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Biocompatibility and bond degradation of poly-acrylic acid coated copper iodide-adhesives

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Abstract

Objective.—To investigate the effect of poly-acrylic acid (PAA) copper iodide (CuI) adhesives on bond degradation, tensile strength, and biocompatibility.

Methods.—PAA-CuI particles were incorporated into Optibond XTR, Optibond Solo and XP Bondin 0.1 and 0.5 mg/ml. Clearfil SE Protect, an MDPB-containing adhesive, was used as control. The adhesives were applied to human dentin, polymerized and restored with composite in 2 mm-increments. Resin–tensile bond strength after 24 h, 6 months and 1 year. Hourglass specimens (10 × 2 × 1 mm) were evaluated for ultimate tensile strength (UTS). Cell metabolic function of human gingival fibroblast cells exposed to adhesive discs (8 × 1 mm) was assessed with MTT assay. Copper release from adhesive discs (5 × 1 mm) was evaluated with UV–vis spectrophotometer after immersion in 0.9% NaCl for 1, 3, 5, 7, 10, 14, 21 and 30 days. SEM, EDX and XRF were conducted for microstructure characterization.

Results.—XTR and Solo did not show degradation when modified with PAA-CuI regardless of the concentration. The UTS for adhesives containing PAA-CuI remained unaltered relative to the controls. The percent viable cells were reduced for Solo 0.5 mg/ml and XP 0.1 or 0.5 mg/ml PAA-CuI. XP demonstrated the highest ion release. For all groups, the highest release was observed at days 1 and 14.

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Significance.—PAA-CuI particles prevented the bond degradation of XTR and Solo after 1 year without an effect on the UTS for any adhesive. Cell viability was affected for some adhesives. A similar pattern of copper release was demonstrated for all adhesives.

Keywords

Adhesive resin; Bond degradation; Biocompatibility; Copper iodide; Cytotoxicity; Ion release; Tensile strength

1. Introduction

Despite significant progress to improve the survival of composite restorations, their long-term durability remains a concern. Bacterial proliferation due to marginal breakdown, and hydrolysis of both resin-based polymers by salivary esterases and demineralized collagen by endogenous proteases have been extensively investigated in the last few decades [1]. Agents with anti-bacterial and anti-proteolytic properties such as benzalkonium chloride [2], glutaraldehyde [3], chlorhexidine digluconate [4], and methacryloyloxydodecyl pyridinium bromide (MDPB) [5] have been proposed.

Nanoparticles with antibacterial properties, such as silver [6] and copper [7], have received considerable attention when incorporated into adhesive materials [8] since bacteria are less likely to acquire resistance against metal nano-particles than to other conventional narrow-target antibiotics [9]. This is presumably because metals are known to act on a broad range of microbial targets, and several mutations would have to occur for microorganisms to resist their antibacterial activity [9]. Recently, adhesive materials containing poly-acrylic acid coated copper iodide (PAA-CuI) particles have demonstrated strong long-term antibacterial properties and reduced collagen degradation [10,11]. We speculate that, in addition to their strong antibacterial properties, PAA-CuI adhesives may help delay bond degradation. The biocompatibility of PAA-CuI containing materials, however, is still of concern since biodegradation of dental materials with the consequent byproduct release into the oral environment, might then initiate local and systemic biological responses. To date, the amount of copper released into the oral environment remains unknown.

Therefore, the purpose of this study was to evaluate adhesives containing PAA-CuI particles for their ability to delay bond degradation. Their effect on the adhesives' mechanical properties, cytotoxicity and copper release was also investigated. Scanning electron microscopy (SEM), X-ray diffraction analysis (EDX) and X-ray fluorescence (XRF) were conducted for microstructure characterization. Specific aims included evaluation of the PAA-CuI adhesives for bond strength after 24 h, 6 months and 1 year of storage, ultimate tensile strength (UTS), cell viability with MTT assay and copper release.

2. Materials and methods

Three commercially available adhesives, XP Bond (XP, Dentsply, York, PA, USA), Optibond Solo Plus (Solo, Kerr, Orange, CA, USA) and Optibond XTR (XTR, Kerr), were modified with 0.1 and 0.5 mg/ml PAA-CuI particles. Clearfil SE Protect (Protect, Kuraray America,

New York, NY, USA) contain-ing MDPB, a known antibacterial agent, was used as positivecontrol.

