Exploring the use of a Ruthenium complex incorporated into a methacrylate-based dental material for antimicrobial photodynamic therapy

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Abstract

Objectives: To evaluate the effects of a blue light photosensitizer (PS), Ruthenium II complex (Ru), on the chemical, physical, mechanical, and antimicrobial properties of experimental dental resin blends.

Methods: The experimental resin (BisEMA, TEEDGMA, HPMA, ethanol, and photoinitiator) was loaded with Ru at 0.00%, 0.07%, 0.14%, 0.28%, 0.56%, 1.12%, 1.2%, 1.5%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% w/w. Samples were evaluated for the degree of conversion (DC) after 30 and 60 s curing-time (n = 6). Selected formulations (0.00%, 0.28%, 0.56%, 1.12%) were further tested for shear bond strength (SBS) (n = 15); flexural strength (FS) (n = 12); and antimicrobial properties (CFUs), in dark and light conditions. These latter tests were performed on specimens stored for 24-h or 2-month in 37°C water. Water sorption (WS) and solubility (SL) tests were also performed (n = 12). Data were analyzed either by a one- or two-factor general linear model (α = 0.05).

Results: Overall, Ru concentration above 1.2% resulted in reduced DC. In SBS results, only the 1.12%Ru resin blend samples had statistically lower values compared to the 0.00%Ru resin blend at 24-h storage (p = 0.004). In addition, no differences in SBS were detected among the experimental groups after 2-month storage in water. Meanwhile, FS increased for all experimental groups under similar aging conditions (p < 0.001). Antimicrobial properties were improved upon inclusion of Ru and application of light (p < 0.001 for both) at 24-h and 2-month storage. Lastly, no detectable changes in WS or SL were observed for the Ru-added resins compared to the 0.00%Ru resin blend. However, the 0.28% Ru blend presented significantly higher WS compared to the 0.56% Ru blend (p = 0.007).

Conclusions: Stable SBS, improved FS, and sustained antimicrobial properties after aging gives significant credence to our approach of adding the Ruthenium II complex into dental adhesive resin blends intended for an aPDT approach.

Keywords
Dental resins, ruthenium, antimicrobial, photodynamic therapy, methacrylate-based resin

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Introduction

Oral bacteria are directly linked to dental caries, which remains one of the most common chronic human diseases.1,2 Dental caries, a multifactorial disease, affects a large proportion of the population around the globe, with 2.5 billion people reportedly dealing with untreated dental caries in 2015.1 It also represents an important burden at many levels, from quality of life and nutrition3 to a massive economic impact with an estimated US$ 545 Billion...
spent in 2015. In parallel, the phase-down of dental amalgam, as proposed by the UN Environment Programme in 2013 due to its high mercury content, has led to a pressing demand for mercury-free, safe, and affordable alternatives to replace it.

To date, little advancement has been made of resin-based dental materials to effectively improve their antimicrobial power. As a result, there remains a significant based dental materials to effectively improve their antimicrobial power.8 As a result, there remains a significant need to design a novel and accessible material-therapy system which is able to assist with the control of this biofilm-dependent disease.1,2,4,9,10 Studies in this field have primarily focused on dental materials loaded with a single antimicrobial agent incorporated into the dental material, such as antimicrobial metallic nanoparticles11–13 or anti-fouling monomers.14–16 However, there has also been interest in combining multiple antimicrobial strategies in order to control pathogenic biofilms.17,18 Even though the aforementioned approaches present positive immediate results to control biofilm, these have so far had limited success with regard to their long-term effects and reactivation potential. In addition, while the microorganisms should not be completely eliminated of the oral environment, as some species are currently being tested as probiotic strain19,20; the development of multifunctional dental materials with specific therapeutic capabilities to locally reduce cariogenic biofilm present in critical areas around teeth restorations will certainly contribute positively to the future of oral, and overall, health management.

