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Hydroxyl radical release from dental resins: Electron paramagnetic resonance evidence

Guillaume Lamblin [a,c,e,1], Julian Leprince [a,b,e,1], Jacques Devaux [b,e], Michèle Mestdagh [c,e], Bernard Gallez [d], Gaëtane Leloup [a,b,e,*]

[a] School of Dental Medicine and Stomatology, Université catholique de Louvain, Avenue Hippocrate 10/5721, Brussels B-1200, Belgium
[b] Institute of Condensed Matter and Nanosciences – Bio & Soft Matter (IMCN/BSMA), Université catholique de Louvain, Croix du sud 1, Louvain-la-Neuve B-1348, Belgium
[c] Unité de chimie des interfaces, Université catholique de Louvain, Croix du sud 1, Louvain-la-Neuve B-1348, Belgium
[d] Unité de résonance magnétique biomédicale, Louvain Drug Research Institute, Université catholique de Louvain, Avenue Mounier 73, Brussels B-1200, Belgium
[e] CRIBIO (Centre de Recherche et d'Ingénierie en Biomatériaux), Avenue Hippocrate 10/5721, Brussels B-1200, Belgium

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ABSTRACT

It is well known that polymeric free radicals remain trapped inside dental resins for a long time after photopolymerization. Moreover, although these high molecular mass compounds have very limited mobility, there is evidence to suggest that they disappear progressively over time. The purpose of this study was to provide new experimental data to help understand this phenomenon. To determine whether low molecular mass free radicals are released by dental composites stored in hydrophilic media, we used electron paramagnetic resonance spectroscopy to perform spin-trapping experiments on experimental and commercial samples stored in ethanol. Under these conditions, ethoxy radicals were produced. Further experiments demonstrated that (1) hydroxyl radicals were released from the methacrylated resin and (2) they reacted with ethanol molecules to produce "secondary" ethoxy free radicals. In addition to the well-known monomer toxicity of methacrylated resins, we may have identified a new source of concern for these biomaterials.

1. Introduction

Resin composites, made of inorganic fillers within an organic matrix, are widely used for dental restoration. The organic matrix is produced by a photoinitiated radical copolymerization of dimethacrylate monomers. Light induces cross-linking of the difunctional monomers, which progressively leads to the vitrification of the material. As a result, two kinds of free radical are trapped in the network: allylic and propagating radicals (Fig. 1) [1]. It is well known that these compounds can remain stable for several months in the polymer network [2–4]. Nevertheless, they disappear more or less rapidly, depending on the storage environment, until they reach undetectable levels on electron paramagnetic resonance (EPR) spectroscopy [5,6]. For instance, Leprince et al. [7] recently investigated the fate of the trapped free radicals in dental resins stored in water, air, oxygen or argon. They demonstrated that the rate of disappearance decreased in the following order: water > oxygen > air > argon.

Given the macromolecular dimensions of these trapped free radicals, their mobility into the polymeric network is strongly limited by entanglement and they cannot diffuse out of the resin. The decrease in trapped free radical concentration can, therefore, only be explained by radical reactions (distinct from typical methacrylate photopolymerization) occurring within the resin. It is then important, especially from a biocompatibility viewpoint, to know whether these reactions can produce and release mobile secondary free radicals into the environment. It has already been demonstrated that unconverted monomers are cytotoxic and can have harmful effects [8,9]. For instance, they can induce tooth pulp damage, mucosal irritation, contact dermatitis and allergic reactions [10]. Similarly, local release of free radicals could be another source of concern.

There are relatively few publications devoted to the stability of trapped free radicals in dental materials, and most of them have focused on the study of postpolymerization (or dark polymerization) [2,5,11,12]. The latter reaction occurs when the illumination is stopped and the resin stored in the dark. In this case, a slow decrease in the concentration of trapped free radicals can be observed due to termination phenomena (the rate of which depends on the cross-link density and on the storage temperature) associated with a certain increase in the degree of conversion [2,6].

