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Determining Optimal Fluorescent Agent Concentrations in Dental Adhesive Resins for Imaging the Tooth/Restoration Interface

Odair Bim Júnior,[1] Marco A. Cebim,[2] Maria T. Atta,[1] Camila M. Machado,[3] Luciana F. Francisconi-dos-Rios,[4] and Linda Wang[1,*]

[1]*Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Bauru, 17012-901 SP, Brazil*
[2]*Department of Inorganic Chemistry, Institute of Chemistry, Universidade Estadual Paulista, Araraquara, 14800-060 SP, Brazil*
[3]*Department of Prosthodontics, Bauru School of Dentistry, University of São Paulo, Bauru, 17012-901 SP, Brazil*
[4]*Department of Operative Dentistry, School of Dentistry, University of São Paulo, São Paulo, 05508-000 SP, Brazil*

Abstract: Fluorescent dyes like Rhodamine B (RB) have been used to identify the spatial distribution of adhesive restorative materials in the tooth/restoration interface. Potential effects of the addition of RB to dental adhesives were addressed in the past, but no further information is available on how to determine suitable concentrations of RB in these bonding agents for imaging in the confocal laser scanning microscope. This study provides systematical strategies for adding RB to viscous dental adhesive resins, focusing on the determination of the lowest range of dye concentrations necessary to achieve an acceptable image of the dentin/adhesive interface. It was demonstrated that optimized images of the resin distribution in dentin can be produced with 0.1–0.02 mg/mL of RB in the (tested) adhesives. Our approaches took into account aspects related to the dye concentration, photophysical parameters in different host media, specimen composition and morphology to develop a rational use of the fluorescent agent with the resin-based materials. Information gained from this work can help optimize labeling methods using dispersions of low-molecular-weight dyes in different monomer blend systems.

Key words: confocal laser microscopy, dental adhesives, dentin bonding, fluorescent dyes, photoluminescence spectroscopy, interfacial morphology

Introduction

In dental research, confocal laser scanning microscopy (CLSM) has been utilized to acquire high-resolution optical sections of the dentin/adhesive interface (Watson & Boyde, 1991; Watson, 1997; D'Alpino et al., 2006*a*; Sauro et al., 2008; Toledano et al., 2014, 2015). This interface is obtained when a dental adhesive—a fluid multi-monomer system used to make the dental composite adhere to the tooth—penetrates an acid-etched dentin surface, entangles the exposed collagen fibrils and undergoes *in situ* polymerization, forming a bio-engineered interlocked structure called the hybrid layer (Spencer et al., 2010; Pashley et al., 2011; Tjäderhane et al., 2013).

Labeling dental adhesives with traditional fluorescent dyes, such as rhodamines and fluorescein salts, became a popular strategy for investigating the resin distribution in the tooth/adhesive interface (Krejci et al., 1999; Ding et al., 2010; Toledano et al., 2013; Ionta et al., 2016). The labeling method is based on a simple dispersion process, rather than covalently attaching the fluorophore to an adhesive monomer (D'Alpino et al., 2006*a*, 2006*b*).

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*Corresponding author. wang.linda@usp.br

Rhodamine B (RB), a xanthene derivative, is the most commonly used dye for adhesive labeling (D'Alpino et al., 2006*a*). Its powder is readily soluble in water and organic solvents, and can be easily mixed with dental adhesives that contain ethanol or acetone (Griffiths et al., 1999; Pioch et al., 2003; D'Alpino et al., 2006*a*). Furthermore, RB presents excellent photophysical properties for a traditional dye. Under the laser scanning microscope, fluorescence from a RB-labeled sample can be induced and then detected, as the sample is scanned point-by-point with a laser line (commonly the 532 nm one) congruent to the dye's absorption band (Semwogerere & Weeks, 2008; Paddock & Eliceiri, 2014).

CLSM for bond integrity analysis is considered a handy, nondestructive, and inexpensive method with regard to dental specimen preparation in comparison with other microscopic techniques (Pioch et al., 1997; Watson et al., 2000). However, variables not shown to be of concern in past investigations may limit the reliable use of dyes for visualizing the tooth/adhesive interface (Watson, 1997; D'Alpino et al., 2006*b*). There is a lack of detailed information on the method of incorporation of fluorescent dyes into dental adhesives, especially in terms of addition of dyes to the nonsimplified systems (when a conditioning primer and the adhesive itself are set in separate bottles) (D'Alpino et al., 2006*a*;

