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Research Article

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Novel Bifunctional Nanofiller (Bioactive\Antimicrobial) for Improving Dental Adhesives Efficacy

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Abstract

Purpose: *The objective of this study was to develop novel bio-composite Nanofiller (Quaternary polyethyleneimine\ Hydroxyapatite, QPEI/HAp), which combines the antibacterial activity of polymeric quaternary ammonium salt and the multi-advantages of Hydroxyapatite nanoparticles for improving biological and physico-mechanical properties of Dental Adhesives.*

Materials and Methods: *Hydroxyapatite (HAp) nano rods were produced by hydrothermal process and coated by Polyethyleneimine (PEI) via electrostatic adsorption, followed by two steps polymeric reaction; tertiary amination and quaternization. The resulting powder was characterized using XRD, FTIR and TEM before and after polymer coating, and bioactivity was evaluated after 7 days soaking in simulated body fluid using XRD analysis. An experimental ethanol-based one-bottle adhesive resin was formulated with 0.2, 0.5, 1, 2 and 5% QPEI/HAp nanofiller. The formulated adhesive resins were evaluated for their colloidal stability, antibacterial activity, Ultimate Tensile Strength, and Micro-Shear bond strength to dentin.*

Results: *Powder characterization confirmed successful surface modification of Hydroxyapatite nanoparticles with PEI polymer; the particles presented a high crystallinity with typical chemical groups and mean size around 20 nanometers. XRD analysis revealed nucleation of apatite crystals on the surface of QPEI/HAp nanoparticles after soaking in SBF; confirming their bioactivity. Lower contents of modified nanoparticles showed little or no aggregation tendency and good colloidal stability in the adhesive solution with Zeta potential of 30.6 mV. Antibacterial outcomes of PEI against S. mutans was significantly higher than that of MDPB in Clearfil Protect bond; a commercial adhesive used as control ($P<0.05$). The addition of 0.2 wt. % modified nano-hydroxyapatite resulted in higher values of Ultimate Tensile and Micro-Shear bond strength than other tested adhesives and commercial Clearfil S^3 Bond (all-in-one adhesive); ($P<0.05$).*

Conclusion: *Incorporation of 0.2 wt. % QPEI-HAp nanoparticles significantly improved the adhesive properties and may be promising multifunctional filler for dental adhesive resin.*

Keywords: Dental adhesive, Nano-Hydroxyapatite, Quaternary Polyethylenimine, Antibacterial activity, Ultimate Tensile Strength, Micro-shear bond strength

Introduction

Despite the fact that success in aesthetic restorations depends greatly upon adhesion success, and despite improvements in dental adhesives over the past decades, the bonding interface remains the weakest

spot of dental restorations. Secondary caries and dental adhesives' poor durability are the two main reasons for the replacement of the resin-based restorations, a situation prompting the need for improvements to obtain multifunctional dental adhesives which are capable of simultaneously improving various performances [1].

Bioactivity is a promising area in which dental adhesives performance can be enhanced. The notion of bioactivity was first given by Hench in 1969; as "A bioactive material is one that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material" [2]. Bioactivity of dental adhesives is achieved through addition of bioactive nanofillers (Hydroxyappatite nanoparticles, Calcium phosphates, and Bioglass), eliciting multiple benefits which includes: remineralization of neighboring tooth material, potential gaps closure between the substance and tooth and less degradation potential thus improved durability of the bond [3,4].

As most of dental tissues are of hard type composed mainly of minerals; the bioactive material should have the same components to mimic the natural one. Hydroxyapatite (Hap; Ca10 (PO4) 6 (OH)) is the main biomineral component of the human hard tissues (accounts for more than 70 wt. % and 90 wt. % of dentin and enamel respectively). It is composed of calcium and phosphorus (Ca/P) in the ratio of 1.67 [5]. Hydroxyapatite can be synthesized via different methods including; hydrolysis, chemical precipitation, hydrothermal process and others. Owing to outstanding properties like biocompatibility, bioactivity, osteoconductivity, non-toxicity and non-inflammatory nature, this bioceramic has conquered variable dental medicine applications [6].

Hydroxyapatite nanoparticles have been used to increase the mechanical properties of bonding agents, as it is easily dissolved in a self-etching adhesive and re-deposited within demineralized enamel substrate and dentin collagen network. Residual Hap also can serve as a base for other chemical interactions with the functional monomer within the adhesive, and thus becomes very essential for the long-term stability of bonded interfaces. The literature reported that; the hydroxyapatite incorporation within polymer adhesives also has significantly enhanced the process of monomer conversion and rate of [7,8]. Therefore, hydroxyapatite nanoparticles will become a promising enhancement for preparing novel dental adhesives with improved properties.

Despite being promising; hydroxyapatite's higher aggregation tendency, brittleness, low colloidal stability and poor bonding to polymeric materials; are the main problems hindering its wide application in dentistry. Surface modifications that can be achieved by surface adsorption and grafting of polymers into Hap, provides effective means to manipulate nanoparticle's surface properties [9,10]. When nanoparticles (n-Hap) and polymers form a composite, provided that homogeneous dispersion of the nanofiller is achieved at the microscopic level, the mechanical properties are expected to be improved and/or new unexpected features might appear [11,12].

Residual bacteria inside the oral cavity will remain at the interface of tooth restoration, mainly owing to contaminated smear layer, that becomes partly incorporated within the hybrid coating. In-vitro research showed that self-etching adhesive systems cannot abolish micro-leakage and bacterial infiltration, causing secondary caries, which is the prevalent reason for dental restorative failure [13]. Thus, the ability of controlling oral bacteria will be beneficial for the elimination of such risks, of demineralization and cavitation. Therefore, the antibacterial properties of adhesive materials are of great importance and must be put in consideration [14].

Till our present time, the most commonly used antibacterial agent in dental materials is methacryloyloxydodecyl pyridinium bromide (MDPB) providing a long-lasting antibacterial activity [15]. In addition, incorporation of quaternary ammonium salts (QAS) within polymers has been also used in dental materials [15]. QAS have the advantages of being nonvolatile, chemically stable, and have long-term antimicrobial activity due to their copolymerization with resin forming a covalent bond with the polymer network [16,17]. Polyethylenimine (PEI) is the antimicrobial polymer of choice in our study forming biocomposite. Owing to the collecting effect of the antibacterial agents on the carrier surfaces (Hap), higher and long lasting antibacterial efficiency with a smaller dosage is expected with a lower cytotoxicity and at the same time without changing the bioactive properties of Hydroxyapatite (Hap).