2.1. Preparation of the PAA-CuI adhesive resins

Both synthesis of PAA-CuI particles and generation of PAA-CuI adhesives was conducted according to Sabatini et al. [11]. Briefly, 10 mg of PAA-CuI powder was admixed with 1 ml of one of three adhesives, XP, Solo and XTR, to yield a concentrated solution (10 mg/ml). To ensure uniform dissolution of the particles, a probe tip sonicator (Sonic Dismembrator 100, Fisher Scientific, Waltham, MA, USA) was used for 15 s under dark conditions in an iced-water bath. Immediately after, 10 or 50 μ l of the concentrated solution was micro-pipetted into amber vials containing either 990 or 950 μ l, respectively of the appropriate stock adhesive to yield a final working tration of 0.1 or 0.5 mg/ml of PAA-CuI adhesive. The following study groups were evaluated: 1) XP; 2) XP-0.1 CuI; 3) XP-0.5 CuI; 4) Solo; 5) Solo-0.1 CuI; 6) Solo-0.5 CuI; 7) XTR; 8) XTR-0.1 CuI; 9) XTR-0.5 CuI and 10) Protect. All procedures were performed at controlled room temperature ($23 \pm 2^\circ\text{C}$) and humidity conditions.

2.2. Micro-tensile bond strength (μ TBS)

Thirty recently extracted, healthy human molars, obtained under a protocol approved by the State University of New York's institutional review board (IRB ID No. 00000133), were used to obtain dentin substrate. The teeth were equally and randomly assigned to ten groups. A flat, transversely cut surface of occlusal superficial dentin was obtained using a water-cooled lab trimmer (Whip Mix, Louisville, KY, USA), and a standardized smear layer created with 320-, 400- and 600-grit silicon carbide abrasive paper (SiC paper, Buehler, Lake Bluff, IL, USA). All materials were applied and polymerized following manufacturer's recommendations (Table 1) with LED light-curing unit (VALO, Ultradent, South Jordan, UT, USA) with a power density of 1400 mW/cm². Resin composite (Filtek Z100, 3M ESPE, Saint Paul, MN, USA) was applied in increments less than 2 mm to the bonded surface and polymerized for 40 s. After distilled water (DW) storage at 37°C for 24 h, sixty resin-dentin beams ($0.9 \pm 0.1 \text{ mm}^2$) per group were obtained according to the non-trimming technique [12], and incubated in DW for μ TBS evaluation after 24 h, 6 months or 1 year ($n = 20$). Individual beams were mounted on a stabilizing jig with cyanoacrylate (Zapit, Dental Ventures of America, Corona, CA, USA) and stressed to failure with a universal testing machine (Bisco, Schaumburg, IL, USA) at a cross-head speed of 1 mm/min. The load required to fracture the specimen was expressed in megapascals (MPa).

2.3. Ultimate tensile strength (UTS)

Hourglass-shaped specimens ($10 \times 2 \times 1 \text{ mm}$) were fabricated by placing the mold over a polyester strip, which was placed on top of a microscope glass slab. The adhesives were inserted carefully into the mold with care not to incorporate voids. A polyester strip and a microscope glass slab were placed on top of the mold, the specimens polymerized with LED unit (VALO) for the designated time, and polished with SiC abrasive papers (320–2400). Ten specimens per group ($n = 10$) were incubated in DW at 37°C for 24 h, and then mounted and stressed to failure as described above.