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Francisconi et al., 2009; Sampaio et al., 2011). Literature also indicates a major issue with the lack of standardization of the concentrations of RB and other fluorescent dyes in labeled adhesives (D'Alpino et al., 2006*a*). With regard to this matter, D'Alpino et al. (2006*b*) investigated potential effects of RB on some chemical/mechanical properties of a commercial dental adhesive. However, the authors have only demonstrated that specific concentrations of the dye can affect the adhesive resin properties, with no further analysis of how to determine suitable concentrations of RB for the CLSM analysis. In addition, the photophysical characteristics of RB-labeled adhesive systems have not been addressed. The fluorescence emission of rhodamines and other dyes can be influenced by the polarity, viscosity, and pH of the host medium (microenvironment) and by the concentration of the dye itself (Valeur, 2001; Wagner, 2009; Kristoffersen et al., 2014). It is well known that overloading certain solutions with RB can even lead to quenching of the fluorescence, instead of boosting its intensity (Arbeloa et al., 1989; Petit et al., 1993; Watson, 1997). Ultimately, the confocal image formation process involves many parameters that should be optimized, including the dye (type and concentration), specimen morphology and composition. Parameters such as the laser line selection, laser intensity [usually controlled by the acousto-optical tunable filter (AOTF)], the voltage and offset on the photomultiplier tube (PMT), emission waveband, pinhole size, and scan speed need to be set up properly for optimal images (Pawley, 2000; Tsien et al., 2006). Choosing a suitable RB concentration for adhesive labeling is an essential part of defining optimal experimental conditions for the CLSM technique.

Given the above, this work provides systematical strategies for adding RB to viscous dental adhesive resins, focusing on the determination of the lowest range of dye concentrations necessary to achieve an acceptable image of the dentin/adhesive interface. Moreover, this study aimed to determine if (i) the emission spectra of the RB-labeled resins are influenced by the dye concentration and host medium, (ii) RB exhibits fluorescence quenching in the polymerized methacrylates, (iii) a semi-direct staining technique, using ethanol/RB solutions, can be used for preparing viscous resin samples containing low concentrations of RB.

Materials and Methods

Table 1 shows the main materials utilized in the experiments and their characteristics. Note that each dental adhesive system—Adper Scotchbond Multi-Purpose (MP) and Clearfil SE Bond (SE)—consists of a primer solution and the adhesive itself (the polymerizable bonding component). The primers were used as received, not being labeled. The adhesives were labeled with RB at decreasing concentrations by means of an ethanol/RB solution technique as described below. The fluorescent behavior of the RB-labeled adhesives (polymerized samples) was investigated by photoluminescence spectroscopy and CLSM.

Ethanol Solutions of RB

A volume of 100 mL of a 2.5 mg/mL ($\approx 5\ 10^{-3}$ mol/L) stock solution of RB in high-purity ethanol was prepared. Five serial dilutions were then performed, obtaining other 25 mL ethanol/RB solutions with dilutions of 1:5, 1:25, 1:125, 1:625, and 1:3,125.

Labeling of Adhesives Using Ethanol/RB Solutions

Adhesives MP and SE (uncured resins) of 1 mL amounts were labeled with RB at five decreasing concentrations (mg/mL) of dye: 0.5, 0.1, 0.02, 0.004, and 0.0008. The addition of RB to the neat adhesives was performed by means of small ethanol/RB aliquots extracted from the ethanol/RB solutions. The aliquot volumes were calculated according to equation (1) (below), drawn with micropipettes (Transferpette S; BRAND GmbH + CO KG, Wertheim, Germany) and then released inside standard micro test tubes with lid closures. All tubes containing specific amounts of ethanol/RB were then placed with lids open in a laboratory incubator (80°C) and monitored until complete ethanol evaporation was detected for each tube. Thus, only the mass of RB predetermined for labeling remained inside the tubes before the addition of the adhesives. After the tubes had cooled down to room temperature, 1.0 mL of either adhesive MP or SE was individually delivered to the corresponding tubes for each of the five different concentrations of dye. Homogenization was carried out using a dental air motor (speed range up to 20,000 rpm) and a straight handpiece equipped with a #10 round bur, to stir the resin samples inside the tubes for 3 min, or until no RB cluster could be seen on the tubes' internal walls. Care was taken to avoid resin exposure to white light and touching the tube's walls with the rotating bur.

Three parameters were mandatory in this labeling method: (i) the volume of adhesive to be labeled, V_{AD}, which was set at 1.0 mL in this experiment; (ii) the desired concentration of RB in the adhesive, C_{AD}, that matched each of the five decreasing concentrations previously mentioned; and (iii) the required volume of the ethanol/RB aliquot, V_{AL}, containing the mass of dye to be incorporated into the adhesive sample. As the first two parameters were set, the volume of the aliquot was calculated by the following equation:

$$V_{AL} = \frac{C_{AD} \cdot V_{AD}}{C_{AL}}, \quad (1)$$

where C_{AL} was the concentration of RB in the aliquot, which matched the concentration of the ethanol/RB stock solution (or the concentration of a diluted solution, if needed).