Therefore, supported by the newest research developments in the field of different antimicrobial polymers and adhesive dentistry, we have focused our efforts on developing novel biocomposite nanofiller (QPEI/Hap); that combines the antibacterial activity of polymeric quaternary ammonium salt and the multi-advantages of Hap nanoparticles; which include superior surface area and bioactivity in addition to excellent mechanical strength and, importantly, low cost. The null hypothesis of the study is that by incorporating this new bifunctional filler into experimental dental adhesive will improve its efficiency and overcome most of the problems associated with them.

Materials and Methods

Polyethylenimine (Branched PEI, Mw ~ 25,000 determined by light scattering, Sigma-Aldrich Co., Ltd. USA). Di-ammonium hydrogen phosphate and Calcium nitrate tetrahydrate were used for synthesis of Hap nanoparticles (Sigma-Aldrich Co., Ltd. USA). Sodium hydroxide, 1-chlorobutan, Methyl iodides, Sodium bicarbonate, all are of analytical grade and purchased from Sigma-Aldrich (USA). Monomers used in preparation of the experimental adhesive are illustrated in Table 1, and used as purchased without further purification. Simulated body fluid (SBF) was prepared by us and reagents used in its formulation are illustrated in Table 2. Water used here was distilled water.

Table 1: The formulation of dental adhesive used in this study

Component	w/w %
* 2, 2-bis [4-(2-hydroxy-3-methacryloyloxy-propoxy)-phenyl] propane (bisphenol A diglycidyl methacrylate). (Bis-GMA)	14 %
* 4-methacryloyloxyethyl trimellitate anhydride. (4-META)	12 %
** Trimethylolpropane trimethacrylate. (TMPTMA)	8 %
** Camphorquinone. (CQ)	0.5 %
* Dimethaminoethyl methacrylate. (DMAEMA)	0.5 %
***4-dimethylaminobenzoic acid 2-n-butoxyethyl ester. (DBB)	0.5 %
** Hydroxyethyl methacrylate. (HEMA)	26 %
** Ethanol	38.5 %

* Purchased from Shanghai SynFarm Pharmaceutical Technology Co., Ltd. China.
** Purchased from Sigma-Aldrich Co., Ltd. USA.
*** Purchased from Tokyo Chemical Co., Ltd. Japan.

Table 2: Composition of Simulated Body Fluid (SBF)

Reagent	Amount [g]	Ion concentration Cation	[mmol / l] Anion
*NaCl	11.994	Na^+	-
*$NaHCO_3$	0.525	213.0	HCO_3- 6.3
*KCl	0.336	K^+	-
*K_2HPO_4	0.342	7.5	HPO_4^{2-} 1.5
*$MgCl_2$	0.458	Mg^{2+}	-
		2.3	
*HCl 1M	60 cm^3	-	Cl- 221.7
*$CaCl^2$	0.417	Ca2+	-
		3.8	
*Na_2 SO_4	0.107		SO_4^{2-} 0.8
*$(CH_2OH)_3$ -C-NH_2	9.086	-	-
*HCl 1M		pH= 7.2-7.4	pH= 7.25

*All are analytical grade reagents (Sigma-Aldrich Co., Ltd. USA)

Commercial dental adhesives (Clearfil S^3 Bond and Clearfil Protect Bond, Kuraray Medical Inc, Okayama, Japan) were used in this study for comparing results, also dental composite "Admira" (Voco-Cuxhaven, Germany) was used, their composition is illustrated in Table 3.

Table 3: Commercial materials used in this study

Materials	Category	Composition
Clearfil S^3 Bond (Kuraray Medical Inc, Okayama, Japan)	All-in-one, single *bottle self-etch* adhesive	- 10- Methacryloyloxydecyl dihydrogen phosphate, bisphenol A- diglycidylmethacrylate, 2-hydroxyethyl methacrylate, Hydrophilic aliphatic dimethacrylate, Hydrophobic aliphatic methacrylate, Colloidal silica, dl-Camphorquinone, ethanol, Accelerators, Initiators, Water.
Clearfil Protect Bond (Kuraray Medical Inc, Okayama, Japan)	Two step, *self-etch antimicrobial* adhesive	-Primer: 10-MDP, MDPB, HEMA, hydrophilic dimethacrylate, photoinitiator, water - Bond: 10-MDP, HEMA, colloidal SiO_2, surface treated sodium fluoride crystals, hydrophilic dimethacrylate, photoinitiator
Admira.(Voco-Cuxhaven, Germany)	Ormocer composite. A3 body shade	- Matrix: Additive aliphatic And aromatic dimethacrylates. -Filler: 78% inorganic filler material (=56% vol. microfillers). radiopaque glass ceramic (0,7 µm) , pyrogenic micro fillers (0,04 µm).

Experimental Procedure

Synthesis of Quaternary Polyethylenimine/Hydroxyapatite (QPEI/Hap) Nanoparticles Followed By Incorporation into Experimental Adhesive Solution in Different Concentrations

Synthesis of Nanosized Quaternery Polyethylenimine/ Hydroxyapatite (QPEI/Hap) Powder

A nanosized hydroxyapatite powder (with molar ratio of Ca/P kept at stoichiometric amount, i.e. its ratio inside hydroxyapatite is 1.67) was synthesized from Calcium nitrate tetrahydrate and Di-ammonium hydrogen phosphate using a Hydrothermal process, to be used for further modification [18]. Polyethylenimine/Hydroxyapatite (PEI/Hap) powder was obtained by coating Hap nanoparticles, with a proportion of 40% by weight PEI, and the net percentage of this polymer in the adhesive was not exceeding 1% to avoid any cytotoxicity.

The adsorption reaction is precisely the following (Figure 1): 20 g of Hap were put in suspension in a 300-ml solution of 0.5% by weight PEI in methanol. This mixture was stirred (1700 rpm at room temperature) for 24 hours, and so the adsorption process is assumed to be completed. Then, the solution was filtered, and the PEI-Hap precipitate was rinsed with 270 ml methanol to remove excess PEI molecules that were not incorporated into the adsorption step, then lastly dried for 48 hrs at 60°C [19].