2.4. Cell viability

Human gingival fibroblasts (HGFs) were isolated from discarded healthy gingival tissue collected from subjects undergoing surgical procedures at the University at Buffalo clinics, using an explant outgrowth method fresh tissue under a current IRB protocol at the University at Buffalo (IRB proto-col 663292–1). Tissue pieces were collected immediately and washed in minimum essential Media (MEM) containing 10× antibiotics, followed by washing three times in media containing 1× antibiotics. Tissue pieces were minced into small pieces and allowed to attach to wells of a 6-well plate for 30 min, followed by the addition of 1 ml of fresh MEM + 10% FBS with antibiotics. Explants were incubated at 37°C, 5% CO₂ until migration of cells from the tissue explant could be observed, with replacement of fresh media every 3 days. Once 60% confluence was achieved, cells were detached using 0.25% trypsin/0.05% EDTA and reseeded into larger flasks. For routine culture, HGFs were grown to 80% confluence in MEM + 10% FBS, and passaged using trypsin/EDTA. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Sigma–Aldrich, St. Louis, MO, USA) was used to assess the cell metabolic function based on mitochondrial dehydrogenase activity. Three disc specimens (8 × 1 mm) per group (n = 3) were fabricated by micro-pipetting the adhesive into a circumferential polytetrafluoroethylene (PTFE) mold against a polyester strip, which was placed on a microscope glass slab and polymerized from top and bottom with LED unit (VALO) for the designated time. The discs were polished with SiC abrasive papers and sterilized by glow discharge sterilization for 15 min. Discs corresponding to the ten groups were incubated in 24-well plates in 1 ml of gingival fibroblast growth medium (alpha-MEM + 10% FBS + 1% antimycotic; pH 7.4; Gibco, Life Technologies, Grand Island, NY) for 24 h. This incubation time was selected since the most toxic effects are normally observed during the first 24 h as the resin is still undergoing polymerization [13]. An additional group, containing gingival fibroblast medium only and no disc, was used as control, whose survival rates were set to represent 100% viability. For the viability assay, HGFs (20,000 cells/well) were grown in 96-well plates until 80% confluent, after which the culture media was removed, replaced with 200 µl of filtered (0.2 µm filter, EMD Millipore, Billerica, MA, USA) disc-conditioned media and further incubated 48 h. The MTT assay was performed by aspirating the disc test media, followed by incubation of the HGFs with 200 µl MEM (no phenol red) and 13 µl MTT assay reagent for an additional 4 h at 37°C in 5% CO₂, then replacement with 200 µl of DMSO at the end of the incubation period and measurement of the absorbance at 540 nm (Flexstation 3, Molecular Devices, Sunnyvale, CA, USA).

2.5. Copper release

Three disc specimens (5 × 1 mm) per group (n = 3) were fabricated as described above, and sterilized with 95 v/v% ethanol for 5 min. Because even small amounts of contamination can lead to large errors when determining trace elemental concentrations, the vials used for specimen's storage were acid-cleaned with an aqueous solution of 0.3 mol/l HNO₃ for 12 h [14]. Specimens were individually stored in a cylindrical screw-top polyethylene test tube containing 5 ml of 0.9% sodium chloride (NaCl) buffered to pH 7.0 using 50 mmol/l of HEPES [15]. The test tubes were incubated in an oscillating tray at 37°C for 30 cycles/min. A nylon monofilament (Fishing line, VWR, Radnor, PA, USA), which passed through a small hole in the center of the specimen, was used to allow specimen retrievability without

contaminating the solution. The ratio of specimen surface area to volume of solution was 0.1 cm²/ml, less than the range of 0.5–6.0 cm²/ml recommended for biological studies of medical devices by ISO Standard 10993 [16]. This, however, was deemed appropriate since no biological studies were performed. Test tubes containing storage solution only and no disc were incubated under the same conditions as control. To determine the maximum absorption wavelength of copper, a standard solution was first generated by adding 100 µg/ml of copper (Sigma–Aldrich) to 3% nitric acid (EMD Millipore,) in a 1 cm quartz cell. A wavelength scan was performed with UV–vis spectrophotometer (ND-1000, Nano-drop. Wilmington, NC, USA) between 250–700 nm to detect changes in the molecular structure of copper as determined by a change in the UV–vis spectrum. Copper standard solutions (0.03125, 0.0625, 0.125, 0.25, 0.5, 1 µg/ml) in 3% nitric acid were prepared, and a calibration curve created by plotting the absorbance values against the concentration of copper. Copper release from the adhesive specimens into the media was measured with UV–vis spectrophotometer after immersion for 1, 3, 5, 7, 10, 14, 21 and 30 days. At each of the testing periods, the discs were retrieved from the test tube and placed in a fresh NaCl solution to incubate for the next time period. The regression equation derived from the calibration curve was used to calculate copper concentration (µg/ml) in the unknown samples based on their absorbance values.

2.6. Scanning electron microscopy (SEM), energy dispersive X-ray microanalysis (EDX) and X-ray fluorescence (XRF)

A disc (5 × 1 mm) per group was rinsed with DW, air dried, and placed on aluminum stubs with conductive tape, carbon coated and observed under SEM (SU70, Hitachi, Tokyo, Japan) using backscattered electron signal. Energy dispersive X-ray microanalysis was used for elemental composition analysis. An area of 20 × 15 µm was selected for 2D analysis, which included both resin matrix and filler particles. Another disc of the same dimensions was used to quantify trace copper amounts in each adhesive using XRF (Innov-X Alpha 2 portable X-ray Fluorescence) with an analysis area of 1 mm and expressed in parts per million (ppm).