Photoluminescence Spectroscopy of RB-Labeled Adhesives

Disk-shaped specimens (5 mm in diameter and 0.8 mm thick) of each labeled adhesive (MP and SE with RB at those five concentrations) were prepared as follows. The labeled

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Table 1. Main Commercial Materials Used in This Study.

Materials	Manufacturer/Batch	Classification	Composition
Adper Scotchbond Multi-Purpose	3M ESPE, St. Paul, MN, USA/lot N322814 (primer) and N342538 (adhesive)	Etch-and-rinse 3 steps adhesive system	Primer: HEMA, polyalkenoic acid copolymer, water Adhesive: BisGMA, HEMA, CQ
Clearfil SE Bond	Kuraray Medical Inc., Japan/lot 01090A (primer) and 01628A (adhesive)	Self-etching 2 steps adhesive system	Primer: MDP, HEMA, CQ, water Adhesive: MDP, HEMA, BisGMA, hydrophobic dimethacrylates, submicron silica fillers, *N*,*N*-diethanol-*p*-toluidine, CQ
Filtek Z250 A2	3M ESPE, St. Paul, MN, USA/lot N327127BR	Microhybrid restorative filler	Resin-based matrix: BisGMA, BisEMA, UDMA, CQ Inorganic fillers: zirconia/silica particles, 60% by volume with 0.6 μm average particle size
Rhodamine B for Fluorescence	Sigma-Aldrich Chemie GmbH, Gillingham, New Road, UK/lot 33901062	Fluorescent dye (powder)	9-(2-Carboxyphenyl)-3,6-bis(diethylamino) xanthylium chloride Molar mass: 479 g/mol

HEMA, 2-hydroxyethyl methacrylate; BisGMA, Bisfenol diglycidyl dimethacrylate; CQ, camphorquinone; MDP, 10-methacryloyloxydecyl-dihydrogen phosphate; BisEMA, bisfenol-A glicidil metacrilato etoxilato; UDMA, Urethane dimethacrylate.

adhesive was poured into a cylindrical plastic mold until it was completely filled. A polyester strip was then placed over the uncured resin and compressed with a glass slide. The sample was light-cured with a light emitting diode (LED) curing unit (Radii-cal®, SDI Limited, Bayswater, VIC, Australia) for 30 s. Photophysical characteristics of these cured resin disks were analyzed by photoluminescence spectroscopy (Spex Fluorolog FL3-222, Horiba Jobin Yvon S.A.S., Longjumeau, France). Emission spectra (525–700 nm bandwidth, 516 nm excitation wavelength, 0.2 s integration time, 0.2 nm increment, 0.8 nm bandpass slit) and excitation spectra (275–575 nm bandwidth, 587 nm emission wavelength, 0.2 s integration time, 0.2 nm increment, 0.8 nm bandpass slit) were recorded individually using a 450 W continuous illumination xenon lamp. In addition, 1.5-mL samples of the five ethanol/RB solutions were transferred into quartz cuvettes and tested.

Preliminary CLSM of RB-Labeled Adhesives

Complementary to the spectroscopic study, a drop of each RB-containing adhesive was individually placed between a microscope slide and a cover slip, polymerized for 10 s with a LED curing unit and then inspected by CLSM (Leica TCS SPE; CMS Leica Microsystems, Mannheim, Germany). In order to compare the fluorescence intensity, according to the five decreasing RB concentrations, all samples were evaluated under the same standardized settings: 532 nm laser excitation wavelength, 10% laser intensity (AOTF control), 600 V gain on the PMT, −4.0 offset of PMT, emission bandwidth from 550 to 675 nm, 275 × 275 μm physical length, ×40/1.15 NA (numerical aperture) in oil. The laser excitation wavelength (laser line of 532 nm; diode-pumped solid state laser) was chosen on the basis of the excitation spectra recorded by fluorescence spectroscopy in advance. The axial resolution (*Z*) was variable, in order to reach a focal plane with the most homogeneous fluorescent sign as the sample's surface can present irregularities.