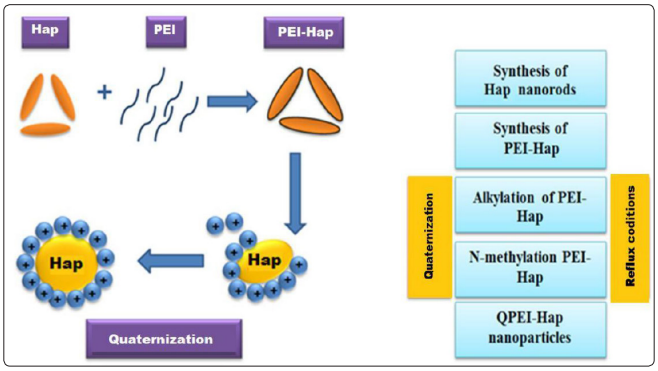

Figure 1: Schematic representation of Synthesis process of QPEI-Hap nanofiller

Quaternary ammonium Polyethyleneimine-Based (QA-PEI) Nanoparticles were formulated by N-Alkylation followed by N-methylation. N-alkylation was done by dissolving 10 g of anhydrous PEI-Hap powder in 40 ml absolute ethanol. Then 1-chlorobutan was added at 1 M ratio (primary amine of PEI monomer units/chlorobutan). The alkylation was performed under reflux conditions (60°C and magnetic stirring) for 24 hours. Excess $NaHCO_3$ (1.25 equimolar of butyl halide) was slowly added to neutralize the released acid. Neutralization reaction continued for 24 hours at the same conditions [20,21].

After that, N-methylation was conducted by adding 43 mL of methyl iodide (0.69 M) to the same flask at 1:3 M ratios (monomer units of PEI/methyl iodide), at 42°C for 48 hours, during this step yellow precipitates were formed. An equivalent amount of sodium bicarbonate (0.23 M) was slowly added using funnel dropper to accumulate the released HI from the methylation process. Neutralization was continued with same conditions for an additional 24 hours and the formed NaI salt and excess of unreacted

$NaHCO_3$ were discarded by decantation, and the liquid portion was precipitated in 300 mL of double distilled water (DDW), then washed with hexane to remove traces of unreacted butyl halide, methyl iodide and to remove inorganic salts, and finally dried under pressure using vacuum evaporator [20,21].

Adhesive Preparation

An experimental ethanol-based, light cured one-bottle dentin bonding was prepared by mixing the components illustrated in Table 1, using ultrasonic bath (Digital ultrasonic cleaner, SH80-2L, USA) for 1 hour in dark place; to obtain homogenous mix [22,23]. The synthesized QPEI-Hap nanoparticles were incorporated, in weight ratios of 0.2%, 0.5%, 1%, 2% to 5%, into the adhesive solution and homogenized by ultrasonication for 5 minutes [18].

Evaluations

Characterization and Evaluation of the Synthesized Powder of QPEI-Hap Nanoparticles

Transmission Electron Microscopic Observations (TEM)

TEM analysis was performed to observe the morphological changes of the samples (Hap NPs; before modification with PEI and after) and to determine their particle size.

X-ray Diffraction (XRD)

The QPEI/Hap nanoparticles were characterized using X-ray diffractometer (XRD, Model: GNR APD-2000 PRO), with CuKa (1.540598) radiation over the 2-theta range of 10-55° with a step size of 0.03°. The peaks obtained were compared to standard references outlined in Joint Committee for Powder Diffraction Standards (JCPDS) file, which is readily available for hydroxyapatite in the device's software (Powder Diffraction File 'PDF' name: 09-0432).

Fourier-Transformed Infrared Spectroscopy (FTIR)

The functional groups of the QPEI-Hap were identified by FTIR Infrared Spectroscopy (FT-IR, Model: EQUINOX 55, BRUKER, Germany) using KBr pellet procedure. The fraction of the crystalline phase in Hap nanoparticles, was determined according to the following equation:

$$X_c = 1 - \frac{V112/300}{I300}$$

Where $I300$ represents the intensity of 300 diffraction peak, and $V112/300$ represents the intensity of the valley between the peaks of 112 and 300.

Bioactivity Testing

Bioactivity of QPEI-Hap nanoparticles was evaluated *in vitro* using simulated body fluid, first synthesized by Kokubo, et al. [24]. It is a cellular and non-protein solution, containing various salts that simulate the composition, concentration and pH of human plasma to reproduce reactions similar to the real biological conditions [25,26]. The composition of SBF solution with 1.5 N concentration; is revealed in (Table 2) [27]. The QPEI-Hap powder was immersed for 7 days in the SBF, by static method, at temperature of 37°C and an initial pH of 7.2.

Evaluation of the Experimentally Prepared Adhesive

Colloidal Stability Evaluation

Colloidal stability was performed as described in previous literature [28]. Briefly, sedimentation behavior of the adhesive containing QPEI-Hap nanoparticles with different concentrations (0.2, 0.5, 1, 2, and 5 wt. %), was measured using visible light in spectrophotometer (1102 UV-VIS spectrophotometer, Techcomp, China), at 420 nm wavelength, the transmission % was recorded every 30 minutes for minimum 12 hours. Also, Zeta potential was measured by an electrophoretic technique using Zetasizer instrument (Malvern Zetasizer 3000 HS, UK). The electrophoretic mobility data was transformed into z potential values through Helmholtz-Smoluchowski model. The reported value for z potential is the average of at least six measurements.

Antimicrobial Activity Evaluation

Agar Diffusion Test (ADT)

Streptococcus mutans (S. mutans) and *Escherichia coli (E. coli)* were isolated from patients admitted into dental clinics (Faculty of Dentistry), and were authenticated by Microbiology Department (Faculty of Pharmacy), Tanta University. Each of the species was grown in brain heart infusion broth (BHI, Becton-Dickinson, USA) and each bacterial suspension turbidity was adjusted to 0.5 McFarland standard (approximately 10^8 cfu/mL) [29].

ADT was performed using 50 microliters of experimental adhesive (0.2, 0.5, 1, 2 and 5%) and the commercial Clearfil Protect Bond adhesive for comparison. The diameter of each zone (including hole diameter) was measured and recorded in mm, the measurement was done on the three plates and the mean was computed for each tested material [30].