* Corresponding author. Address: School of Dental Medicine and Stomatology, Université catholique de Louvain, Avenue Hippocrate 10/5721, Brussels B-1200, Belgium. Tel.: +32 493 24 88 10.
E-mail address: gaetane.leloup@uclouvain.be (G. Leloup).
[1] These authors contributed equally to this work.

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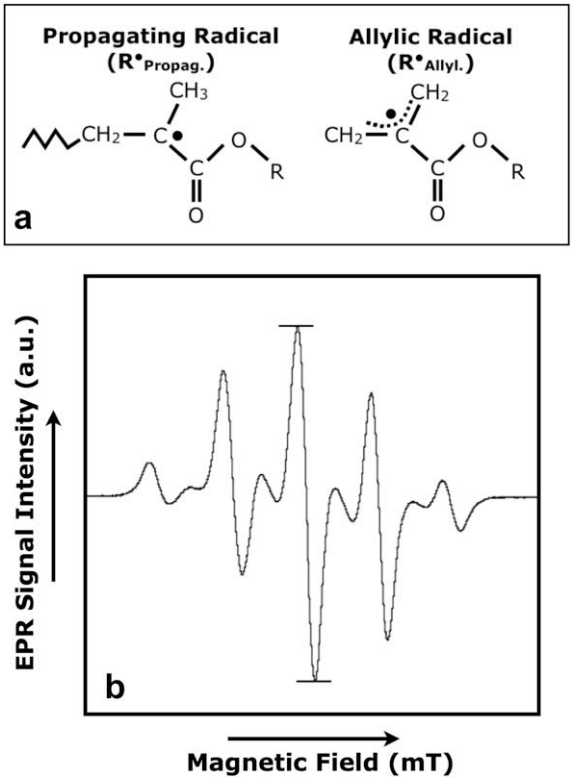


Fig. 1. (a) Chemical structure of both trapped radical species (R represents either an end group or a connection to the network). (b) Nine-line EPR spectrum of the experimental resin after polymerization. Plain line on the spectrum corresponds to the position (allylic radical) where the intensity was measured for kinetic experiments.

In addition to postpolymerization effects, Leprince et al. studied the impact of the storage environment on the kinetics of the trapped free radicals [7]. Twenty-four hours after irradiation, oxidation was the main phenomenon involved in the decrease in radical concentration. Moreover, conditions influencing oxygen diffusion inside the polymer network (e.g. swelling in solvent) have an impact on radical kinetics. This effect of atmospheric oxygen was also noted by Pavlinec et al. [4]. Nevertheless, the exact mechanism involved has not yet been described. A generally accepted first step of the reaction leads to the formation of peroxyl radicals (Eq. (1)):

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (1)$$

Unfortunately, most studies have not detected these species by EPR spectroscopy despite the fact that evidence of the existence of peroxides has been published [7]. For example, Pavlinec et al. [4] recently noted, using chemiluminescence, that accumulation of these compounds occurred concomitantly with trapped free radical decay. Leprince et al. [7] hypothesized that low molecular mass radicals may terminate trapped species by recombination or disproportionation. In line with this hypothesis, Lee et al. [13,14] proposed that hydroxyl radicals could be released by hydroperoxidation of the methacrylated double bonds. If this hypothesis is valid, spin-trapping experiments are required to detect the hydroxyl radicals.