CLSM of the Dentin/Adhesive Interface

Based on the results of the preliminary CLSM of the labeled adhesives, two target RB concentrations (mg/mL) were preselected for imaging the dentin/adhesive interface: 0.1 and 0.02. The dye concentration of 0.004 mg/mL was additionally selected for further testing. In total, 24 freshly extracted noncarious third molars were obtained under a protocol approved by the Ethics Committee of the Bauru School of Dentistry, University of São Paulo (118/2011-FOB). After cleaning, the teeth had their occlusal enamel cut as described previously (Foxton et al., 2008) and roots were cut transversally in the middle third as well. The flattened dental crowns were randomly assigned into two groups according to the adhesive system (MP or SE). Then, crowns of each group were assigned into three subgroups (n = 4) matching the concentration of RB (mg/mL) in the labeled adhesives: 0.1, 0.02, or 0.004. The adhesive systems were applied in accordance to the manufacturers' guidelines, in terms of time and mode of application. The primers were used in their genuine composition (without addition of RB). Resin composite Filtek (3M ESPE, St. Paul, MN, USA) Z250 was applied in one horizontal increment (≈2.0 mm thickness) and light-cured for 40 s. After 7 days of storage in distilled water (37°C), the restored crowns were cut mesio-distally in 1.0-mm thick slices, parallel to the tooth's long axis, using an ISOMET Low-Speed Saw (Buehler, Lake Bluff, IL, USA) and a diamond wafering blade (102 × 0.3 × 12.7 mm, Extec Corp., Enfield, CT, USA). One slice of each crown was randomly selected and evaluated by CLSM using the following settings: 532 nm laser excitation wavelength, 10–30% laser intensity (AOTF control), gain on the PMT at the range of 600–800 V, emission bandwidth from 550 to 675 nm, 275 × 275 μm physical length, ×40/1.15 NA in oil.

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Results

Photoluminescence Spectroscopy of RB-Labeled Adhesives

The normalized emission spectra of RB in the adhesives (polymerized samples) are shown in Figure 1. The labeled adhesives fluoresced in a wavelength range of visible light from the green to the red color region (≈550–675 nm). The fluorescence emission maxima of RB at 0.5 mg/mL in MP and SE were found at about 587 and 599 nm, respectively. The emission maximum shifted to shorter wavelengths (blueshift) with decreasing dye concentration in the adhesives.

Figure 2 presents the integrated emission intensity as function of the RB concentration in the adhesives and in ethanol solutions. In the adhesives MP and SE, the fluorescence intensity increased accordingly with increasing the RB concentration. In ethanol, the fluorescence intensity decreased when the dye concentration was increased to >0.04 mg/mL.

The excitation maxima of RB (all concentrations) in the adhesives were detected at ≈555–565 nm (green color region).

Preliminary CLSM of RB-Labeled Adhesives

Figure 3 shows serial photomicrographs recorded from cured adhesive samples containing RB at decreasing concentrations. For both adhesives, MP and SE, the fluorescence intensity seemed similar at the same dye concentration. The highest RB concentration (0.5 mg/mL, Figs. 3a–3f) produced a very intense sign comparable with that of 0.1 mg/mL (Figs. 3b–3g). For the next decreasing RB concentrations, the fluorescence intensity gradually decreased until a black field (no fluorescence, as in Figs. 3e–3j). Based on this initial analysis, the lowest range of concentrations of RB in MP and SE that can produce optimized preliminary images should lie between 0.1 and 0.02 mg/mL. Further investigation was then carried out using labeled adhesives in dentin specimens.

CLSM of the Dentin/Adhesive Interface

Representative CLSM photomicrographs of dentin/MP and dentin/SE interfaces are shown in Figure 4. The images confirm that the lowest range of RB concentrations (in MP and SE) necessary to achieve acceptable images of the dentin/adhesive interface is between 0.1 and 0.02 mg/mL. The adhesives prepared with RB at 0.1 mg/mL produced intense fluorescence (processed as red color) in the dentin/adhesive interface (Figs. 4a–4d). Resins prepared with 0.02 mg/mL resulted in a more balanced intensity and background (Figs. 4b–4e), even though the dye concentration was reduced to 1/5th of the previous images. Adhesives with RB at 0.004 mg/mL—which showed almost no fluorescence in the preliminary CLSM analysis (Figs. 3d–3i)—delivered a faint visualization of the resin distribution in dentin (Figs. 4c–4f), when the laser intensity (AOTF control) was increased to 30% and the gain at the range of 800 V.

Discussion

Photoluminescence Spectroscopy of RB-Labeled Adhesives

For the first time, the fluorescent behavior of RB in dental adhesive resins was investigated by photoluminescence spectroscopy—a method suited for monitoring the fluorescence emission and excitation spectra of luminescent materials (Lakowicz, 2006; Vergeer, 2007). The normalized emission spectra (Fig. 1) revealed that the emission bands of RB in the adhesives MP and SE (cured resin samples) occurred in a similar broad range of wavelengths of visible light. Information from the normalized emission spectra was used for determining an optimal detection channel—i.e., a spectral band that collects probe-specific emission—in the confocal microscope to image the RB-labeled samples (see arrows in Fig. 1). The detection channel, set from 550 to 675 nm, was selected to encompass the most of the emission bands of RB in MP and SE, taking into account the blueshift observed with decreasing the dye concentration (Fig. 1).