Minimum Inhibitory\Minimum Bactericidal Concentration (MIC and MBC) Measurements for the Most Efficient Antibacterial Complexes against Test Microorganisms

MIC and MBC of the most efficient antibacterial experimental adhesive, as evidenced by ADT, and the commercial antibacterial adhesive CPB, for the two bacteria species used in our study were assessed by serial microdilution assay (in triplicate), as reported previously [29]. The MIC value was determined by visual examinations as the lowest concentration of the adhesive mixture that was visually clear (i.e. no bacterial growth) in the well. This was confirmed by further sub-culture of the well's contents; via spreading on BHI agar plates, this should also show no observable bacterial growth. Then, the plates were anaerobically incubated for 48 hours, and the MBC value was determined as the lowest concentration of the adhesive mixture which gave no colonies, in the three replicates, on BHI agar. In addition, scanning electron microscopy (JEOL, JSM, 5200 LV, Japan) was used to visually observe the antibacterial effect of the filler nanoparticles on the bacteria.

Evaluation of the Mechanical Properties

Diametral Compression Test for Tensile Strength

Diametral compression was performed by adopting the procedures of ANSI/ADA, described for light cure resins in specification no. 27 (American Dental Association) and according to previously described method [31,32]. Briefly, the solvent of the tested adhesives was vacuum-evaporated (IKA RV 8V, Germany) then the solvent-free adhesive mixtures were cured using a LED light-curing unit (400 mW/cm2, the critical limit for safe polymerization) for 40s through glass from each side. After that, the specimens were maintained in DD-H_2O for 24 hours before testing.

Five different samples were tested for each formulation (experimental adhesive with filler concentrations of 0.2, 0.5, 1, 2 and 5% and the commercial Clearfil S^3 Bond adhesive for comparison). Diameter

and height of each specimen were recorded using digital micrometer before the test, and the universal testing machine (LRX 5kN, Lloyd Ltd., UK) was used utilizing 5 kN load cell and 1 mm/min cross-head speed.

Micro-Shear Bond Strength Test

Thirty extracted caries-free human premolars (five for each tested group) were used following written agreement from the patients and approval of the protocol by Research Ethics Committee, Faculty of Dentistry, and Tanta University. Teeth were prepared as previously described [29].

Each tooth was embedded in self-cure acrylic resin cylinders (1.5 cm diameter and 2 cm height), and randomly allocated into the six experimental groups of adhesive mixtures, as following: groups 1-5 (0.2%, 0.5%, 1%, 2%, or 5% QPEI-Hap, respectively), or group 6: Clearfil S3. The bonding agent was applied using a micro-brush for 10 seconds, rubbed for 5 seconds, and then the solvent was gently evaporated using low-pressure air stream until a homogeneous shiny layer was observed on the surface (except in group 6; brushing for 20 seconds then air dried, as in manufacturer's instructions), a second layer was applied in a similar manner.

The remaining process resembled previously published protocol, with slight modifications [33]. In brief, after the adhesive application, two clear cylindrical silicon tubes, 0.8 mm internal diameter and 2 mm height, were positioned on the flat dentin surface and then underwent 10 seconds light curing. Then, each tube was filled with Ormocer resin composite (Admira A3, Voco, Cuxhaven, Germany); and irradiated for 40s. After that, the specimens were placed in distilled water at room temperature for one hour, and then the silicone tubes were detached and underwent microscopic examination at 10X magnification (ML 9300; MEIJI TECHNO, Saitama, Japan) for integrity evaluation of the resin–dentin interface. After storage in water at 37°C for 1 week, a thin steel wire (2 mm diameter) was coiled between the resin cylinder and tooth surface. Then, a 1 mm/min cross-head speed force was applied until failure. The maximum load/force necessary to separate the cylinder from tooth surface divided by the attached area was denoted as micro-shear bond strength. The form of failure that occurred at the tooth/adhesive-system/restoration-interface was examined under a 20X magnification stereomicroscope (Stemi 2000C, Carl Zeiss, Canada), and the interface microstructure was examined using SEM (JEOL, JSM, 5200 LV, Japan).

Statistical Analyses

The raw data was collected, tabulated and analysed using one way analysis of variance test (ANOVA, using SPSS v17, USA), followed by Paired T-test for Antibacterial activity, and followed by Turkey HSD test for mechanical properties (Ultimate Tensile and Micro-Shear bond strength). Statistical outcomes were denoted significant at $P \leq 0.05$.

Results

Characterization of the Synthesized Powder of QPEI-Hap Filler Nanoparticles

Transmission Electron Microscopy (TEM) Observation

TEM showed no significant difference between the images of Hap and PEI-Hap in primary crystals (Figure 2a and 2b), clear rod-shaped nanoparticles were obtained with size-range of 50-70 nm showing signs of agglomeration. After quaternization; spherical shaped nanoparticles with size-range of 16-27 nm (Figure 2c) were obtained with a mean size of 20.8 nm and no agglomeration at all was observed.

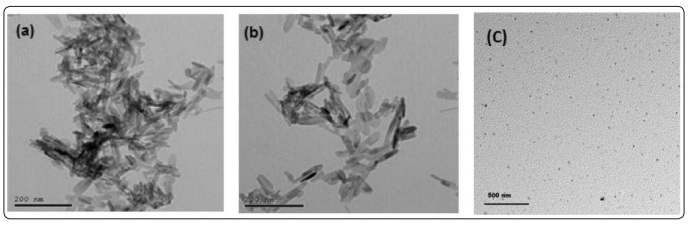

Figure 2: Transmission electron micrographs of (a) Hap nanoparticles, (b) PEI-Hap nanoparticles, and (c) QPEI-Hap nanoparticles

X-ray Diffraction (XRD)

According to ICDD (International Centre for Diffraction Data) standard (PDF no. 09-0432); the XRD pattern of all tested powders (unmodified Hap, PEI-Hap and QPEI-Hap) revealed the main (h k l) indices associated with Hap crystals: 002, 211, 112, 300, 310, 202, 222 and 213, (Figure 3). The XRD patterns of PEI-Hap and QPEI-Hap nanoparticles; showed broadening of the peaks at 2θ from 15 to 25 degrees in both spectra; corresponding to the amorphous polyethylene imine polymer.