2.7. Statistical analyses

A two-way analysis of variance (ANOVA) followed by Tukey's test were used to analyze the effect of 'treatment', 'time' and their interaction on µTBS. A Kruskal Wallis ANOVA on ranks followed by Tukey's test were used to identify differences in mean UTS values among groups. The effect of 'treatment' on cell viability was evaluated by one-way ANOVA and Tukey's test. Friedman's two-way ANOVA on ranks followed by Tukey's test were used to evaluate the effect of "treatment", "time" and their interaction on copper release. A significance level of $p < 0.05$ was used for all tests. All the analyses were performed with statistical software (SigmaStat version 3.5, San Jose, CA, USA).

3. Results

3.1. Micro-tensile bond strength

A significant effect of 'treatment' and 'time' ($p < 0.001$), but no effect of their interaction ($p = 0.063$) was demonstrated. No significant variations were observed after 6 months for any

of the groups relative to their 24 h values. Except for Protect, all other control adhesives demonstrated significant bond degradation after 1 year ($p = 0.027$, $p < 0.001$ and $p = 0.027$ for XTR, Solo and XP, respectively) (Fig. 1). Neither XTR or Solo with PAA-CuI degraded after 1 year regardless of the concentration, whereas XP Bond did ($p < 0.001$ and $p = 0.003$ for 0.1 or 0.5 mg/ml PAA-CuI respectively). Comparisons among groups means at each test period individually revealed a trend. While Solo demonstrated no significant variations, relative to their counterparts modified with PAA-CuI, XTR demonstrated lower values and XP higher values when modified with copper relative to their corresponding controls. No differences were shown between any of the PAA-CuI-modified adhesives and Protect at any of the test periods. The only exception was XP 0.1 mg/ml PAA-CuI, which demonstrated significantly lower μ TBS than Protect when evaluated after 1 year ($p < 0.001$).

3.2. Ultimate tensile strength

A significant effect of the “treatment” was demonstrated ($p < 0.001$). While no differences between adhesives modified with PAA-CuI and their corresponding controls were evidenced, XP as a group, exhibited significantly lower values than the other groups, including Protect (Fig. 2).

3.3. Cell viability

A significant effect of the “treatment” was observed ($p = 0.001$). While the percent viable cells for XTR 0.1 and 0.5 mg/ml and Solo 0.1 mg/ml PAA-CuI remained unaltered relative to their corresponding controls without copper, Solo 0.5 mg/ml and XP 0.1 and 0.5 mg/ml PAA-CuI showed significantly lower values than their corresponding controls ($p = 0.008$, $p = 0.005$ and $p < 0.001$, respectively) (Fig. 3). XP as a group revealed significantly lower cell viability values than all other groups ($p < 0.05$).

3.4. Copper release

A significant effect of the “treatment”, “time” and their interaction was demonstrated ($p < 0.001$). XP 0.5 and 0.1 mg/ml PAA-CuI had the highest copper release, with values which were significantly higher than all other adhesives ($p < 0.001$). XTR 0.1 and 0.5 mg/ml PAA-CuI followed, with values which were significantly higher than Solo 0.1 mg/ml PAA-CuI ($p < 0.001$ and $p = 0.008$, respectively) but not different from Solo 0.5 mg/ml PAA-CuI. The control adhesives demonstrated significantly lower copper release values (0.248, 0.235, 0.224 and 0.198 μ g/ml for XTR, Solo, Protect and XP, respectively) than PAA-CuI adhesives ($p < 0.001$). In general, copper release was high at day 1 (0.750 μ g/ml) followed by a decrease at days 3 (0.534 μ g/ml) and 5 (0.382 μ g/ml), a subsequent increase at days 7 (0.476 μ g/ml) and 14 (0.822 μ g/ml) and plateauing at days 21 (0.686 μ g/ml) and 30 (0.750 μ g/ml). Overall, release values at days 1 and 14 were significantly higher than all other periods ($p < 0.001$). The release value at day 30 was no different from days 1, 14 and 21, but was significantly different from days 3, 5 and 7 ($p < 0.001$). The lowest release values were observed at day 5 with a significantly lower value than all other periods ($p < 0.001$). Fig. 4 summarizes the copper release values in μ g/ml for each adhesive at the different incubation periods, as well as the multiple comparisons among groups. In addition, the mean copper release per day for the different copper-modified adhesives at days 1, 3, 5, 7, 14, 21 and 30 were calculated as follows: 1.27, 0.38, 0.26, 0.30, 0.15, 0.13 and 0.12 μ g/ml for XTR

0.1 mg/ml PAA-CuI; 0.82, 0.20, 0.26, 0.22, 0.20, 0.16 and 0.13 µg/ml for XTR 0.5 mg/ml PAA-CuI; 0.94, 0.18, 0.21, 0.24, 0.15, 0.10 and 0.10 µg/ml for Solo 0.1 mg/ml PAA-CuI; 0.72, 0.34, 0.25, 0.31, 0.16, 0.11 and 0.10 µg/ml for Solo 0.5 mg/ml PAA-CuI; 1.11, 0.58, 0.38, 0.33, 0.17, 0.16 and 0.13 µg/ml for XP 0.1 mg/ml PAA-CuI; 1.27, 0.69, 0.34, 0.65, 0.20, 0.19 and 0.14 µg/ml for XP 0.5 mg/ml PAA-CuI.