EPR spectroscopy and, more precisely, spin-trapping methods indeed enable the detection and identification of unstable free radicals [15,16]. This technique was first employed in the 1960s and has become increasingly popular because of the key role of radicals in many biological mechanisms and in deleterious processes in disease and ageing [17]. An unsaturated non-radical (diamagnetic) molecule, M, called the spin-trap, reacts with the unstable free radical, R•. The product, called the spin-adduct, M–R•, has a longer half-life than R• [18,19], allowing its detection by EPR. The hyperfine constants of the adducts (M–R•) are characteristic, enabling them to be identified. Nevertheless, due to the variable half-lives of spin-adducts, which depend on many parameters (nature of the trapped free radical and spin-traps, solvent, pH, temperature, etc.), detection may be more or less difficult. Typical spin-traps are nitroso compounds, like 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) or phenylbutylnitrone (PBN). Currently, research is being conducted to synthesize new spin-traps with longer spin-adduct half-lives [20,21]. For instance, the spin-traps 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (EMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrrolyne-*N*-oxide (DEPMPO) have been synthesized recently [22–25].

The objective of the present paper was to assess the potential release of free radicals from dental resin using spin-trapping methods. Ethanol was used as a solvent for immersion as it induces better swelling of methacrylated polymer than water, thus maximizing the diffusion of species in and out of the material. Because of the long half-lives of their spin-adducts, EMPO and DEPMPO spin-traps were chosen to characterize the free radicals released.

2. Materials and methods

2.1. Sample preparation

Experimental dental resins were prepared from a blend of bisphenol A glycidyl dimethacrylate (Bis-GMA, from Heraeus Kulzer, Dormagen, Germany) and triethylene glycol dimethacrylate (TEGDMA, from Sigma–Aldrich) in a 70:30 weight ratio. A photoinitiator system (1 wt.% of camphorquinone and tertiary amine obtained from Sigma–Aldrich) was added to initiate the polymerization. Point 4 (Kerr, Orange County, USA) was used as the commercial resin composite, which contains inorganic fillers. The resins (experimental or commercial) were inserted in moulds (7 mm long, 1 mm wide, 1 mm thick) and photopolymerized with a visible light device (Curing Light XL 3000, 3M-Espe, St. Paul, MN, USA). A constant intensity of 450 mW cm^{-2} was applied for 40 s to one side of the samples. The mould allows not only the same sample geometry to be used for each experiment but also for the same amount of resin to be placed in the EPR cavity. These dimensions were chosen to ensure that the full area of the sample was covered by the diameter of the optic fibre of the lighting device.

2.2. EPR spectroscopy

The X-band EPR spectra were recorded at room temperature, using a Magnettech MINISCOPE MS200 spectrometer, equipped with a rectangular cavity (TE102). For kinetics experiments, the following instrument settings were applied: magnetic centre field, 336.7 mT; microwave frequency, 9.5 GHz; microwave power, 0.5 mW (23 dB); modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; field sweep 19.8 mT, sweep time, 120 s; receiver gain, 100. For the spin-trapping experiments, all the parameters were identical except the sweep and centre of the magnetic field, which were adjusted to 9.8 and 335.6 mT, respectively. For all observations, the microwave irradiation power and the modulation amplitude were set to avoid signal saturation and were kept constant. Three samples were analysed for each experimental condition. Except for experiments with reduced oxygen pressure, the linewidth of the spectra was constant. The radical concentration

could, therefore, be assessed by measurement of the peak height. In cases where the linewidths changed, the free radical quantity was determined by the double integration of the peaks.

2.3. Kinetic decay of trapped free radicals in ethanol and water

Experimental samples were removed from the mould and inserted in an EPR tube for measurement 5 min after the end of the illumination process to ensure the same zero time for all kinetics analyses. Then samples were stored in the appropriate environment, ethanol or water, in the dark, at 25 °C. The EPR spectra were recorded at different times after photopolymerization until complete extinction of the EPR signal intensity.

2.4. Spin-trapping experiments

2.4.1. Spin-adduct detection with EMPO and DEPMPO

An average of 30 min after photopolymerization, two rods of resin were immersed in ethanol (170 ml, HPLC grade, Sigma–Aldrich) which contained dissolved EMPO or DEPMPO (50 mM, Alexis Biochemicals) spin-traps. The presence of spin-adduct peaks was noted 15 min after immersion by sampling the spin-traps solution with a capillary tube. For the kinetic study of released free radicals, the measurement was extended to 1 and 2 h. The content of the capillary tube was replaced in the spin-traps solution after each ESR measurement. The intensity was determined by measurement of the first peak of the spectrum.