The use of an antimicrobial photodynamic therapy (aPDT) approach as a strategy to reduce cariogenic species has been of increasing interest in this field.21,22 The aPDT strategy consists of a photosensitizer (PS), the application of light, and presence of oxygen to effectively target bacteria. The PS absorbs a photon light of specific wavelength and transfers energy to the oxygen to form a singlet oxygen (1O2).23,24 Recent studies have shown the efficiency of aPDT on oral biofilms involving caries-related microorganisms and, as a result, may be beneficial as a conservative therapy to control dental caries. However, the studies specifically investigated aPDT with the PS freely available in an aqueous solution and in direct contact with the oral microorganisms, either in planktonic or biofilm forms. To date there have been no thorough short or long-term investigation of PS-loaded dental materials reported in the literature.25–28

A potential photosensitizer of interest is Ruthenium (Ru), a safe transition metal in group VIII.30–33 It has been applied in several biomedical fields, showing in vitro34,35 and in vivo16 biocompatibility. This metallic compound presents two main oxidation states, specifically +2 for Ru(II) complexes and +3 for Ru(III) complexes. Ru(II) complexes are used as favorable light-responsive compounds.30–33 The absorption peaks of Ru(II) complexes are in the 440–463 nm range, representing an ideal match with the activation spectrum on the blue light wavelength. In addition, Ru(II) complexes based on the 4,7-diphenyl-1,10-phenanthroline ligand present several benefits, including their solubility in most polar organic solvents, combined with high values of quantum yields of singlet-oxygen production in those solvents, they can be excited in a wide range of wavelengths and do not undergo photo-chemical or secondary dark reactions, and their rate constant of singlet-oxygen quenching is low.37

The light sources available for PDT include lasers, halogen lamps, or light emitting diodes (LEDs). The light wavelength is selected upon considering the two main factors: the ability of the light to penetrate into the substrate, and the optimum PS absorption peak. LEDs have become a particularly favored for PDT option due to low cost, small size, durability, and safety.38–40 The use of LED blue light sources is already incorporated in routine dental practices for photo-curing procedures; therefore, they can potentially be used as an high energy light device for an aPDT approach in dental offices.38–40 Furthermore, blue light by itself has been shown to have an antimicrobial effect.38,39,41

In this study we aim to address the knowledge gap and evaluate the chemical, physical, mechanical and antimicrobial properties, immediately and after aging, of a novel, metal-doped, photosensitive methacrylate-based dental material to be associated with aPDT. The overarching goal is to explore long-term antimicrobial properties of resin based dental materials that may bring potential for periodic reactivation with the aPDT associated with blue light in future investigation and clinical application.

**Methods**

**Experimental resin blends formulations**

An experimental resin blend (RB) consisting of 50 wt% ethoxylated (4) bisphenol A dimethacrylate (BisEMA; Esstech, Essington, PA, USA), 30 wt% tetraethylene glycol dimethacrylate (TEEGDMA; Esstech), 14 wt% 2-Hydroxypropyl methacrylate (HPMA; Esstech), 4 wt% ethanol was developed containing 2 wt% photoinitiator system (camphorquinone and ethyl 4-(dimethylamino) benzoate; Sigma–Aldrich, St Louis, USA) was prepared. Then, different concentrations of a blue light photosensitiser, an Ruthenium II complex of Tris(4,7-diphenyl-1, 10-phenanthroline)ruthenium(II) bis(hexafluorophosphate) (Ru(dpp)(PF$_6$)$_2$ ) or “Ru,” were added to the experimental RB at 17 different concentrations (0.0%, 0.07%, 0.14%, 0.28%, 0.56%, 1.12%, 1.2%, 1.5%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% w/w) and kept under magnetic stirring for 24 h at room temperature in a dark room.
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Degree of conversion (DC)

Each formulation of RB loaded with Ru was chemically analyzed on a Fourier Transform Infrared (FTIR) spectrometer (IRPrestige-21 FTIR-8400S, Shimadzu, Kyoto, Japan) operating in Transmittance mode with peaks presented in absorbance setting. For that, 20 µL of each formulation (n=6) was applied between two transparent polyacetate films (3 M; London, ON, Canada) and placed in a sample holder. Following a background scan of the polyacetate films alone, a spectra of the uncured sample was obtained over a range of 4000 to 400 cm\(^{-1}\) (36 times; resolution of 4 cm\(^{-1}\)). Next, samples were light cured for either 30 or 60 s with a LED light curing unit (Bluephase, Ivoclar Vivadent AG, Schaan, Liechtenstein) operating at 650 mW/cm\(^2\). The reading of cured samples was assessed using the same parameters of the uncured sample. The DC was calculated using the ratio between the aliphatic carbon-carbon double bond absorbance (at 1638 cm\(^{-1}\)) and the aromatic component (at 1608 cm\(^{-1}\)) for the uncured and cured samples (n=6) as per the equation: 

\[
\%DC = 100 \times \frac{1-R_{\text{cured}}}{R_{\text{uncured}}} 
\]

where the ratio R = Abs(1638 cm\(^{-1}\))/Abs(1608 cm\(^{-1}\)). The Figure 1 shows a representative FTIR spectra for the control (0.00% Ru) resin blend with the DC-relevant peaks indicated.