3.5. Scanning electron microscopy (SEM), energy dispersive X-ray microanalysis (EDX) and X-ray fluorescence (XRF)

The SEM micrographs (Fig. 5) reveal the crystallinity and size polydispersity of the PAA-CuI particles. The particle sizes ranged from 1–3 µm for the individual particles to 150–250 µm for the CuI agglomerates. The EDX spectrum of the PAA-CuI particles shows a ratio of Cu (K-line) to I (L-line) of 40:60, confirming its composition as copper iodide. Except for Solo 0.1 mg/ml PAA-CuI, the presence of copper was confirmed with SEM and EDX in all other adhesives (Fig. 6). The EDX spectrum verified the inorganic filler content as silicon dioxide for Optibond XTR, barium aluminoborosilicate and silicon dioxide for Solo, and primarily silica for XP. XRF demonstrated the presence of copper in all PAA-CuI containing adhesives, which was proportional to the concentration of PAA-CuI in the adhesive (Table 2). XRF values were 167 and 597 for Solo 0.1 and 0.5 mg/ml, respectively; 150 and 516 for XP 0.1 and 0.5 mg/ml, respectively; and 81 and 555 for XTR 0.1 and 0.5 mg/ml, respectively. No copper was detected for any of the control groups. The values for control groups, Solo, XP, XTR and Protect, 67, 45, 49 and 38, respectively, represent the threshold above which the element would be detected by the software for each group.

4. Discussion

This study provides original information on the effects of PAA-CuI modified adhesives on bond degradation, tensile strength, and biocompatibility as per evaluation of the cytotoxicity and ion release. No degradation was observed for any adhesive after 6 months. Six months has previously been reported as insufficient time to detect the effects of hydrolytic degradation [17]. After 1 year, however, all the control groups, except Protect, showed significant degradation with self-etch adhesives degrading less [XTR (25%) and Protect (10%)] than etch-and-rinse [Solo (66%) and XP (63%)]. The dual adhesive mechanism of self-etch adhesives, that is the chemical adhesion to hydroxyapatite in addition to the micro-mechanical interlocking, has been reported to make these adhesives stable even in aqueous environments [18], perhaps contributing to explain our findings. These interfaces may then better be able to withstand the hydrolytic breakdown of its components, with the consequent improvement in bond durability [19]. Only XP showed signs of degradation after 1 year regardless of the concentration of copper. The lower performance of XP relative to other adhesives has been previously documented [20], and it may be the result of its lower filler content, which was confirmed by our SEM/EDX observations. Conversely, XTR and Solo demonstrated stable bonds after 1 year irrespective of the concentration of PAA-CuI (Table 2). This may be the result of a myriad of factors. Our group has recently found that copper can reduce both degradation of collagen [10] and esterase activity (unpublished observations). In addition, copper is also known to be a catalyst for HEMA radical polymerization [21]. We speculate that an increased degree of monomer

conversion may have rendered some of the copper-containing adhesives more resistant to hydrolytic degradation and thus less susceptible to bond degradation.

Incorporation of PAA-CuI particles into the adhesives did not affect their UTS. Similarly, XP exhibited significantly lower UTS values than all other adhesives regardless of the presence of copper. A recent study by Stanislawczuk et al. also reported low UTS values for XP (7.2 MPa) relative to other adhesives [22].

Incorporation of PAA-CuI to XTR did not seem to affect cell viability regardless of the concentration indicating that copper was not cytotoxic to gingival fibroblasts. Protect did not demonstrate reduced cell viability either. Higher monomer concentrations are known to lead to lower cell survival rates [23]. We speculate that the higher survival rates observed for specimens of self-etch adhesives, XTR and Protect, may be attributed, at least partially, to an overall reduced monomer concentration since these were fabricated with a solution of adhesive only, as opposed to specimens of etch-and-rinse adhesives, Solo and XP, which were fabricated with a solution of primer and adhesive. The cell viability of Solo was significantly decreased with addition of 0.5 mg/ml, but not 0.1 mg/ml PAA-CuI. As a group, XP demonstrated the lowest cell survival rates, with cell viability values which were lower than the main control representing 100% viability. Other authors have previously reported the low survival rates of XP Bond, with cell viability values of 32% [24], 30% [25] and even 10% [26]. This has been attributed to the higher levels of UDMA, TEGDMA, HEMA and camphorquinone present in the mixture relative to other adhesives. Both, monomers [27–29] and camphorquinone [30–33] in the matrix of contemporary adhesives, are known to be cytotoxic.