Additional experiments were also performed with 3-month-old rods instead of freshly polymerized ones.

For experiments under limited pressure of oxygen, nitrogen gas was bubbled in the pure unpolymerized resin and in the ethanol spin-trap solution prior to photopolymerization and EPR analyses, respectively.

To assess the possible effect of methacrylated double bond concentration on the detection of spin-adducts, three poly(methyl methacrylate) (PMMA) (Oroglas) rods or unpolymerized resins were introduced in place of the two freshly photopolymerized rods.

Three specific reactions were performed with EMPO only. Firstly, some experiments were performed with methanol in place of ethanol for spin-adduct detection. Secondly, a Fenton reaction was conducted by adding $FeCl_2$ (1 mM, Acros) to ethanol containing EMPO (50 mM) and H_2O_2 (1 mM, Merck). Finally, a hydroxyl radical scavenger, *N*,*N*-dimethylthiourea (50 mM, Sigma–Aldrich), was introduced at a given time in the spin-trapping kinetics experiments.

2.4.2. Spectrum simulation

Computer simulations were performed using hyperfine constants found in the literature. Matlab software was used and upgraded with the "Easyspin toolbox".

3. Results

3.1. Kinetic decay of trapped free radicals in ethanol and water

It can be observed in Fig. 2 that the free radicals trapped in the dental resins disappeared much more quickly in ethanol than in water. In ethanol, the rate of decrease of free radicals was nearly linear in the logarithmic representation and complete extinction is obtained after 4 days. In water, two steps could be clearly identified in the kinetics and the EPR signal was undetectable after a few months.

3.2. Spin-trapping experiments in ethanol

3.2.1. Free radical release by dental resin

The signal obtained with EMPO for experimental resins is shown in Fig. 3a. This six-peak signal has the following characteristics: peaks are fairly broad and the hyperfine constants are aN = 1.29 mT and aH = 0.68 mT. A similar signal was obtained with methanol as a solvent, i.e. a six-peak signal with approximately the same hyperfine constants: aN = 1.31 mT, aH = 0.71 mT (data not shown) corresponding to $MeO^{\cdot}$ radical. This is in accordance with previous work [26], and contrary to what was initially expected, did not enable hydroxyl radical spin-adducts to be detected directly. The same signal was obtained with a commercial resin, Point 4 (Fig. 3b). These characteristics are similar to recently published values [26] for ethoxy radicals ($EtO^{\cdot}$), spin-adducts of EMPO (Table 1), and were used to simulate a spectrum (Fig. 3c), which was very close to the experimental one. Finally, Fig. 3d shows the EPR spectrum of $EtO^{\cdot}$ produced by the Fenton reaction, which was very similar to both experimental and simulated spectra.

Fig. 4 shows the results of similar experiments conducted with the DEPMPO spin-trap on experimental resin. A 14-peak signal with the following hyperfine constants was obtained:

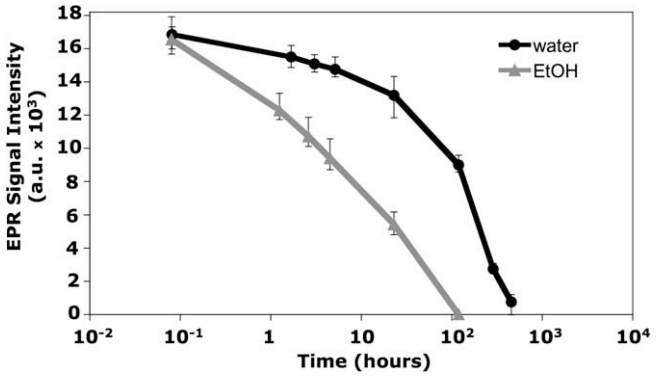


Fig. 2. Change in the concentrations of the trapped allylic radical over time for experimental dental resin stored in water or ethanol. Time is presented on a logarithmic scale.