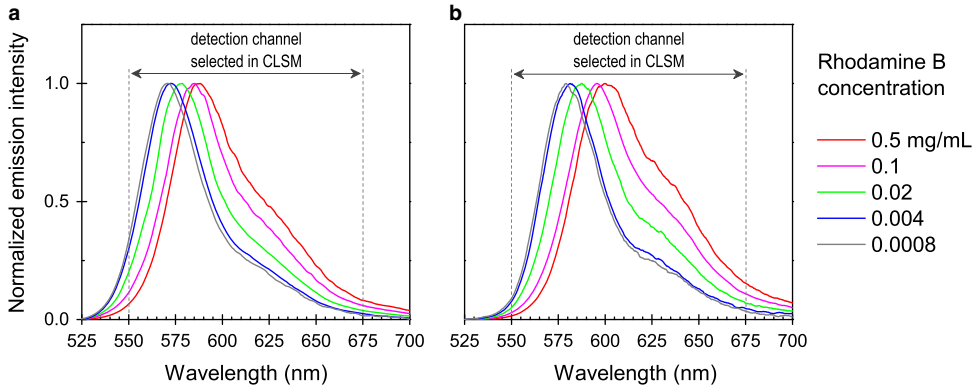

Figure 1. Photoluminescence spectroscopy. Normalized emission spectra of Rhodamine B at decreasing concentrations in the dental adhesives (cured samples) Adper Scotchbond Multi-Purpose (**a**) and Clearfil SE Bond (**b**). Light excitation wavelength of 516 nm.

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It was important to leave a gap (18 nm) between the laser excitation wavelength on the confocal microscope (laser line of 532 nm) and the start of the emission detection channel (550 nm), to prevent the inclusion of any residual excitation light during the spectral imaging (Garini et al., 2006). Besides, knowing the emission spectrum of a labeled sample—in particular its emission maximum—is key in determining the different detection channels for multi-staining fluorescence imaging (Watson & Wilmot, 1992; Griffiths et al., 1999; Zimmermann et al., 2003). When using combinations of two or more fluorescent dyes in the same sample, different detection channels are set up to avoid potential crosstalk, due to spectral overlap between dyes' emission bands.

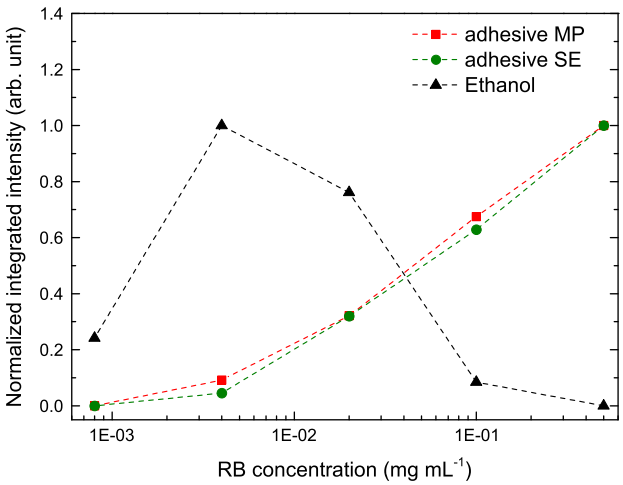

Figure 2. Integrated emission intensity as function of Rhodamine B (RB) concentration in the adhesives [Adper Scotchbond Multi-Purpose (MP) and Clearfil SE Bond (SE), cured samples] and in ethanol solutions.

RB can be often used in low amounts for labeling, due to its very high molar absorptivity and quantum yield (D'Alpino et al., 2006*a*). This means that RB molecules can strongly absorb light of specific wavelengths and transform almost all the absorbed energy into light emission (of lower energy, longer wavelengths). But the photophysical behavior of a fluorescent dye can vary with its concentration and also with chemical properties of the host medium (Valeur, 2001; Kristoffersen et al., 2014). This fact can be clearly observed in Figure 2, by comparing the integrated emission intensity as function of the RB concentration in the adhesives and in ethanol solutions. In the nonsimplified adhesives (polymerized samples), which are hydrophobic nonpolar media, the fluorescence intensity always increased with increasing the RB concentration. On the other hand, in ethanol, a hydrophilic and polar solvent, the fluorescence intensity decreased at high RB concentrations, indicating the occurrence of fluorescence quenching. Quenching refers to a variety of processes that can decrease the fluorescence intensity of a labeled sample (Penzkofer & Lu, 1986; Valeur, 2001). In ethanol, for example, RB at high concentrations can form monomer dimmers and higher aggregates that restrict the fluorescence emission intensity (Fikry et al., 2011). In the nonsimplified adhesives, the higher viscosity of the media reduces the molecular diffusion, constraining the movement of dye molecules necessary to allow specific molecular interactions and nonradiative energy loss that lead to quenching. So, in spite of some previous concerns suggesting the occurrence of fluorescence quenching in labeled dental adhesives (Watson, 1997), the results in Figure 2 show that RB is not likely to quench in polymerized adhesive samples.