Differences in copper release values were also observed. Overall, XP with 0.1 and 0.5 mg/ml demonstrated the highest release, and Solo 0.1 mg/ml the lowest release. Both filler particle size and volume are known to play a role on ion release [34]. Our SEM/EDX observations indicate that the filler content in XP is minimal relative to XTR and Solo. The increased matrix volume in XP may have led to a greater ion release, relative to the other adhesives. Ion release from resin-based materials is also known to be dependent on pH [15] and hydrophilicity of the resin matrix [35]. We further speculate that the higher levels of hydrophilic monomers (e.g. HEMA) in the matrix of XP, compared to the other adhesives, may have also contributed to the observed increased rate of copper release. The highest release values shown for XP, relative to other groups, may thus help explain the highest cytotoxicity effect observed for this group. The control groups, interestingly, showed some copper release. This could be attributed either to the presence of trace amounts of copper unreported by the manufacturer, or different elements reactive at the same wavelength used to detect copper (245 nm). Our XRF observations demonstrating the presence of minimal quantities of copper in the control adhesives, confirmed the former speculation. Analysis of the copper release over time demonstrated a consistent pattern for all adhesives. Days 1 and 14 demonstrated the highest release and days 5 and 7 the lowest release. An initial high release at day 1 was followed by an immediate gradual decrease at days 3 and 5, another increase at days 7 and 14, finally reaching a plateau at days 21 and 30. The initial surge is likely derived from the additional copper released from the top and bottom surfaces of the specimens. A correlation between the exposed surface of the sample and the release of

specific components has been previously reported [36]. The gradual decrease in release values at days 3 and 5 may be a more accurate representation of the true release pattern once the superficial copper has been exposed and released. A decrease in ion release values after an initial surge has been previously reported in previous studies [34,37]. The second surge may be the result of the exposure and release of additional copper particles as the resin matrix began to undergo swelling and hydrolysis during the first 14 days of incubation. Early effects of water absorption have been previously reported [38]. Water aging is known to increase filler particle pull out on the exposed surface, possibly due to a breakdown of the silane bonds between the resin and filler particles [39]. Moreover, since there is no silane coating present to promote adhesion of the copper particles to the matrix, these are expected to be much more susceptible to dislodgement.

Because element release plays a critical role in the material biocompatibility, it was critical to investigate, for the first time, the copper release pattern of PAA-CuI-containing materials. Our in vitro model, though it hardly resembles a clinical situation characterized by a rapid and continuous flow and removal of saliva, provides a simplified test set-up to allow for an initial estimation of the rate and quantity of copper expected to be released from resin matrices without the undue influence of mechanical and enzymatic factors. Metals are known to be more vulnerable to electrochemical degradation than other materials [40]. Copper modified adhesives have recently demonstrated excellent long-term antibacterial properties [11]. Ideally, antibacterial particles should be fixed within the restorative material to prevent undesirable side effects to the host tissues as well as the potential loss of its antibacterial properties [41]. Overall, the copper release values over the 30-day period were minimal ranging from 0.198 to 1.238 $\mu\text{g/ml}$. The allowed daily dietary intake of copper has been reported to be 11 $\mu\text{g/kg}$ [42,43]. Ion release studies normally present data cumulatively or additively for each incubation period. In this study, the data is presented additively for easier visualization of the amount of copper released at each interval. However, calculations of the mean release per day at each incubation period revealed that even the highest release value per day (1.27 $\mu\text{g/ml}$) was still well below the reported toxicity threshold. This indicates that a systemic toxic effect from copper containing adhesives is highly unlikely.