Water sorption (WS) and solubility (SL)

Following DC analysis, RB containing 0.00, 0.28, 0.56 or 1.12 (w/w %) Ru were selected to evaluate the WS and SL of the resin disks (n=12). The homogeneous mixture of the resin blends was confirmed in an optical microscope (Nikon Microscope with Intensilight, C-HGFI attachment; Melville, NY, USA) at 2X and 20X magnification. The goal was to ensure the Ru was fully dissolved into the mix, without any precipitation or dispersed particles. Thus, disk specimens made of different RB-Ru formulations and measuring 6 mm of diameter and 1 mm of thickness were produced. Using PVS molds, blends were light cured for 60 s at both the top and bottom surfaces using a LED light curing unit at operating at 650 mW/cm\(^2\) (Valo\textsuperscript{TM}, Ultradent Products Inc., South Jordan, UT, USA). The freshly produced specimens were placed in a desiccator and transferred to an oven at 37°C. The specimens were weighed in an analytical balance (ATX 124, Shimatzu, Kyoto, Japan) daily until a constant mass (variation <0.2 mg) was obtained (M1). The dimensions of the specimens (thickness and diameter) were measured (Mitutoyo, Digital Caliper, Aurora, IL, USA) to calculate the volume (V) of each specimen (in mm\(^3\)). Each disk was then transferred to sealed tubes with 1 mL of Milli-Q\textsuperscript{®} water at 37°C. Over a 14-day period, at frequent time points, the specimens were washed, gently wiped, weighed and then returned to the tubes containing 1 mL of fresh Milli-Q\textsuperscript{®} water until a constant mass (variation <0.2 mg) was achieved. Thus, after a final recording of disk mass at 14 days (M2), the specimens were stored in a desiccator to dry the samples until a constant mass (variation <0.2 mg) was obtained (M3). WS and SL after 14-days of water storage were calculates according with the following equations: 

\[
WS = \frac{(M_2-M_3)}{V}\]

\[
SL = \frac{(M_1-M_3)}{V}\]

Flexural strength (FS)

As with WS and SL analysis the RB containing 0.0%, 0.28%, 0.56%, and 1.12% Ru were selected to evaluate the FS (n=12). First, FS bars (2 × 2 × 25 mm) were produced.
in a Teflon® matrix following light curing with a LED unit operating at 650 mW/cm² (Valo™, Ultradent Products Inc., South Jordan, UT, USA), applied to three different sections across the 25 mm length, for 60 s each. The same light curing protocol was repeated on the opposite side of sections across the 25 mm length, for 60 s each. The same Inc., South Jordan, UT, USA), applied to three different operating at 650 mW/cm² (Valo™, Ultradent Products Inc., South Jordan, UT, USA) was positioned over the pre-treated dentin surface to build the resin composite (Filtek Z350, 3M ESPE) specimen. Each cylinder specimen (2.0 mm height) and a bonding clamp (Ultradent Products, Inc., South Jordan, UT, USA) operating at 650 mW/cm². The Ultradent jig consisting of a polytetrafluoroethylene mold insert (2.4 mm internal diameter) and a bonding clamp (Ultradent Products, Inc., South Jordan, UT, USA) was positioned over the pre-treated dentin surface to build the resin composite (Filtek Z350, 3M ESPE) specimen. Each cylinder specimen (2.0 mm height) was light cured for 40 s with the same LED light curing unit. Then, the mold was carefully removed and a second resin composite specimen was built on the same dentin surface. Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer was applied actively (10 s) and gently dried with air (5 s). Adhesive was applied and light cured for 10 s. Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer (from SBMP) was applied actively (10 s) and gently dried with jet of air (5 s). RB was applied and light cured for 30 s. Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer (SBMP) was applied actively (10 s) and gently dried with jet of air (5 s). RB + Ru was applied and light cured for 30 s.