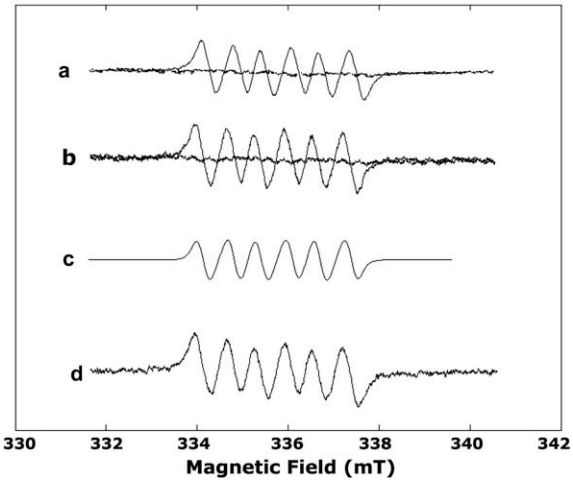


Fig. 3. EPR spectrum obtained with EMPO spin-trap: (a) EMPO (50 mM) and experimental dental resin incubated 15 min in pure ethanol (baseline: without resin); (b) same as with commercial resin (baseline: without resin); (c) simulation of the ethoxy radical spectrum with aN = 1.29 mT, aH = 0.68 mT; (d) EMPO incubated in a Fenton system in pure ethanol.

Table 1
Comparison of the EPR parameters of ethoxy radicals trapped by EMPO and DEPMPO.

Radical	Spin-trap	*trans*/*cis*	*aP* (mT)	*aN* (mT)	*aHβ* (mT)	*aHγ* (mT)	Source	Reference
EtO·	EMPO		–	1.265	0.685	–	Fenton system in ethanol	This work
EtO·	EMPO		–	1.29	0.68	–	Dental resin in ethanol	This work
EtO·	EMPO	*trans* 50%	–	1.375	1.088	–	Fenton system in ethanol	[26]
		cis 50%	–	1.375	0.808	–		
EtO·	DEPMPO		4.56	1.27	0.5–0.7	–	Dental resin in ethanol	This work
EtO·	DEPMPO	*trans* 85%	4.621	1.296	0.69	–	NO· bubbling in EtOH	[27]
		cis 15%	3.824	1.307	0.782	–		

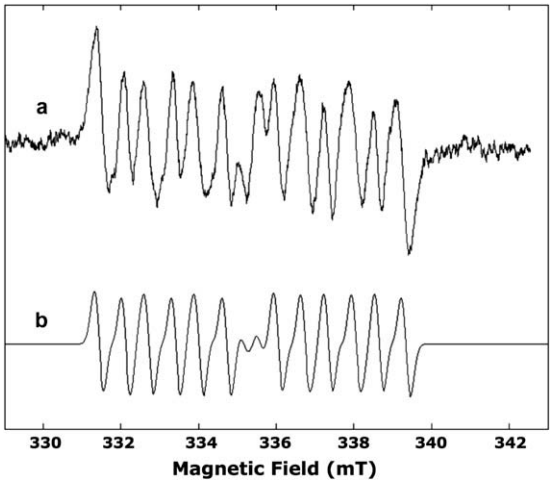


Fig. 4. EPR spectra obtained with DEPMPO spin-trap: (a) DEPMPO (50 mM) and experimental dental resin incubated 15 min in pure ethanol; (b) simulation of the ethoxy radical spectrum with aP = 4.56 mT, aN = 1.27 mT, aH = 0.6 mT.

aP = 4.56 mT, aN = 1.27 mT and aH = 0.6 mT. As for EMPO, these values were very similar to published ones [27] for ethoxy free radicals (Table 1). The computer simulation based on these values led to a spectrum (Fig. 4b) similar to the experimental one (Fig. 4a).