In MP and SE samples containing dye at the same concentration, small differences in the emission maxima of RB (Fig. 1) and fluorescence emission intensity (Fig. 2) may be due to specific dye–medium interactions as some

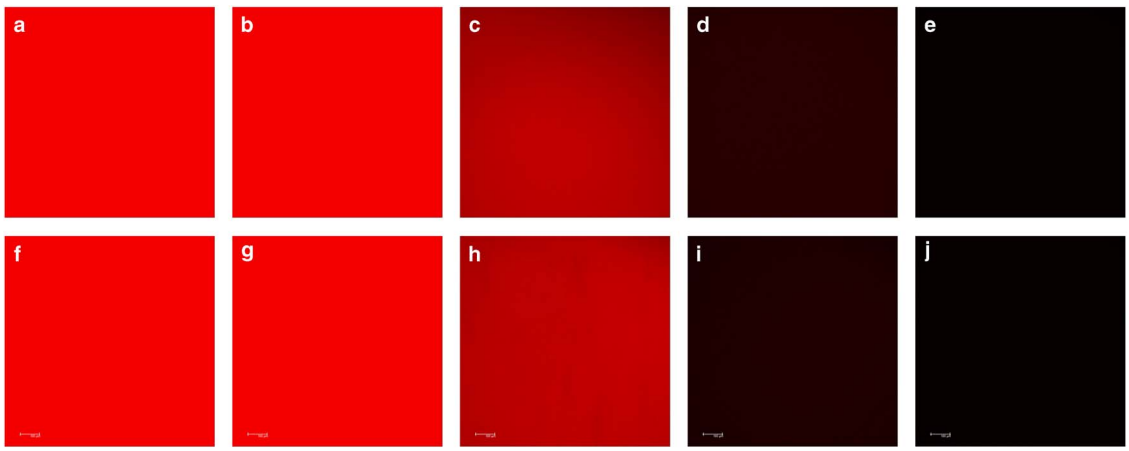

Figure 3. Confocal laser scanning microscopy (CLSM). **a–e:** Representative photomicrographs of the adhesive Adper Scotchbond Multi-Purpose labeled with Rhodamine B (RB) at 0.5, 0.1, 0.02, 0.004, and 0.0008 mg/mL, respectively. **f–j:** Photomicrographs of the adhesive Clearfil SE Bond labeled with RB at those same decreasing concentrations. Laser excitation wavelength of 532 nm, 10% laser intensity (acousto-optical tunable filter control), 600 V gain on the photomultiplier tube, ×40/1.15 NA (numerical aperture) in oil.