**Shear bond strength (SBS) to dentin**

**Dentin surface preparation:** Sound human third molars with intact crowns were obtained immediately after extraction with the approval of the Research Ethics Board (#H14–02189). All tissue debris and calculus were removed mechanically prior to storing the teeth in 0.1% thymol solution (Sigma Aldrich) at 4°C. The occlusal enamel was cut off using a metallographic cutter (SYJ-150, MTI Corporation, Richmond, BC, Canada) coupled to a diamond blade (Buehler, Lake Bluff, IL, USA) to obtain a flat dentin surface. Teeth were individually embedded in a round polyvinylchloride (PVC) mold using a self-cure acrylic resin (Caulk Orthodontic Acrylic Resin, Dentsply, York, PA, USA). Subsequently, a standard smear layer on the flat dentin surfaces was created by polishing it for 60 s using 320-grit silicon carbide paper under constant water-cooling (Unipol-1210 Precision Lapping, CYKY, Henan, China). Then, the debris on the surface of the samples were removed with an ultrasonic bath in distilled water for 2 min.45

**Bonding procedure and SBS testing:** Adper™ Scotchbond™ Multi-Purpose (3M ESPE, St Paul, MN, USA) was chosen as a control group of the bonding procedure. Also, RB containing 0.0, 0.28, 0.56, and 1.12 (w/w%) Ru were selected to evaluate the SBS (n=15). All prepared dentin surfaces were treated as per manufacturer’s recommendation (control group) or according to the experimental procedure (Table 1). The adhesive layer was light cured using a LED light curing unit (Valo™, Ultradent Products Inc., South Jordan, UT, USA) operating at 650 mW/cm². The Ultradent jig consisting of a polytetrafluoroethylene mold insert (2.4 mm internal diameter) and a bonding clamp (Ultradent Products, Inc., South Jordan, UT, USA) was positioned over the pre-treated dentin surface to build the resin composite (Filtek Z350, 3M ESPE) specimen. Each cylinder specimen (2.0 mm height) was light cured for 40 s with the same LED light curing unit. Then, the mold was carefully removed and a second resin composite specimen was built on the same dentin surface.45 The samples were stored in MiliQ® water (replaced every week) at 37°C for 24 h and 2 months before testing. In each period of analysis, one specimen from each dentin surface was randomly selected and tested. The SBS test was performed by Bisco Shear Bond tester (Bisco Inc., IL, USA) at a crosshead speed of 0.5 mm/min. The SBS results (MPa) were given by the values of the maximum stress supported (N) by the dentin/material bond area (mm²).45

**Microbiological analysis**

The experimental RB containing 0.28, 0.56, or 1.12 (w/w%) of Ru photosensitizer was used to produce the resin disks of 6 mm diameter and 1 mm thickness following the same protocol described for water sorption and solubility tests (Section 2.3). Unloaded RB (i.e. 0.00% Ru) disks served as the study control.

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**Table 1.** Application mode of materials employed in the control and experimental groups.

| Material | Composition | Treatment |
|----------|-------------|-----------|
| Commercial control: Adper Scotchbond Multi-Purpose Adhesive System (SBMP; 3M ESPE) | Primer: HEMA, polyalkenoic acid copolymer and water. Adhesive: Bis-GMA, HEMA and initiators | Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer was applied actively (10 s) and gently dried with jet of air (5 s). Adhesive was applied and light cured for 10 s. |
| Experimental resin blend only (RB) | RB: BisEMA, TEEGDMA, HPMA, ethanol and initiators | Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer (from SBMP) was applied actively (10 s) and gently dried with jet of air (5 s). RB was applied and light cured for 30 s. |
| Experimental resin blend loaded with Ruthenium II complex (RB + Ru) | RB + PS: BisEMA, TEEGDMA, HPMA, ethanol, initiators and Ruthenium II complex | Dentin surface was etched with 35% phosphoric acid (15 s), followed by rinsing (10 s). The excess of water was removed by blotting. Primer (SBMP) was applied actively (10 s) and gently dried with jet of air (5 s). RB + Ru was applied and light cured for 30 s. |