These results show that ethoxy radicals are released by dental resins; their kinetic of release is shown in Fig. 5. The addition of a specific hydroxyl radical scavenger (*N*,*N*-dimethylthiourea) [28,29] resulted in a significant decrease in signal intensity (Fig. 6).

3.2.2. Supplementary experiments

Supplementary experiments were conducted with experimental resins to examine the influence of three factors on the production of ethoxy radicals: trapped free radicals, oxygen concentration, and double bonds. Replacing freshly photopolymerized samples with old (>3 months) samples had no effect on the shape

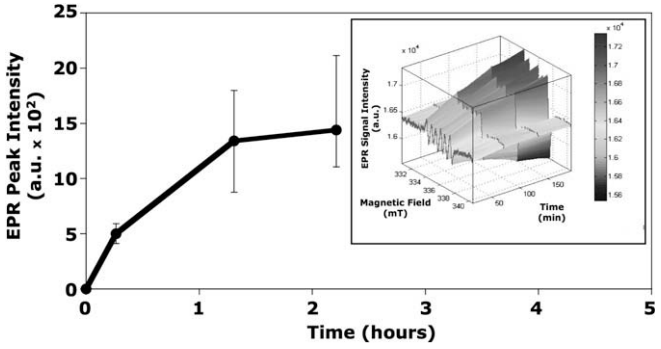


Fig. 5. Change in the concentration of ethoxy radical EMPO spin-adducts (50 mM) over time. Inset: evolution of the spectrum over time in a 3D representation.

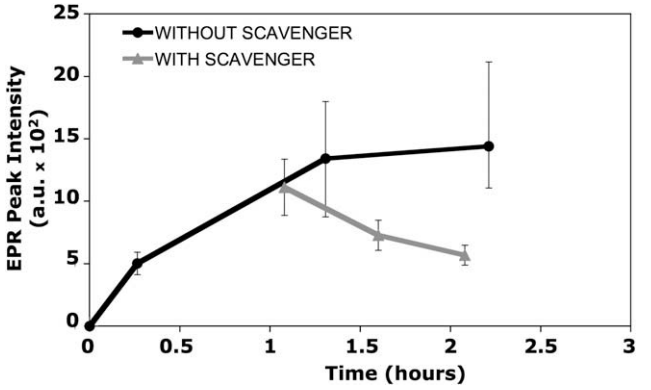


Fig. 6. Change in the concentration of ethoxy radical spin-adducts of EMPO (50 mM) over time, with and without hydroxyl radical scavenger.

or on the intensity of the six-peak signals (data not shown). This 3-month ageing period has been shown to lead to the extinction of the EPR signal attributed to the trapped free radicals [7]. Consequently, it can be stated that EtO· release is independent of the presence of trapped free radicals. When oxygen concentration was reduced (in photopolymerized resins and in spin-trap solutions) there was a significant change in the spectrum (Fig. 7): the six peaks were narrower and split, and their double integral was reduced by half (Table 2). This latter observation demonstrates the key role of oxygen: without oxygen, no ethoxy radicals would be released. The signal is only reduced but also not completely extinguished because deoxygenation was not complete, being technically more difficult to achieve. The effects of methacrylated

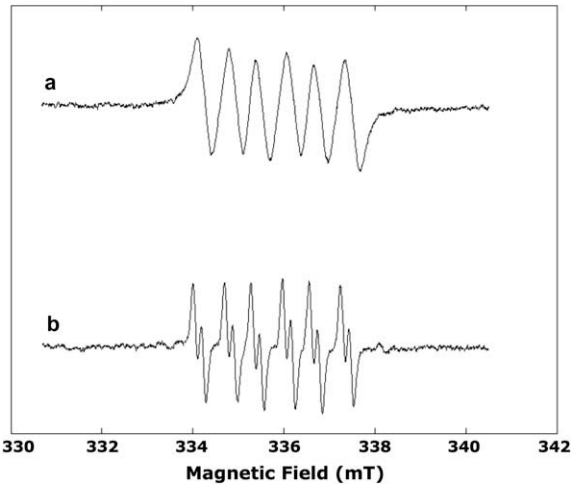


Fig. 7. EPR spectrum obtained with EMPO spin-trap: (a) EMPO (50 mM) and experimental dental resin incubated 150 min in pure ethanol; (b) same as (a) except that oxygen concentration has been reduced.