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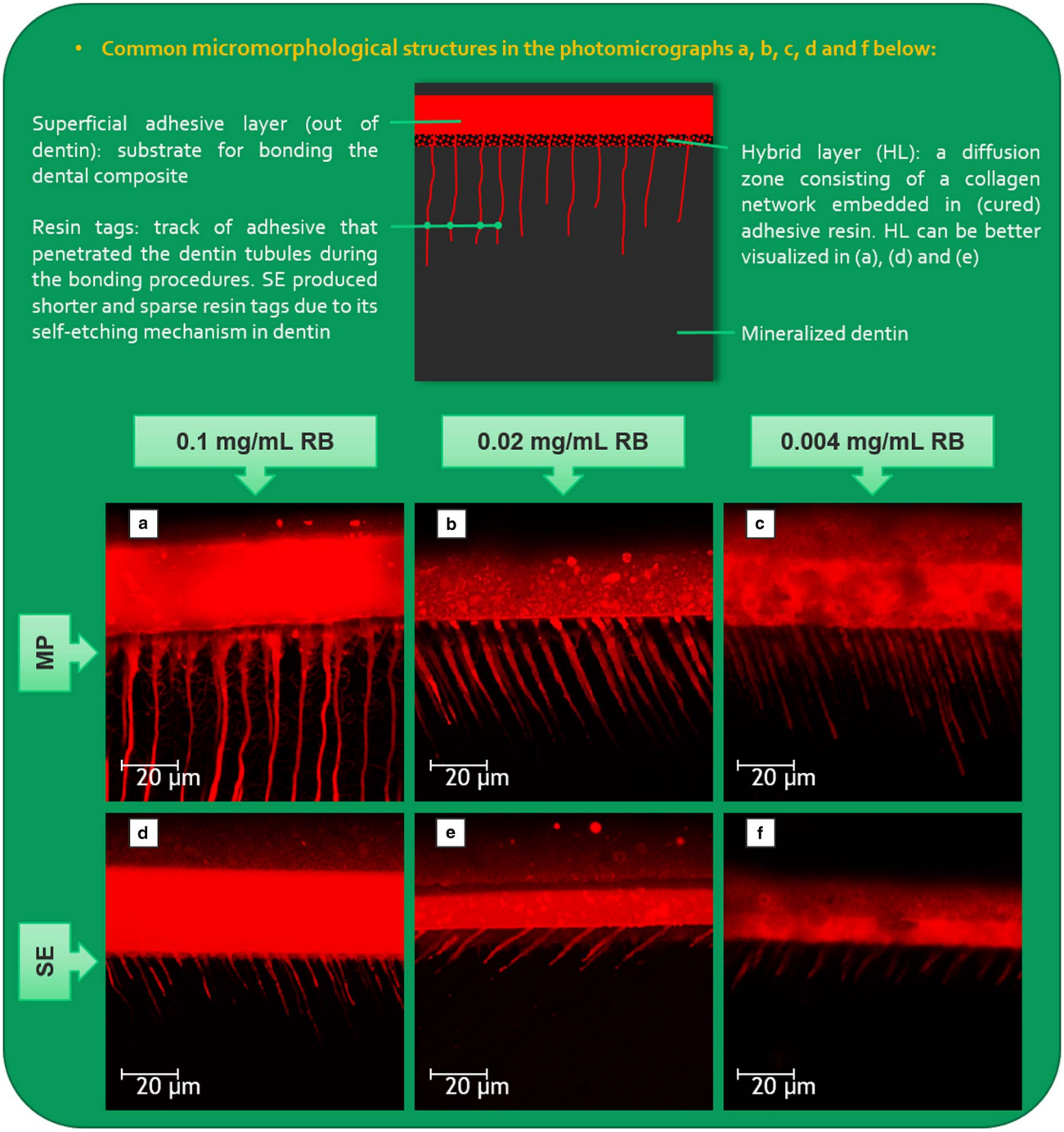

Figure 4. Confocal laser scanning microscopy (CLSM). Representative photomicrographs of the dentin/adhesive interface, using the adhesives Adper Scotchbond Multi-Purpose (MP) and Clearfil SE Bond (SE) labeled with Rhodamine B (RB) at different concentrations (0.1, 0.02, and 0.004 mg/mL). Laser excitation wavelength of 532 nm, ×40/1.15 NA oil. Laser intensity (acousto-optical tunable filter control): 10% (**a** and **d**), 17% (**b** and **e**), and 30% (**c** and **f**).

photophysical parameters of rhodamines react to the chemical characteristics of the medium, like the pH (Valeur, 2001). It should be noted that both adhesives share common mono and dimethacrylates, but SE is a self-etching adhesive containing acidic methacrylate phosphates (Table 1). In aqueous solution and certain organic solvents, the carboxyl group in the RB molecule undergoes typical acid-base equilibria that can affect fluorescence (Beija et al., 2009). Such acid-base interactions probably occur to a lesser extent in the nonsimplified adhesives, which contain no solvent to facilitate dissociation or protonation.

Preliminary CLSM of RB-Labeled Adhesives

Complementary to the results in Figure 2 (spectroscopic data), the CLSM images in Figure 3 provided the first visual

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information, regarding RB fluorescence in the cured adhesives MP and SE. This preliminary analysis aimed to preselect target RB concentrations (out of the five initial ones) for imaging the dentin/adhesive interface as a next step. To compare the effect of RB concentration on fluorescence intensity, standardized microscopy settings were adopted for the preliminary CLSM. The settings, listed in the methods section, were chosen taking into account a combination of laser intensity (by AOTF control), gain and offset (on the PMT) that produces preliminary images with balanced intensity and background noise.

CLSM of the Dentin/Adhesive Interface

Figure 4 shows that RB, as a fluorescent dye for the micromorphologycal analysis of the dentin/adhesive interface, can be incorporated into dentals resins at concentrations rather lower than what has been utilized before (D'Alpino et al., 2006*a*; Francisconi et al., 2009; Sampaio et al., 2011). The most favorable concentration range of RB in the adhesives MP and SE was verified between 0.1 and 0.02 mg/mL. This should be the lowest range of concentrations of the dye that still can produce acceptable images of the bonding interface with balanced CLSM settings. Low dye concentrations are desirable, as there is still a lack of evidence of the effect of RB on a series of resin properties related to dental bonding (D'Alpino et al., 2006*b*; Wang et al., 2016).