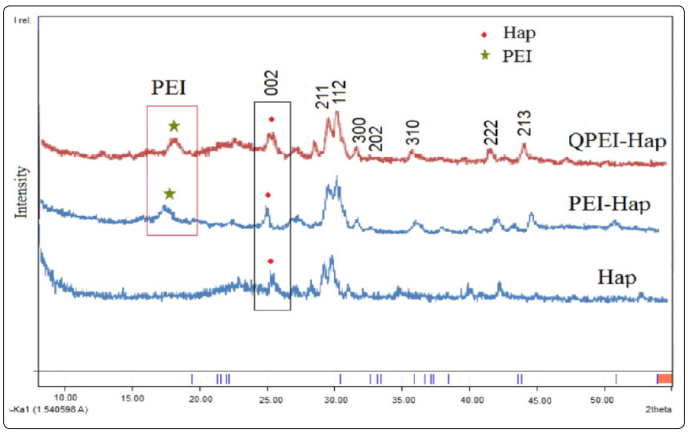

Figure 3: XRD of synthesized Hap nanoparticles, PEI-Hap and QPEI-Hap nanoparticles

Fourier-Transformed Infrared Spectroscopy (FTIR)

Hydroxyapatite nanoparticles FTIR-absorption spectra before and after combination with antimicrobial PEI polymer and after further quaternization are shown in Figure 4 (a, b and c, respectively). The characteristic PO4 peaks appeared at 1092, 1032, 601, and 564 cm^{-1} (Figure 4a), show that the nanoparticles should be Hap. After combination with the amorphous PEI polymer, the characteristic Hap peaks were still present (Figure 4b); except for the appearance of some new peaks characteristic for PEI like that at 3426 cm^{-1} which is indicative of the stretching mode of N-H group of PEI, while N-H vibration bend peak appeared at 1622 cm^{-1}. Also, the peak at wave number 2959 cm^{-1} is attributed to C-H stretching vibration mode, while the C-H bending mode appeared at 1461 cm^{-1}. After quaternization of PEI-Hap nanoparticles, the characteristic peaks of Hap and PEI were still observable (Figure 4c); except for the appearance of characteristic quaternary amino-group absorption band at 961 cm^{-1}.

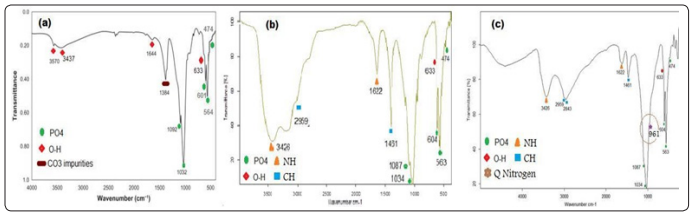

Figure 4: (a) FTIR curve of synthesized Hap nanorods, (b) PEI-Hap nanoparticles and (c) QPEI-Hap nanoparticles

X-ray Diffraction Analysis for Bioactivity

X-ray diffraction patterns of QPEI-Hap nanoparticles, before and after incubation for 7 days in simulated body fluid (SBF) at 37°C and pH=7.25 are shown in Figure 5. Un-soaked nanoparticles showed characteristic spectrum of amorphous material (PEI), while after incubation in SBF solution; diffraction peaks located at 25, 92 2θ (002) and 39, 83 2θ (310) in accordance with ICDD-PD2: 00-009-0432.

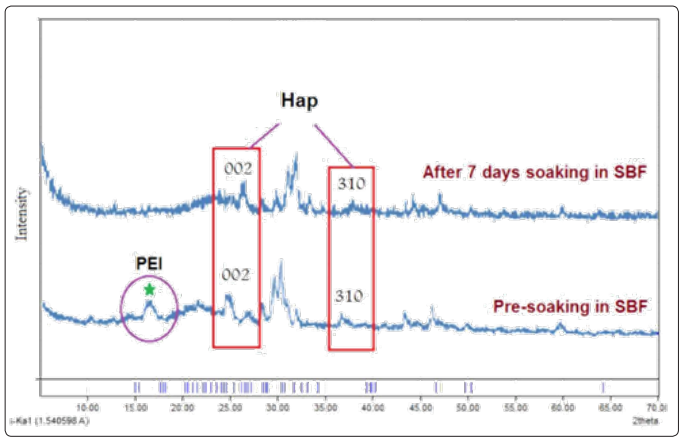

Figure 5: X-ray diffraction patterns of QPEI- Hap nanoparticles before and after incubation in simulated body fluid

Evaluation of the Experimentally Prepared Adhesive Evaluation of Colloidal Stability

The sedimentation behavior, measured by Spectrophotometer, of QPEI-Hap nanoparticles suspension in experimental one step self-etch adhesive with different concentrations is shown in (Figure 6). There was increased particles sedimentation rate with time within the solution of the bonding system. The transmission of the adhesive with 0.2 and 0.5 wt. % QPEI-Hap filler nanoparticles, although particle settling increased steadily, did not reach 100% transmission during the 12 hours, However; the sedimentation rate increased dramatically after nearly the third hour for 1, 2, 5 wt. % QPEI-Hap NPs containing adhesives, with complete precipitation of the filler particles (i.e. reached 100% transmission) after six hours for 1 and 2 wt. % concentrations, and in less than three hours for 5 wt. % nanofiller concentration. Positive Zeta potential value (+30.6 mV) of QPEI-Hap nanoparticles was proved by zeta sizer.

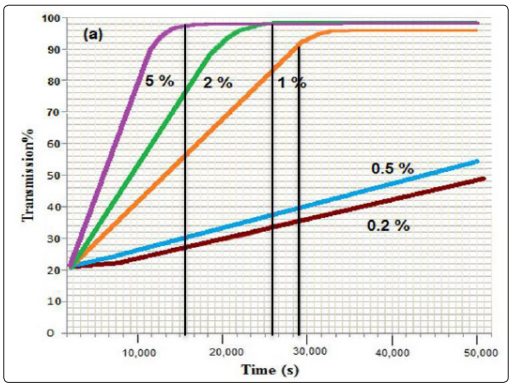

Figure 6: The sedimentation behavior of the experimental adhesive with different filler concentrations

Evaluation of the Antibacterial Activity

Mean diameters in mm and standard deviation values of antibacterial inhibition zones of the tested materials (the experimentally prepared adhesive with different filler concentrations, and the commercial all in one antimicrobial dental adhesive; Clearfil protect bond [CPB]; as a control) are shown in Table 4 and Figure 7 and 8.