Table 2
Comparison of the concentrations of ethoxy radical EMPO spin-adducts in ethanol and in partially deoxygenated ethanol.

Experiment	Double integral (a.u.)
EMPO in ethanol	15 ± 6
EMPO in deoxygenated ethanol	7.6 ± 2

double bond concentration were also examined. Using completely converted PMMA (no remaining double bonds), no peak was detected. On the contrary, an ethoxy spectrum was detected when unpolymerized experimental resin (maximum double bond concentration) was used.

4. Discussion

4.1. Free radicals trapped in the dental resin network disappear in ethanol

As is clearly shown in Fig. 2, the trapped free radicals disappeared most rapidly when dental resins were stored in ethanol. This is not surprising as ethanol is well known to induce better swelling of methacrylated polymer than water. Therefore, as briefly stated above, the diffusion mass transfers in and out of the resin are fast. In view of the spin-adduct half-lives of EMPO and DEPMPO spin-traps, ethanol clearly offers the better option for detecting spin-adduct accumulation.

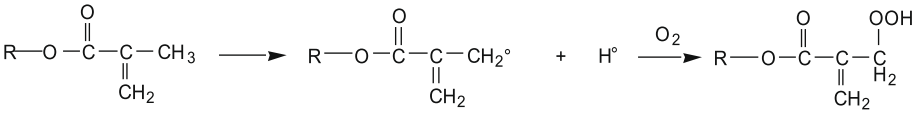
4.2. Dental resins produce ethoxy radicals in ethanol

Comparison of the different spectra obtained with EMPO (Fig. 3a–c) clearly demonstrates for the first time that dental resins release ethoxy free radicals (EtO·) when they are stored in ethanol. This is confirmed by the DEPMPO spectra (Fig. 4a and b). Interestingly, the signals obtained with EMPO for commercial and experimental resins were similar, supporting the fact that the organic part of the dental resin is the only part involved in the release of ethoxy radicals.

4.3. Ethoxy radicals are secondary products from the reaction of hydroxyl radicals with ethanol

When considering the kinetics of release of EtO·, the major observation was the significant decrease in the double integral when a hydroxyl radical scavenger was introduced (Fig. 6). This suggested that ethoxy radicals are secondary products of the reaction between hydroxyl radicals and ethanol molecules. The results obtained with the Fenton system (Fig. 3d) in ethanol support this assumption. Indeed, ethanol is known to be an efficient scavenger of hydroxyl radicals, which are rapidly converted into ethoxy-derived free radicals [30–32]. For example, ethanol was used to demonstrate the decomposition of superoxide radicals into hydroxyl radicals, based on the fact that ethyl alcohol reacts only with hydroxyl radicals to form EtO·, which is not the case with superoxide radicals. Therefore, the simple detection of ethoxy radicals suggests the release of hydroxyl radicals. The results obtained in methanol supports these conclusions as they produce methoxy radicals, which was also reported for Fenton system in methanol.

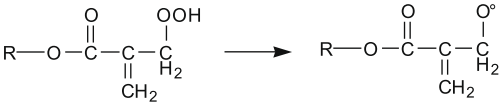
The release of hydroxyl radicals (OH·) by dental resins has already been suggested in the literature. Lee et al. hypothesized that OH· production could explain the chemical degradation of dental composites stored in a food simulating environment (water/ethanol (25/75)) [13,14]. These authors proposed the hydroperoxidation of the methyl group in the position of a methacrylate group (Eq. (2)):



(2)

It has to be noted that the allyl radical produced in the first step of the reaction described in Eq. (2) is the same as the allyl radical trapped in the resin. However, the one produced by the reaction described in Eq. (2) is accompanied by a H· abstraction which can give a hydroperoxide. The trapped allyl radical can only do so with the assistance of another "mobile" hydrocarbon.

