail to penetrate). This study aimed to induce both antibacterial and remineralizing potential to dental adhesives by incorporation of phosphate-based glass microspheres doped with titanium dioxide that is known with its antibacterial action. The remineralizing potential of these microspheres will be considered in our future work.

This study investigated silanized glass microspheres and their effect on properties of AdperTM Single Bond 2 Refill adhesives. The size range of microspheres was bigger compared to the starting glass particles. This was expected due to the possible fusion of smaller particles during melting and spheroidization process. The silane treated microspheres showed the ζ-potential value reduced to -7 (±2) mV, indicating neutralization of the surface charge due to the incorporation of the silane group on the negatively charged glass surface. The silanization has been also confirmed by FTIR through the presence of amide group associated peaks at 1500-1600 cm‑1 and bands at 1380, 1096 and 780 cm‑1 due to C-N, Si-O-Si and Si-O-C groups from APTES, respectively. The silanization has been carried out to ensure proper integrity of the glass microspheres into the adhesive resin matrix (28) and this was also confirmed by FTIR.

The antibacterial action of glass microspheres-filled adhesives on *S. mutans* biofilm was investigated. *S. mutans* was chosen as it has been known as the most cariogenic bacteria; *S. mutans* metabolizes the carbohydrates and produces acids that causes demineralization of tooth structure with the subsequent inititation of caries (29). The results of this study showed a significant reduction in CFU with the AdperTM Single Bond 2 Refill adhesive that was used as a positive control. Since this adhesive does not contain any antibacterial agent, the presented reduction in CFU could be attributed to the toxic effect of residual monomer that could be present as the polymerization reaction is never complete. Incorporation of glass microspheres into the chosen adhesive produced further significant reduction in CFU when compared with the control adhesive. This could be due to the release of titanium ions (12) that have been known with their antibacterial action (30). Non-silanized microspheres showed higher reduction in CFU compared to silanized microspheres. This was expected since the presence of silane coupling agent around the glass microspheres could relatively reduce the diffusion of fluid into the filler hence low titanium ion release would result (31). The silane coupling agent is a bifunctional molecule used to coat the fillers and enhance the bonding with the resin matrix. It has been observed that the composite reinforced with silanized dicalcium phosphate anhydrous (DCPA) nanoparticles showed less ion release than the composite reinforced with unsilazed DCPA nanoparticle (31). With the use of silanized fillers more strengthening of the adhesive will be expected than with non-silanized fillers. Generally, the incorporation of glass microspheres into dental adhesives could be a viable option to enhance the antibacterial action of two-steps total-etch dental adhesives hence a promising approach to prevent recurrent caries. Generally, according to the result of antibacterial study, there is an enough evidence to reject the null hypothesis.

Dentin wettability by the adhesive resin has been investigated by measuring the contact angle (32). As observed, glass microspheres did not adversely affect dentin wettability by AdperTM Single Bond 2 and this could be related to the hydrophilic nature of these glasses. As reported previously titanium dioxide-doped phosphate glasses showed contant angle with water lower than 90◦, it varied from 33.7±3.6◦ to 34±5.1◦ according to titanium dioxide content (5-15 mol%) incorporated into the glass (12). The measured contact angle of titanium dioxide-doped phosphate glasses with diiodomethane varied from 40.7±2.5◦ to 46.8±1.4◦ according to titanium dioxide content (5-15 mol%) incorporated into the glass. The lower contant angels obtained with polar liquid (water) than that with non-polar liquid (diiodomehane) has been related to the polar characteristics of P-O-P bonds in these glasses (12). Accordingly, the adhesive efficiency (ie, bond strength) would not be affected (32) since lower contact angle ensures better interaction with dentin substrate. This finding was supported by the non-significant increase in microtensile bond strength after the addition of glass microspheres into AdperTM Single Bond 2. Similar findings were also observed by Careneiro et al., (33) who found that incorporation of 40 wt% silanated or non-silanated niobophosphate bioactive glass (NbG) microfiller into commercial etch and rinse adhesives (One Step and Prime & Bond) has no significant effect on microtensile bond strength to dentin. The non-significant increase in microtensile bond strength could be due to the high variability of data as expected with dentin obtained from different patients. The non-significant increase in microtensile bond strength could also be related to the variability in surface roughness of different dentin samples that would directly affect the wetting ability of the adhesive and indirectly the bond strength. In our future work, to eliminate the impact of surface roughnes on the obtained data, a standaridized protocole for sample preparation (eg, use of mould for composite build-up) will be followed to ensure that all samples are consistent and within the tolearance range in term of flatness and surface roughness before any testing will be carried out. According to the results of contact angle and microtensile bond strength, there is no enough evidence to reject the null hypothesis. Despite the promising findings, further studies are required to test the efficacy of these modified adhesives in a more complex environment where a diversity of bacterial biofilms is present (34). Furthermore, the effect of these glass microspheres on the bonding quality of these adhesives to dentin will be also considered.

**5. Conclusion**

All tested adhesives showed higher inhibitory effect on *S. mutans* biofilm growth than the NFM negative control. Glass microspheres modified adhesives furthere inhibited the biofilm growth. Incorporation of 5 wt% of glass microspheres produced significantly higher inhibitory effect than 10 wt%. Even though silanization of glass microspheres reduced the inhibitory effect, both silanized and non-silanized glass microspheres produced significantly higher inhibitory effect compared to the control adhesive. The wettability of the adhesive was not adversely affected by incorporation of glass microspheres accordingly the penetration of adhesive into demineralized dentin would not be adversely affected. The microtensile bond strength of AdperTM Single Bond 2 however was improved by the addition of glass microspheres. Therefore, the modified adhesive would be promising to reduce the possibility of recurrent caries around restorations.

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