During the image optimization process, the microscopy settings are as relevant as the dye concentration itself. A particular advantage of CLSM is the flexibility to determine different settings based on the dye concentration, specimen morphology, and composition (Paddock & Eliceiri, 2014). It should be noted that while the interfaces in Figures 4a, 4b, 4d, and 4e were recorded with balanced laser settings (10–17% laser intensity by the AOTF and ≈600 V on the PMT), weakly stained interfaces as in Figures 4c and 4f required increased laser intensity of 30% and gain at the range of 800 V to be visualized. Too high a gain (voltage on the PMT) can amplify weak signal, thus delivering better fluorescence intensity, but also amplifies noise and increases the background (Pawley, 2000). Besides, increasing the amount of excitation energy to deal with weakly stained samples can lead to faster photobleaching—a photochemical process involving irreversible covalent modifications that convert the fluorophore to a non-fluorescent product (Ghauharali & Brakenhoff, 2000; Bernas et al., 2005). To avoid photobleaching during the laser scan, approaches which include increasing the dye concentration, decreasing the laser intensity, and the exposure time have to be used.

The dentin bonding substrate may be very heterogeneous from sample to sample (Spencer et al., 2010). With the bonding protocols utilized in this investigation, the channels between the demineralized dentin collagen fibrils were filled with water and/or with (unlabeled) primer solution. When the corresponding adhesive was applied as a last step, RB molecules probably leached from the labeled bonding agent to the primed demineralized dentin, resulting in some dilution that was not likely to be controlled by the researchers (Watson, 1997). Furthermore, RB can also leach from cured resin samples stored in different solutions (D'Alpino et al., 2006*a*, 2006*b*). To compensate the decrease of fluorescence intensity in the bonding interface, due to dye dilution/leaching, parameters such as the laser intensity and gain were optimized as discussed above.

Considerations About the Labeling Procedures

It has been difficult to find in the literature detailed instructions of the dispersion of fluorescent dyes in different types of adhesive systems (D'Alpino et al., 2006*a*). The simplest way to label a resin-based material is to disperse a low-molecular-weight dye, usually a fine powder, in the monomer blend (Thomas et al., 2014). The homogenization of the system can be achieved by stirring with a vortex mixer for example. This can be straightforward when labeling blends dissolved in organic solvents, like ethanol, acetone, or chloroform, because many dyes are readily soluble in such solvents (D'Alpino et al., 2006*b*; Khalilzadeh et al., 2008; Arrais et al., 2009). However, the addition of a dye directly to solvent-free viscous monomer blends can be problematic. The adhesive resins labeled in this study consist of solvent-free, hydrophobic and viscous methacrylate blends (Van Landuyt et al., 2007; Pashley et al., 2011). In a pilot experiment, we added samples of either MP or SE to micro test tubes containing specific amounts of RB and stirred the components using a vortex mixer followed by an orbital shaker. After stirring, clusters of dye could still be observed in the samples, especially on the tubes' internal walls. Adding a solvent like ethanol to the neat adhesives to facilitate the dispersion of the dye was not an option here, because that could affect the degree of conversion, structure, and mechanical properties of the bonding agents (Ye et al., 2007). To force the dispersion of RB in the adhesives, a large round bur adapted to a dental air turbine handpiece was used, serving as a powerful mechanical stirrer (up to 20,000 rpm) for low-volume samples. With this method, no RB cluster was observed with the naked eye in the resin samples and on the tube's walls after a few minutes of stirring.

In contrast to the labeling method in which amounts of dye are added directly to the supplied adhesive bottle (D'Alpino et al., 2006*b*; Arrais et al., 2009), a semi-direct method has been used for measuring the masses of RB in the sub-milligram range. Small aliquots of ethanol solutions containing RB were transferred to test tubes. The solvent served as a medium for transporting specific masses of dye, being completely evaporated before the addition of the adhesives. Using the semi-direct method, low volumes of uncured resin (≈1.0 mL) could be labeled with RB at very low concentrations, like 0.004 and 0.0008 mg/mL. The masses of RB required to bring these 1-mL samples to such low dye concentrations could not be directly weighted in a standard analytical balance, due to limitation of the readability.

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Summary

Our approaches took into account aspects related to the dye concentration, photophysical parameters in different host media, specimen composition, and morphology to develop a optimal use of the fluorescent agent with the resin-based materials. The lowest range of RB concentrations in the adhesives MP and SE necessary to achieve an acceptable image of the dentin/adhesive interface has been determined: 0.1–0.02 mg/mL. Photophysical parameters of RB reacted to the dye concentration and, to a lesser extent, to the host polymer. RB did not show quenching of fluorescence in the host polymers. The semi-direct staining technique, using ethanol/RB solutions, permitted the preparation of low-volume viscous resin samples containing very low concentrations of RB.

This was a preliminary study focusing on optical aspects and further investigations are being carried out on the effect of RB on a series of resin properties.

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