HEMA: 2-hydroxyethyl methacrylate; Bis-GMA: bisphenol A diglycidyl ether dimethacrylate; BisEMA: ethoxylated (4) bisphenol A dimethacrylate; TEEGDMA: tetracethylene glycol dimethacrylate; HPMA: 2-hydroxypropyl methacrylate.
A preliminary study (data are not shown) was performed to isolate the potential antibacterial effect of the blue light irradiation, demonstrating no significant antibacterial effect at power density of 650 mW/cm² for 15 min. Therefore, the colony forming units (CFU) experiment was performed without the control for blue light irradiation. Then, the disks were then randomly distributed in two subgroups for microbiological tests: immediate or 2-month artificial aging in in MiliQ® water. Disk specimens for each testing time were autoclaved (Castle, M/C 3333) for 45 min at 125°C in normal gravity prior to the antimicrobial activity evaluation. A Streptococcus Mutans (S. mutans) strain (ATCC 33535) was used for the evaluation of the antimicrobial activity of the photosensitive Ru-resin disks. Sterile disks were first placed at the bottom of the 24-well tissue culture plates (TCP) before adding 1 mL of a LB solution containing 10⁵ to 10⁶ S. mutans to each well and incubating the plates anaerobically for 4 h at 37°C and 5% CO₂. The disks were then removed from the wells, carefully washed with sterile PBS to remove any non-adherent bacteria, and glued on the same side at the bottom of a new TCP and topped with 1 mL of fresh sterile PBS. A blue LED light source, designed to fit the 24-well TCP and capable of specifically activating the Ru photosensitizer, was applied and the disks were irradiated for 15 min at an average power density of 650 mW/cm². A second group of Ru-loaded disks was also evaluated under dark (no light applied) conditions. Following treatment (i.e. light or no light application) the disks were removed from the TCP wells, transferred to Eppendorf tubes containing 1 mL of sterile PBS, and sonicated in an ultrasonic bath (Vevor, Digital Pro, China) for 5 min to remove the adherent bacteria from the disk surfaces. Then, collected bacteria was serially diluted in PBS by factors of 10 and 10 µL drop of the dilutions added to LB agar plates before placing the dishes under anaerobic conditions with 5% CO₂ at 37°C. After 96 h the CFUs were counted for each dilution and the CFU/mL calculated using the following equation CFU/mL=[(# of colonies) × (1/dilution factor) × 1000 µL/mL)/(10 µL). The log of the determined CFU/mL is reported.

**Statistical analysis**

All statistical analysis was conducted using IBM® SPSS® Statistics v28 software package. The data was analyzed with a multi-factor univariate general linear model and post-hoc Tukey (α=0.05). Data was presented as the mean ± one standard deviation. Statistical significance was accepted as $p<0.05$.

**Results**

**Degree of conversion**

The inclusion of Ru in to the experimental RB detectably impacted the DC at 30 and 60 s ($p<0.001$ for both; Figure 2). As expected, the longer light curing time allowed higher DC for all groups and as Ru concentration increased the DC of the RB detectably decreased. For 30 s light-cured blends, the addition of more than 3% of Ru resulted in a detectable decrease in DC compared to the control blend ($p<0.001$). Meanwhile, for the 60 s light-cured blends, this decrease in DC was observed consistently for concentrations higher than 4% of Ru ($p<0.001$). Lastly, it was observed that the DC had a significant reduction between 3% and 4% for both 30 to 60 s light cured samples ($p=0.018$ and 0.033, respectively). However, the 30 s

Figure 2. Degree of conversion (DC) for the experimental resin blend as a function of Ru concentration. Data reported as average ± one standard deviation (n=6). *Indicates a statistically significant change from previously reported w/w% of Ru added to the RB ($p<0.05$).
light-cured also showed a preceding drop, which was significant between 2% and 3% ($P < 0.001$). Owing to these DC findings and early sample fabrication observations, three concentrations of Ru (0.28, 0.56, and 1.12 w/w%) were selected for further investigation in this study.

**Water sorption and solubility**

The addition of Ru in to the experimental resin blend detectably affected WS ($p=0.010$), but did not impact SL ($p=0.089$) (Figure 3). However, the impact of Ru concentration on WS was minimal as the only detectable pairwise difference was between the 0.28% and 0.56% Ru-loaded experimental blends ($p=0.007$).

**Flexural strength**

The incorporation of 0.28, 0.56 or 1.12 w/w% of Ru in the RB did not detectably impact flexural strength for both storage periods analyzed ($p > 0.05$; Figure 4). However,
all groups showed a detectable increase of FS after 2 months of water storage compared to those tested after only 24 h in water ($p < 0.001$).

**Shear bond strength**

Overall, the shear bond strength of the resin (Figure 5) was not detectably impacted by the incorporation of Ru for both aging periods ($p > 0.05$). However, the addition of the highest concentration of Ru (1.12%) significantly reduced the shear bond strengths of the experimental resin to dentin at 24 h ($p = 0.019$). The commercial control group (SBMP) shows the highest values of SBS at 24 h of storage compared to all resins investigated ($p < 0.001$). Meanwhile, the 2-month aging did not greatly affect the SBS reported for all sample and control groups ($p = 0.581$). After 2-months of storage there was no detected difference between the commercial control (SBMP), the experimental control (0.00% Ru), and the experimental Ru 0.28% ($p > 0.05$). Meanwhile, 0.56% and 1.12% Ru added resin blends had detectably lower SBS reported than the commercial control after 2-months storage ($p < 0.001$).