The hydroperoxide produced can then decompose into hydroxyl and acyl radicals (Eq. (3)). The latter could provide new cross-linking or decompose into aldehyde species:



(3)

Hydroperoxidation of the methyl group in a methacrylate, therefore, depends on two major parameters, the presence of oxygen and of unconverted double bonds. This is exactly what was highlighted in the supplementary experiments conducted with EMPO to investigate the mechanism of EtO· release. Firstly, ethoxy radical release was shown to be independent of the presence of trapped free radicals in resin rods, but dependent on oxygen. Secondly, the qualitative experiments conducted with unpolymerized experimental resin and fully converted methacrylated polymers revealed the dependence of ethoxy radical release on the concentration of double bonds.

The release of hydroxyl radicals could explain the long term decay of trapped free radicals in dental resins. It is indeed conceivable that, once created, small OH· radicals diffuse then combine with trapped radicals by addition. The decreased kinetics of the trapped free radicals would then depend on the diffusion of oxygen in the sample. This was demonstrated in an earlier paper [7], in which water was shown to accelerate radical decay more than oxygen and air. It therefore seems reasonable that hydroperoxidation may also explain the decrease in trapped free radicals when dental materials are stored in other environments, such as air and water.

However, as already mentioned, this mechanism involving OH· is not the most likely. A more obvious mechanism would start by direct oxidation of the trapped radicals, leading to peroxyl radicals (Eq. (1)). Nevertheless, no EPR signal corresponding to peroxyl radicals has been detected, in the present study or in other papers on dental resins. Two hypotheses can then be proposed. Firstly, this may be explained by their transformation into hydroperoxide compounds, as detected by Pavlinec et al. [4]. This transformation from peroxyl radicals to hydroperoxides requires the intervention of "mobile" hydrocarbons, e.g. those present on neighbouring "free" monomers (only slightly mobile under T_g). Secondly, it is well

known that the amount of non-converted pendant methacrylated functions is higher than the one of trapped radicals in the resin. Indeed the degree of conversion of dimethacrylate resins is limited as it only reaches 60–65% maximum [2], and therefore contains many unreacted methacrylate functions after photopolymerization. As a consequence, oxygen molecules are much more likely to react with a methacrylate double bond than with free radicals trapped in the polymer. While the detection of free radical release is of prime interest in the understanding of material ageing process, it should not be misinterpreted in terms of potential toxicity. Indeed, this paper only displays qualitative work, demonstrating that small free radicals are released by dental resin. Even if the quantity of the released free radicals is probably small, the precise determination of their concentrations was not in the scope of this paper. Though very tempting to evaluate the amount of released radicals, the direct comparison of the concentrations of trapped free radicals (Fig. 2) and hydroxyl radicals (Figs. 5 and 6) must be considered cautiously for several reasons: different spectrometer settings, different physical environments – respectively, liquid for hydroxyl radicals and solid for trapped radicals – and intrinsic variability of spin-trapping experiments, which prevents accurate quantitative measurements. Moreover, as explained by Schmaltz and Arenholt-Bindslev [33], toxic reactions depend not only on the dose of the chemical species, but also on other parameters like the distance and the nature of the structures between release location and potential target tissue. Therefore, the present results do not enable us to come to any conclusion regarding potential biological effects. In vitro investigations are required to determine the impact of the released hydroxyl radicals on the local biological environment of dental restorations.

5. Conclusion

The results of this paper present sufficient evidence to affirm that dental resins release hydroxyl radicals. This gives new insight into the resin ageing process and should be taken into consideration in biological studies.

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