The SEM/EDX micrographs demonstrated the presence of copper particles in all adhesives except in Solo 0.1 mg/ml. We speculate that copper particles being relatively dense (8.96 g/cm^3) compared to other elements in the periodic table, may tend to agglomerate toward the center of the specimen, especially when added in low concentrations, explaining why these analytical techniques were unable to detect copper particles in Solo 0.1 mg/ml. Because SEM provides only a visual analysis of the surface and EDX an analysis to a depth of 2 μm , it is unlikely that the particles would remain suspended on the surface and available for detection at all times. Moreover, EDX has a detection level of 1%, which is higher than the concentration of copper in our experimental adhesives. The presence of copper particles was confirmed by XRF in all groups, including Solo 0.1 mg/ml, validating thus our assumption concerning the particles density.

The present study provides evidence in support of the beneficial role of PAA-CuI particles as an additive to dental adhesives to help improve the bond stability of resin-dentin bonds without an adverse effect to the adhesive's UTS, and with the release of only minimal levels

of copper that remain under the accepted toxicity level. Additional studies using different cytotoxicity models, adhesives of different composition and concentrations of the additive need to be undertaken to determine the optimal balance between antibacterial properties without an adverse effect on the material's bond strength, mechanical properties and toxicity to the host. A pH of 7 was used in the present study for the incubation solutions as a starting point. However, ion release from dental materials has shown to be highest at pH 4, decreasing at pH 5, and becoming minimal at pH 7 [44,45]. Because adhesives may be able to act as "smart" materials increasing copper release at cariogenic low pH ranges (pH 5.5–6.5) during a biofilm acid challenge, future studies should also investigate ion release patterns at lower pH ranges to evaluate whether they remain under the reported toxicity thresholds.

5. Conclusion

Within the limitations of this study, it can be concluded that incorporation of PAA-CuI particles was able to prevent bond degradation after 1 year for XTR, Solo, but not XP. No bond degradation was observed for any of the adhesives after 6 months. The UTS remained unaffected for all adhesives regardless of the concentration of PAA-CuI particles. Cell viability was not affected with the incorporation of 0.1 and 0.5 mg/ml PAA-CuI to XTR or 0.1 to Solo, but it was reduced when incorporating 0.5 mg/ml to Solo or 0.1 and 0.5 mg/ml PAA-CuI to XP. XP demonstrated the highest copper release of all adhesives. A similar pattern of copper release was observed for all adhesives with a high release at day 1, followed by a gradual decrease at days 3 and 5, another increase at days 7 and 14 and release values plateauing at days 21 and 30.

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Fig. 1 –.

Mean micro-tensile bond strengths of PAA-CuI particles containing adhesives and corresponding control groups at 24 h, 6 months and 1 year. Bars represent the mean values; brackets indicate the SD values. N = 20. Groups identified by different letters are significantly different (Tukey's test; $p < 0.001$). Upper case denotes differences among test periods for each treatment group. Lower case denotes differences among treatment groups for each test period.

Fig. 2 –.

Mean ultimate tensile bond strengths (UTS) of PAA-CuI particles containing adhesives and corresponding control groups. Bars represent the mean values; brackets indicate the SD values. N = 10. Groups identified by different letters are significantly different (Kruskal Wallis; $p < 0.001$).

Fig. 3 –.

MTT assay results of cytotoxicity against human gingival fibroblast cells obtained from polymerized resin discs. Adhesives with PAA-CuI particles were compared with control adhesive groups and untreated cells (Control), whose survival rates were set to represent 100% viability. Results are expressed in percentage of viable cells. Bars represent the mean values; brackets indicate the SD values. $N = 3$. Groups identified by different letters are significantly different (Tukey's test; $p < 0.05$).

Fig. 4 –. Copper release values (µg/ml) for the different adhesives modified with PAA-CuI particles and corresponding controlgroups at the different test periods. N = 3. For each adhesive-concentration group, different superscript letters indicatesignificant differences among test periods (days-horizontal) (Tukey’s test, p < 0.05).

Fig. 5 –. SEM micrograph and EDX spectrum of PAA-CuI particles; magnification 250 $\times$ (A), 3500 $\times$ (B) and 10,000 (C). Elements identified by energy dispersive X-ray spectroscopy microanalysis for the PAA-CuI particles (D).

Fig. 6 –.
SEM micrograph and EDX spectrum of PAA-CuI adhesives and corresponding control groups; magnification 1500×. Optibond XTR (A. Control, B. 0.1 mg/ml, C. 0.5 mg/ml), Optibond Solo (D. Control, E. 0.1 mg/ml, F. 0.5 mg/ml), and XP Bond (G. Control, H. 0.1 mg/ml, I. 0.5 mg/ml).

Table 1 –

Study materials, including composition and application protocol, as per manufacturer's recommendations.