**Microbiological analysis**

All groups demonstrated significant antibacterial activity following light treatment (Figure 6). The highest degree of antimicrobial photodynamic action was observed for 1.12% (w/w) Ru experimental resin blend, which resulted in greater than two LOG CFU/mL of photokilling effect compared to the dark control (0.00% Ru RB). However, relative to the dark control the application of light on each Ru-loaded RB disk showed a detectable photokilling effect of at least one LOG CFU/mL. For dark toxicity effect, with the exception of the 1.12% (w/w) group tested after 2 months water storage, no other experimental group showed any significant reduction in CFU counts compared to the respective dark control. The experiments for both immediate as well as 2 months storage were repeated at least twice ($n > 3$).

**Discussion**

Many metals have found use as antimicrobial agents and/or surfaces owing to their toxicity to bacteria and yeast. The mechanisms of metal toxicity can involve: I. production of ROS associated with antioxidant depletion; II. loss of protein function and enzymatic activity; III. damage on the membrane function; IV. compromise in the nutrient assimilation; and V. genetic damages. Among the metal-based antimicrobials, Ru is of particular interest since its mode of action allows remarkable applications in the biomedical and chemical fields. Additionally, various types of ruthenium-complexes have been explored as a potential photosensitizer (PS) for antimicrobial photodynamic therapy (aPDT). For example, some ruthenium-complexes have exhibited antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* (gram-positive bacteria), either in the dark or light conditions, being the stronger antibacterial effect observed when exposed to a blue LED light. It was also observed that a noninnocent...
ligand-based Ru(II) complex 3, where the oxidation state is unclear, can enable an efficient DNA damage mediated by OH• upon excitation at 649 nm and inactivate *Escherichia coli* (gram negative bacteria). Phototoxicity of Ru has been further demonstrated against other gram-positive and gram-negative species, suggesting that their wide spectrum could include antimicrobial activity against cariogenic-specific bacteria. As a result, in the present study, different concentrations of Ru (II) complex were incorporated in an experimental dental resin blend to evaluate the Ru dependence on the chemical, physical, mechanical and antibacterial properties of the new methacrylate-based dental material designed for aPDT.

In the present study, the experimental blend was loaded with Ru(II)-complex in 17 different concentrations (0–10% w/w) and initially screened regarding their degree of conversion (DC). While longer polymerization time resulted in an increase in the DC, higher concentrations of Ru complex incorporated in the resin blends decreased the DC. Similarly, a previous study added different concentrations of two PS in the blue wavelength absorption peak (Riboflavin and Rose Bengal) into an adhesive resin. The results of the aforementioned study corroborate with our findings, in which higher concentrations of PS in the blue region resulted in a decrease in the DC. These particular findings can be explained on the photoinitiator system used in light curable dental resins, as the majority of restorative dental materials use a camphorquinone and amine photoinitiator system, suitable for light curing. Camphorquinone, a bright yellow compound, absorbs blue light at 400–500 nm wavelength due to the n,π* transition of the α-dicarbonyl chromophore. The excited compound removes a hydrogen atom from the tertiary amine [e.g. ethyl 4-(dimethylamino) benzoate] generating amine-primary radical and attacking the carbon double bonds. The processes triggers a chain of reactions that result in free radicals and initiate the polymerization. Thus, it is likely that the Ru(II)-complex added to the experimental resin blend competes with the photoinitiator system for the same wavelength peak. This has a more significant impact when the Ru-complex is incorporated in higher concentrations, resulting in reduced DC. Furthermore, as the solubility of the Ru(II) complex in the resin blend is relatively poor, just above ~1% w/w. As such, there may be some small amount of scattering of the light due to immiscible Ru particles which would also contribute to a reduced DC. Considering that an important requirement for resin based dental material relies on an adequate degree of conversion, subsequent evaluations of the experimental blends loaded Ru(II)-complex was limited to a maximum of 1.12 w/w% Ru.