Table 4: Mean diameter of antibacterial inhibition zones of the tested materials on both *S. mutans* and *E. coli* species, including one way ANOVA and pairwise T-test for comparing antibacterial effect of tested materials on both species

Tested Adhesives	Antibacterial effect			
	S. mutans		*E. coli*	
	Mean (mm)	Change%	Mean (mm)	Change%
0.2% PEI-Hap	40.6±0.88^{*A}	82.06%	25±2.08^{**a}	-13.92%
0.5% PEI-Hap	37.3±0.81^{*B}	67.72%	22.3±1.2^{**b}	-29.43%
1% PEI-Hap	31.6±1.7^{*C}	41.70%	23±1.52^{**c}	-7.21%
2% PEI-Hap	26.3±0.91^{*D}	17.93%	29.3±0.3^{**d}	-7.27%
5% PEI-Hap	22.6±2.9^{*E}	1.34%	29.6±2.8^{**E}	-6.32%
CPB	22.3±0.66^{*F}	-	31.6±0.3^{**f}	-
PEI in Experimental adhesive VS	PEI= 31.7		PEI= 26.2	
MDBP in Commercial adhesive (CPB)	MDBP=22.3		MDBP=31.6	

* One-way ANOVA test showed high significant difference in antibacterial effect against *S. mutans* between different tested materials with (P) value (6.96×10^{-6}).

** One-way ANOVA test showed significant difference in antibacterial effect against *E. coli* between different tested materials with (P) value (0.008749).

Significant difference between groups with capital and small letters using pairwise T-test ($P < 0.05$)

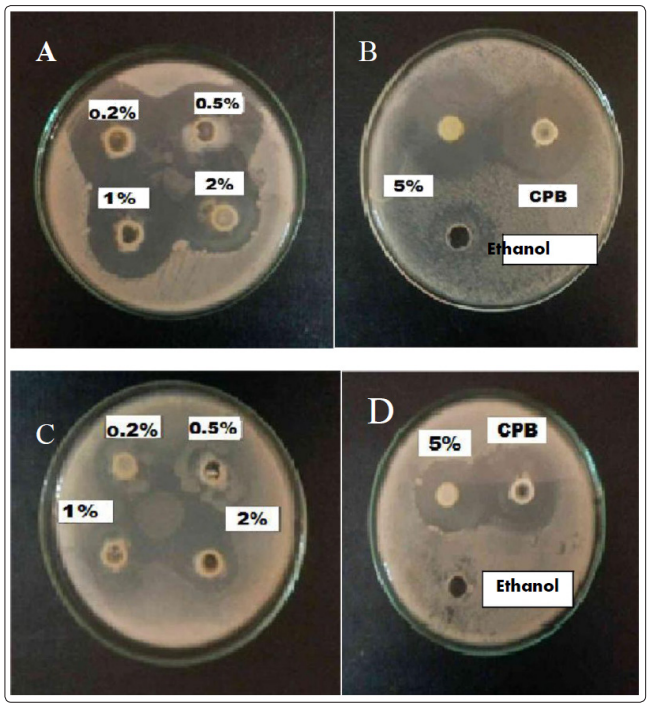

Figure 7: Agar plates showing inhibition zones of antibacterial activity of 0.2, 0.5, 1, 2 and 5% QPEI-Hap and Clearfil S^3 (CPB) tested against *S. mutans* (A&B) and *E. coli* (C&D)

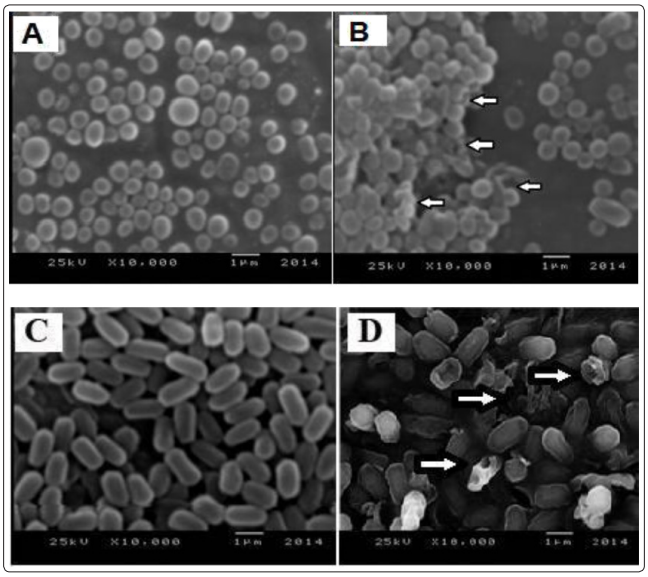

Figure 8: SEM of *S. mutans* and *E. coli* showing normal intact bacterial cells as a control (A&C), and ruptured ones (B&D) as a consequence to the inhibitory agent (QPEI)

According to ADT results; samples with low filler content (0.2% QPEI-Hap) showed a significant bacterial reduction ($p>0.05$) of approximately 82% against *S. mutans*, compared to control group (commercial antimicrobial adhesive CPB), the level of inhibition then steeply decreased with increasing the filler content, the least change percent was recorded for 5% QPEI-Hap nanofiller concentration (1.34%). For *E. coli*, the control group showed greater level of inhibition (6-29%) compared to the experimental adhesive with different filler concentrations. Ethanol had negligible effect on both bacteria species, thus its antibacterial effect was regarded as negative.

Minimum Inhibitory\Minimum Bactericidal Concentration (MIC and MBC) measurements (Table 5); again revealed the potent bactericidal effect of 0.2% QPEI-Hap containing samples on *S. mutans* (0.078/0.155), while greater concentrations were needed for *E. coli* (0.149/1.36) to produce an obvious effect.

Table 5: MIC and MBC values of tested adhesives for *S. mutans* and *E. coli* expressed as a percentage of the original solution

Tested adhesives	MIC\MBC %	
	S. mutans	***E.coli***
0.2% Ex. Ad. 5% Ex. Ad.	0.078/0.155	0.625/1.65
CPB	0.149/1.36	0.452/1.75

Destructive effects of inhibitory agents (QPEI) in the experimentally prepared adhesive against different microorganisms (*S. mutans* and *E. coli*) appear great in SEM images. As seen in Figure 8 B-D, both strains' cells lose their defined cell wall boundaries with extrusion of their intracellular components resulting in bacterial cells coagulation, compared to control images with intact cell morphology (Figure 8 A-C).

Evaluation of Mechanical Properties of the Experimental Adhesive Diametral Compression Test (Ultimate Tensile Strength)

Diametral compression test results showed greater mean values (47±2.19 - 43.2±4.97 MPa) for samples containing 0.2-0.5 wt. % NPs which is about 15-25% higher than that of the tested commercial filler (Clearfil S^3 bond, 37.4±3.28). However, these values significantly decreased by further increasing filler content (1, 2 and 5 wt. % NPs, $p<0.05$), to record 36-65% inferior strength than the control filler (Figure 9).