Material	Composition	Application protocol
Ultra-etch (Ultradent) (Lot # B8RRK)	35% Phosphoric acid	<ul style="list-style-type: none"> • Rinse and dry surface • Apply etchant (20 s) • Rinse thoroughly, dry and apply adhesive per manufacturer's instructions
Z100 (3M ESPE) (Lot #5904A4)	Matrix: Bis-GMA and TEGDMA Filler: zirconia and silica—84.5%–0.6 µm	<ul style="list-style-type: none"> • Place and light cure in 2 mm increments (40 s) with halogen or LED light with minimum intensity of 400mW/cm²
Optibond XTR (Kerr Corp.)	Primer: HEMA (30–50), acetone (25–35), ethyl alcohol (4–15)	<ul style="list-style-type: none"> • Apply SE primer with active scrubbing (20 s)
Two-bottle system Self-etch (Lot # 000045)	Bond: ethyl alcohol (20–30), alkyl dimethacrylate resins (47–68), barium aluminoborosilicate glass (5–15), silicon dioxide (3–10), sodium hexafluorosilicate (0.5–3)	<ul style="list-style-type: none"> • Air thin (5 s) • Apply Bond with active scrubbing (15 s) • Air thin (5 s) • Polymerize (10 s)
Optibond Solo Plus (Kerr Corp.) One-bottle system Etch-and-rinse (Lot #5475754)	Bis-GMA, HEMA, GPDM, ethanol, barium aluminum borosilicate glass, fumed silica (silicon dioxide), sodium hexafluoro-silicate, photoinitiator (CQ)	<ul style="list-style-type: none"> • Etch with 35% H₃PO₄ (15 s) • Rinse & lightly dry • Apply adhesive with active scrubbing (15s) • Air thin (3 s) • Polymerize (20 s)
XP Bond (Dentsply) Two-bottle system Etch-and-rinse (Lot # 141027)	TCB resin, PENTA, UDMA, TEGDMA, HEMA, butylated benzenediol (stabilizer), ethyl-4-dimethylaminobenzoate, CQ, functionalized amorphous silica, t-butanol	<ul style="list-style-type: none"> • Etch with 35% H₃PO₄ (15 s) • Rinse (15s) & blot dry • Apply adhesive • Leave undisturbed (20s) • Air thin (5 s) • Polymerize (10 s)
Clearfil SE Protect (Kuraray Medical Inc.) Two-bottle system self-etch (Lot #AE0027)	Primer: MDPB, MDP, HEMA, hydrophilic dimethacrylate, photo-initiator, water Bond: MDP, HEMA, bis-GMA, hydrophobic dimethacrylate, photo-initiators, silanated colloidal silica, surface-treated NaF	<ul style="list-style-type: none"> • Apply SE primer. Leave in place (20 s) • Evaporate excess solvent with mild air stream • Apply bond with microbrush • Gently air thin leaving uniform layer • Polymerize (10 s)

CQ, camphorquinone; HEMA, 2-hydroxyethylmethacrylate; MDP, 10-methacryloyloxydecyl dihydrogen phosphate; MDPB, 12-methacryloyloxydodecylpyridinium bromide; PENTA, phosphoric acid modified acrylate resin; TEGDMA, triethyleneglycol dimethacrylate; TCB resin, carboxylic acid modified dimethacrylate; UDMA, urethane dimethacrylate; Bis-GMA, bisphenol A diglycidyl methacrylate; GPDM, glycerol phosphate dimethacrylate; TCB, butan-1,2,3,4-tetracarboxylic acid di-2-hydroxyethylmethacrylate ester.

Table 2 –

Summary of all results including micro-tensile bond strength, ultimate tensile strength, ion release, cell viability and X-ray fluorescence.

Group	6-month bond degradation (%)	1-year bond degradation (%)	UTS (MPa)	Cell viability (%)	Overall ion release (µg/ml)	XRF (PPM)
XTR	3	–25	21.9	92.7	0.248	ND < 49
XTR 0.1	12	–19	18.7	94.3	0.871	81 (±20)
XTR 0.5	–12	–19	23.2	96.7	0.827	555 (±39)
Solo	–3	–66	16.2	93.9	0.235	ND < 67
Solo 0.1	–1	–11	16.3	90.9	0.682	167 (±27)
Solo 0.5	0	–28	17.7	85.1	0.745	597 (±35)
XP	–11	–63	9	71.4	0.198	ND < 45
XP 0.1	–17	–112	9.1	62.2	1.015	150 (±22)
XP 0.5	–22	–45	9.5	56.9	1.238	516 (±45)
Protect	–3	–10	18.3	99.2	0.224	ND < 38