It is well known that monomers applied to dental materials can absorb water and chemicals from the surround medium, as well as release compounds in the environment. The moist environment of the oral cavity associated with variations in the composition of such dental

Figure 6. CFU count as a function of Ru concentration, storage time, and application of light. Data reported as average ± one standard deviation (n > 3). Control for this study was the unloaded RB (i.e. 0.00% Ru) after dark treatment. Symbols represent statistically significant differences: *indicates a detected difference from the dark control under similar aging, # a detected change from RB of matching Ru loading under dark treatment, and $ a detected change from RB loaded with 1.12% Ru under similar light treatment (p < 0.05).
materials may accelerate elution of unreacted and under polymerized light cured monomers, promote plasticization of the resin and result in structural weakening. Some studies have shown that the presence of an antibacterial agent incorporated into a methacrylate-based dental material may increase its water sorption and solubility, resulting in deleterious effects on the polymeric structure and compromising its durability. Therefore, this study evaluated the impact of the Ru incorporated into the resin blends in regard to the water sorption and solubility properties. Interestingly, our results demonstrated that the presence of Ru on the experimental RB had no detectable influence on the WS and SL, compared to the control. However, 0.56% Ru blend presented a significantly lower WS compared to 0.28% Ru blend, which could be attributed to the ability of the Ru(II) complex to stabilize the polymeric surface against hydrolytic interaction. Intriguingly, the additional increase in Ru concentration, at 1.12%, did not result in lower WS compared to the control. In fact, future studies are necessary to further investigate the miscibility and dispersion of this particular Ru(II) complex. It should either property be poor, the ability to properly interpret the WS and SL data may be limited.

The innovative Ru-loaded resin blend must also provide sufficient mechanical and physical properties to support the action of occlusal forces and provide adequate wear resistance. These properties, directly linked to flexural strength, may influence the clinical success of the methacrylate-based dental materials. Previous studies have shown that the incorporation of some antimicrobial monomers for polymeric dental restoratives in high concentrations promote a reduction in flexural strength. However, another study evaluating an experimental resin composite (BisGMA, TEGDMA and inorganic filler particles) associated with different amounts of microparticulate bioactive glass (BAG) (0, 5, 10, and 30 wt%) demonstrated an antimicrobial effect against S. aureus, S. mutans and E. coli at 10% BAG, and no significant differences in flexural strength when compared to the control group. Furthermore, Sigusch et al. incorporated a mixture of α-tricalcium phosphate and photosensitizer meta-tetra(hydroxyphenyl)chlorin (mTHPC at 20 wt%) in two light-curable methacrylate-based materials and found an antimicrobial activity on Porphyromonas gingivalis and Enterococcus faecalis under laser irradiation at 652 nm wavelength, without compromises in flexural strength. Also, antimicrobial effect against S. mutans in an aPDT approach was observed when acrylic resin was loaded with 0.5% or 1% of Ulva lactuca, a green seaweed extract, without compromises in the flexural strength. In the current work, the incorporation of different concentrations of Ru on the experimental resin blends did not compromise the flexural strength. Furthermore, it was observed that all experimental groups presented an increase in the flexural strength values after aging. This behavior can be explained based on the storage conditions of the samples (water at 37°C). Studies have shown that increased mechanical properties of polymeric materials after aging under water/thermal conditions might be associated with a further increase in crosslinking and polymerization. Moreover, a greater polymer crosslink density can enhance the flexural strength on resin-based materials. Therefore, the proposed Ru(II)-complex loaded into dental resin blends seems to be a promising alternative for secondary caries management with the additional benefit of maintaining the mechanical properties of the methacrylate-based dental material.

It is well known that dentin bonding is one of the most critical steps in restorative dentistry, as this substrate is heterogeneous and wet in situ. While the immediate shear bond strength test (i.e. 24 h storage) is an important screening method for novel dental adhesive materials, the long-term bond stability also needs to be evaluated to confirm its ability to maintain clinically acceptable bond strength values over time. This study showed that all experimental resin blends evaluated presented SBS stability after 2 month of water storage compared to the 24 h aged specimens. Also, the incorporation of Ru in the RB had a minimal impact on SBS to dentin with only the highest concentration of Ru evaluated (1.12%) showing detectably lower SBS values compared to the 0.00% Ru-RB after 24 h water storage (reduction by ~30%). Other studies have explored the incorporation of PSs in resin-based dental materials for aPDT applications and its influence in the adhesive properties. For instance, one study demonstrated that an increased concentration of Riboflavin or Rose Bengal PSs resulted in improved antimicrobial effects but relatively poor adhesive properties. In contrast, other studies have shown that the highest concentration of PS evaluated were able to maintain clinically acceptable bond strengths after 2 month water storage, combined with an aPDT effect against S. mutans when exposed to blue LED and even in the dark. Furthermore, our study included a commercial control (SBMP) considered the gold-standard for three-step etch-and-rinse adhesive systems. This allowed us to directly compare the adhesive properties of our novel experimental blends with a commercial product extensively studied. Although the experimental resins showed lower SBS values compared to the commercial control, it is important to highlight that all blends presented bond stability over time and SBS values are within the clinically acceptable range. Therefore, this innovative Ru-enriched resinous material could be an excellent alternative in Restorative Dentistry procedures, from a potential low-viscosity infiltrating resin or a more hydrophobic fissure sealant, to an antibacterial bonding agent for direct and indirect restorations and orthodontic brackets.