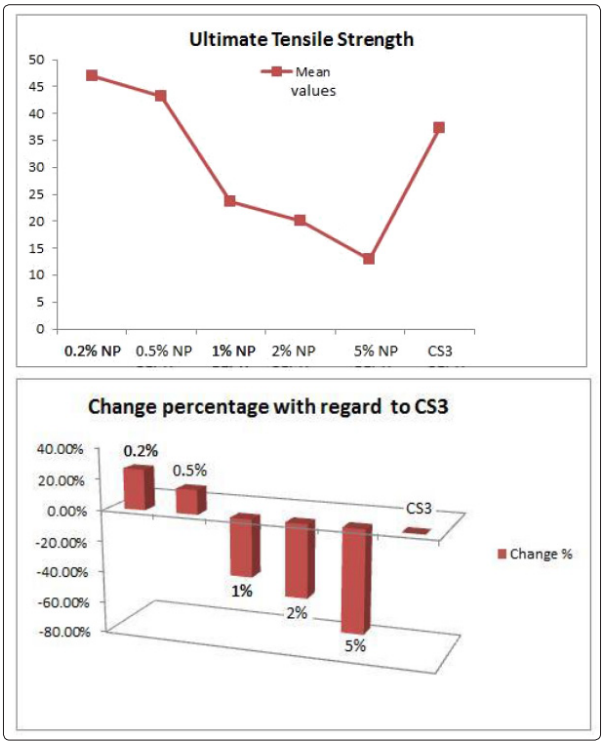

Figure 9: Ultimate Tensile Strength (mean values and change percentage) of the experimentally prepared adhesive with different filler contents compared to the commercial Clearfil S^3 Bond

Micro-Shear Bond Strength

Average micro-shear bond strength values were greatest for the experimentally prepared adhesive with 0.2% and 0.5% QPEI-Hap nanofiller (18.95±2.5, 17.32±0.33, respectively), which is approximately 57-72% higher than that of the control commercial bond CS^3 (11.03±1.843). Whereas, the control group bond strength was comparable to the adhesive with 1% and 2% filler content (10.56±2.5 and 8.99±1.6, respectively, no statistical significant difference $p>0.05$), and significantly higher ($p<0.05$) compared to the adhesive with 5% QPEI-Hap filler concentration (5.77±1.05). Regarding bond failure type distribution, although mixed mode of failure was observed in some cases, adhesive failures predominate, but no cohesive failures were observed at all (Table 6).

Table 6: Micro shear bond strength values, change % and failure modes

Adhesive system	Mean ± SD (change %*)	Mode of failure (n)			
		Adhesive	Mixed	Cohesive in dentine	Cohesive in composite
Gp I: 0.2% QPEI-Hap	$18.95±2.5^A$ (71.8)	9	1	-	-
Gp II: 0.5% QPEI-Hap	$17.32±0.3^A$ (57.02)	8	2	-	-
Gp III: 1% QPEI-Hap	$10.56±2.5^B$ (-4.26)	8	2	-	-
Gp IV: 2% QPEI-Hap	$8.99±1.6^B$ (-18.49)	9	1	-	-
GpV: 5% QPEI-Hap	$5.77±1.05^C$ (-47.68)	8	2	-	-
Gp VI: CS^3	$11.03±1.8^B$ (0)	9	1	-	-

* Compared to the control group (Gp VI: CS^3)
Different letters indicates significant differences between groups (*Turkey's* HSD test, $p<0.05$)

Representative fractographic images are shown in Figure 10. CS^3 specimen (Figure 10 A) showed the dentinal tubules; some were empty and the others were occluded, with exposed intertubular dentin. While, specimens with 0.2% and 0.5% filler concentrations showed an adhesive layer completely covering the dentin surface; with sites of dentinal tubules appear filled with adhesive (Figure 10 B & C). Specimens' images for groups III, IV and V (Figure 10 D, E & F), showed a thick polymer layer with larger particles covering the whole dentin, and the dentinal tubules were hardly visible.

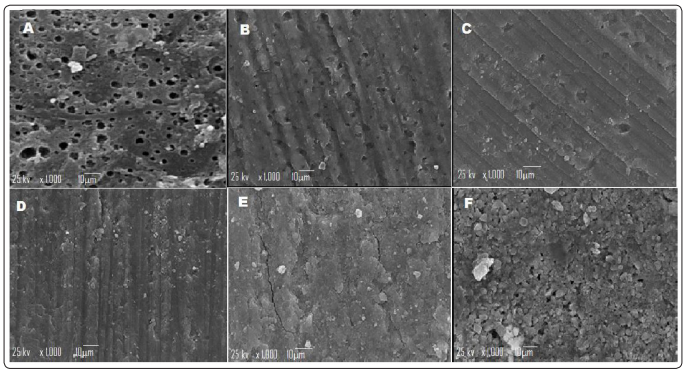

Figure 10: Representative SEM photomicrographs of fracture area of the tested adhesives, A: CS3, B: 0.2% QPEI-Hap, C: 0.5% QPEI-Hap, D: 1% QPEI-Hap, E: 2% QPEI-Hap, F: 5% QPEI-Hap

Discussion

Currently, many researchers perform dental adhesive modifications to achieve new products with better performance. But most of these studies simply focus on the improvement of only one function and scarce studies seek multifunction modifications of dental adhesives that are capable of improving various performances simultaneously [1]. Herein, we reported a promising method for preparing bioactive-antibacterial composites via adsorption of functional polymers with antibacterial activities onto inorganic bioactive particle carriers to be used as filler in dental adhesives.

Overall, FTIR, TEM, X-ray diffraction, and zeta potential measurement confirmed successful surface modification of Nano hydroxyapatite particles with PEI polymer. In TEM images changing the particles rod-shape to spherical after quaternization step and the greatest decrease in dimension was also observed in previous studies; which proved that at higher PEI concentrations, more PEI molecules can cap the inorganic carrier particles decreasing their size. This might be a beneficial new method for synthesis of organic-inorganic nanoparticle composites with smaller size reaching around 20 nm. Also after quaternization; no agglomeration at all was observed, rather the particles were pushed away from each other, mainly due to the positively charged PEI molecules that prevented the aggregating of nanoparticles due to electrostatic repulsion, and these results were in coincidence with that of previous study by Lee and his coworkers [32-34].