The microbiological evaluations in the present study were completed in fresh and aged resin blend specimens,
under light and dark conditions. While all experimental Ru-RBs induced a significant log reduction (CFU/mL) of *S. mutans* under LED irradiation (650 mW/cm²), in fresh and 2-month aged specimens, the greatest antimicrobial potential was observed for the 1.12% (w/w) Ru-RB. Additionally, the 1.12 w/w% Ru-RB was the only formulation presenting antibacterial activity in dark conditions at 2-month storage, compared to the experimental control (0.00% Ru). It is interesting to note that very low concentrations of Ru is required for an aPDT effect against *S. mutans*, compared to other studies exploring other photosensitizers.70,71 For instance, a previous study evaluated a polymeric Ru precursor at different concentrations (0–667 ng/mL) against *E. coli* and *S. aureus* as a photosensitized antimicrobial agent triggered by UV-A irradiation on 365 nm wavelength.61 Corroborating with the current research, it was demonstrated that inhibition of bacterial growth is concentration and irradiation-dependent.61 Similarly, this was also observed in another study with a peptide–ruthenium conjugate applied to inactivation of multidrug-resistant bacteria under 470 nm light over 12 h.76 The mechanism of action of Ru toxicity to bacteria is associated to their capability to produce singlet oxygen species (1O₂) due to their low-energy triplet state when irradiated with a blue light, leading to damage of several biomolecules, disruption of bacterial membranes and microbial death.48,55,61 It can also be associated to their strong DNA binding, causing DNA degradation, even without light radiation.48,76 Microbiological studies have reported that a reduction of 3 log₁₀ are considered clinically applicable and relevant,77–79 according to the American Society for Microbiology and the Clinical and Laboratory Standards Institute (CLSI). In our study, the inclusion of 0.28%, 0.56%, and 1.12% Ru in the experimental blends detectably reduced the log count of *S. mutans* compared to the unloaded control RB. Although 1.12% (w/w) Ru experimental resin blend under light irradiation resulted in greater than 2 log₁₀ CFU/mL reduction compared to the dark control (0.00% Ru RB), future studies are necessary to further explore and optimize the systems, aiming to achieve suitable antimicrobial properties while maintaining or increasing the adhesive, mechanical and chemical properties. Additionally, the 1.12% (w/w) Ru-RB could be of use in some clinical scenarios as a dental material to control pathogenic oral biofilms formation, thus minimizing the progress of dental caries.27

Therefore, the present research demonstrated that having up to 1.12% Ru (II) complex incorporated into a methacrylate-based dental material has a great potential in multiple aspects of Dentistry. The novel approach, designed to control cariogenic bacteria with the use of aPDT, aims to be a in-situ platform for repeated intra-oral light applications in an aPDT to manage oral pathogenic bacteria linked to dental caries. Additional investigations exploring the parameters on the light, the resin blend formulation, and other oral pathogenic microorganisms are needed in this promising area, alongside with their effectiveness on *in situ* and *in vivo* studies.

## Conclusion

The findings of this study demonstrated that the addition of Ru (II) complex in low concentrations (0.28, 0.56, or 1.12 w/w %) into a methacrylate-based formulation is a feasible and promising aPDT approach, particularly concerning the sustained antimicrobial properties. The experimental formulations presented adequate DC, enhanced FS and stable SBS to dentin over time, with no compromises on WS, SL, combined with antimicrobial activity in fresh and aged specimens. The study showed for the first time that this novel approach of incorporating ruthenium-complex as a photosensitizing compound into a methacrylate-based material is a feasible strategy against oral pathogenic bacteria. The proposed concept opens several opportunities for future studies on its use for other dental and medical applications.

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## Author contributions

APM: Conceptualization, Validation, Resources, Writing-Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. MLL: Methodology, Investigation, Writing-Original Draft. PC: Investigation, Formal Analysis, Writing - Review & Editing. CD: Methodology, Investigation. SG: Methodology, Investigation. DL: Conceptualization, Validation, Resources, Writing - Review & Editing, Project administration. NB: Conceptualization, Validation, Resources, Project administration. All authors reviewed and approved the final version of the manuscript.

## Data availability statement

The data that support the findings of this study are available.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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