After incubation in SBF solution; it was found that our modified nanoparticles were able to generate nucleation of apatite crystals deposition on their surfaces, a phenomenon confirmed by characteristic sharp peaks of Hap at (002) and (310) and disappear of PEI amorphous peak at 2θ from 15 to 25 degrees, proving that Hap' bioactive properties did not change by polymer coating.

In the present study polyethyleneimine (PEI) was used as a cationic surfactant coated on Hap nanoparticles instead of silanization process; to induce matrix/filler interaction and enhance dispersion properties of Hap nanoparticles in the organic liquid media (experimentally prepared bonding agent). Although this hypothesis appeared to be rejected due to increased particles sedimentation rate with time, this behavior couldn't be attributed to agglomeration alone, also usage of high molecular weight polymer (Mw= 25.000) in high concentration (40%) is another factor that may result in greater tendency for particle sedimentation regardless filler concentration, this point need to be confirmed by further research.

Adhesive systems with long-lasting antibacterial activity are in excessive demand nowadays to avoid the complications noted in self-etching/priming systems due to contaminated smear layer. Immobilization of antibacterial components can be achieved by altering the resin components or in the filler of the adhesive resins [13,35]. In the present study such modification was done to the filler and the achieved results are promising.

Quaternary ammonium cations are widely used as effective antibacterial agents owing to their permanent positive charge and hydrophobicity. For more efficacies against various Gram-positive and -negative bacteria; the immobilized PEI was N-alkylated with linear alkyl bromides of different lengths. Results from previous studies clearly showed the strong effect of increasing carbon chain length (8-12 carbon atoms) on increasing the antibacterial potency of monomers [36]. This study achieved high antibacterial potency using a carbon chain with only 4 atoms, which may be attributed to lower viscosity and film thickness of the adhesive polymer. And so, the same effect of long chains length in penetrating and disrupting bacterial cell membrane leading to explosion was gained with shorter chain length here. This result is in agreement with Gelman and coworkers regarding biocidal activities of an intermediate backbone spacer that have 4 carbons length between cationic polystyrene units [21].

Agar diffusion test (ADT) demonstrated that all materials tested could inhibit the two bacterial strains (Figure 7). For all strains tested the reduction of living microorganisms using QPEI-Hap nanofiller was significant ($p<0.05$), regardless its concentration, when compared to MDBP used in control group (CPB). This may be partly attributed to strain-dependent sensitivity of species to a material containing immobilized biocide and also to the less diffusability of aromatic MDPB from slight highly viscous adhesive (CPB with 5% MDPB monomer), compared to that of QPEI having loops and tails protruding into the adhesive solution with free movement [29,34,37].

Previous literature explained the destructive effects of QPEI against different strains, as the cationic groups of PEI electrostatically bind to the negatively charged bacterial cell surfaces. Following binding, the PEI amphiphilic structure encourage the insertion of its hydrophobic side-chains into bacterial cells' membrane, leading to the disruption and breakdown of membrane integrity leading to bursting of intracellular components and death [17,38,39]. These findings promote the notion that added PEI nanoparticles to the dental adhesive have bactericidal effects; which is preferred over bacteriostatic ones (MDBP).

Tensile strength is one of the important mechanical properties for performance assessment of brittle dental resins [18]. The present UTS findings greatly suggest a strong interaction between matrix and filler in cured resins, especially with lower filler contents in our study, thus encouraging the idea that PEI surfactant is a better substitute to silane-coupling agents (inorganic fillers) in resin matrix. While at higher concentrations there is greater tendency for flocculation and formation of defect points that hinder complete resin curing which then fails in tension, thus explaining the lower values of ultimate strength at higher filler contents [1,40].

Providing a heavy-duty and stable bond between the tooth and restoration material is the definitive goal of any dental bonding system. So, when investigating adhesive systems performance, micro-shear bond test has been accepted as a reliable and facile method in measuring strength of the bond between tooth structures and dental adhesives [22,41,42). Statistical analyses showed positive effect of filler type and concentration on the strength of dental adhesives bond ($p<0.05$). In the present study, the used nanostructured hydroxyapatite had a mean size of 20.8 nm, greatly encouraging filler penetration into interfibrillar spaces (15-20 nm) of the etched dentin and hence the higher bond strengths reported herein [18,22].

When Hydroxyapatite was used as filler; better reinforcement of the bond strength was expected owing to the perception of bio-mineralization and chemical adhesion to tooth structure [40]. Although; CS^3 contains 10-MDP monomer which is known for its higher tendency to chemically unite with Hap of enamel and dentine, this study proved that the incorporation of bioactive filler is a promising enhancement for effective chemical adhesion of bonding agents to tooth structure, evidenced here by 71.8% higher bond strength values for group I compared to the control group [39,43]. Additionally, coating Hap nanoparticles with PEI surfactant improved its dispersion properties, thus guaranteeing uniform nanoparticles distribution within the adhesive resin, and preventing, to a large extent, their clustering specially at lower concentrations.

The viscosity of the bonding agent is a central factor affecting bond's strength. An inverse relation exists, as increased bond strength was evident with decreasing the bonding agent viscosity, mainly due to the lower angle of contact, resulting in better surface spread of the bonding agent [44]. This explains the lower penetration rate of the adhesive in dentinal tubules, evidenced by SEM, using the commercial adhesive (CS^3 bond; with high viscosity), and lower bond strength than the formulated ones with QPEI-Hap nanofiller (especially in groups I and II).

Incorporation of fillers with larger dimensions than the interfibrillar space of the etched dentine not only increases the adhesive viscosity, but also might cause filler accumulation forming larger spherical clusters over the top of etched dentin surface, which was clearly visible in SEM images of group V (5% filler content, Figure 10 - F) [38]. Therefore, it might reduce the infiltration of the adhesive in the etched dentin and produce defective layer that show lower bond strength.

Conclusion

In this study synthesis and incorporation of bi-functional nanofiller in adhesive solution was successful. The novel dental adhesive formulated exhibited improved stability, physical and mechanical properties (especially at the 0.2% or 0.5% QPEI-Hap nanoparticles concentrations) compared to the tested commercial ones in addition to added antibacterial activities. Further in-vivo and in-vitro studies are necessary to determine the long term antibacterial effect of this new single bottle self-etching dentin bonding system and to determine the benefit of their antibacterial properties for clinical applications. The antibacterial effect of these particles against other bacteria involved in persistent infections, merits also future screening for wide application of such filler in dentistry.

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