From Natural Products to Polymeric Derivatives of "Eugenol": A New Approach for Preparation of Dental Composites and Orthopedic Bone Cements

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Polymers with eugenol moieties covalently bonded to the macromolecular chains were synthesized for potential application in orthopedic and dental cements. First, eugenol was functionalized with polymerizable groups. The synthetic methods employed afforded two different methacrylic derivatives, where the acrylic and eugenol moieties were either directly bonded, eugenyl methacrylate (EgMA), or separated through an oxyethylene group, ethoxyeugenyl methacrylate (EEgMA). A typical Fisher esterification reaction was used for the synthesis of EgMA and EEgMA, affording the desired monomers in 80% yields. Polymerization of each of the novel monomers, at low conversion, provided soluble polymers consisting of hydrocarbon macromolecules with pendant eugenol moieties. At high conversions only cross-linked polymers were obtained, attributed to participation of the allylic double bonds in the polymerization reaction. In addition, copolymers of each eugenol derivative with ethyl methacrylate (EMA) were prepared at low conversion, with the copolymerization reaction studied by assuming the terminal model and the reactivity ratios determined according to linear and nonlinear methods. The values obtained were $r_{EgMA} = 1.48$, $r_{EMA} = 0.55$ and $r_{EEgMA} = 1.22$, $r_{EMA} = 0.42$. High molecular weight polymers and copolymers were obtained at low conversion. Analysis of thermal properties revealed a T_g of 95 °C for PEgMA and of 20 °C for PEEgMA and an increase in the thermal stability for the eugenol derivatives polymers and copolymers with respect to that of PEMA. Water sorption of the copolymers was found to decrease with the eugenol derivative content. Both monomers EgMA and EEgMA showed antibacterial activity against *Streptococcus mutans*, producing inhibition halos of 7 and 21 mm, respectively. Finally, cell culture studies revealed that the copolymers did not leach any toxic eluants and showed good cellular proliferation with respect to PEMA. This study thus indicates that the eugenyl methacrylate derivatives are potentially good candidates for dental and orthopedic cements.

Introduction

Eugenol (4-allyl-2-methoxyphenol) is a major constituent (70−90%) of clove oil from *Eugenia caryophyllata* and occurs widely as a component of essential oils. This compound possesses analgesic and antiinflammatory properties with the ability to relieve pain in irritated or diseased tooth pulp; however, is not a true local anaesthetic.[1] Eugenol exhibits antimicrobial and antiaggregating activity that has been demonstrated in vitro, along with its influence on platelet function ex vivo.[2] It also has a marked antipyretic activity[3] given intravenously and centrally and may reduce fever primarily through a central action. Antianaphylactic properties of eugenol by preventing mast cell degranulation have been reported in the literature.[4] In addition, eugenol can prevent lipidic peroxidation in the initial steps due to the presence of the phenolic group, which can scavenge free radicals.[5]

Eugenol has been used in dental materials[6] since at least the 19th century. Presently, it is commonly used in combination with zinc oxide (ZOE) as temporary pulp capping agents and as filling systems in root canals, where the eugenol functions as an obturation agent producing a soothing effect on the pulp. These cements produce some adverse effects in vivo probably due to the release of unreacted eugenol, which, in some concentrations, can produce tissue irritation and induce inflammatory reactions over the oral mucous membrane. The main drawbacks of ZOE cements are their weak mechanical properties and the fact that they interfere with the polymerization reaction of acrylic resins due to remaining free eugenol.[7−9] The latter effect can cause a detrimental effect on the physical and mechanical properties of the overlying permanent dental composite resins that cure mainly by free-radical polymerization. Light-activated composites seem to be influenced to a greater degree in their polymerization by adjacent lining materials than chemically activated ones.[10]

The aim of this work was to focus on the modification of the chemical structure of eugenol by the incorporation of a polymerizable group, e.g., the methacrylic group. This approach will allow the eugenol derivative to participate in polymerization reactions rather than to inhibit them and will be more efficacious in the field of dental materials. The new derivatives could be incorporated to permanent restorative materials, giving to the macromolecular chains the bactericide effects of eugenol. To date, these type of systems have not been reported yet.

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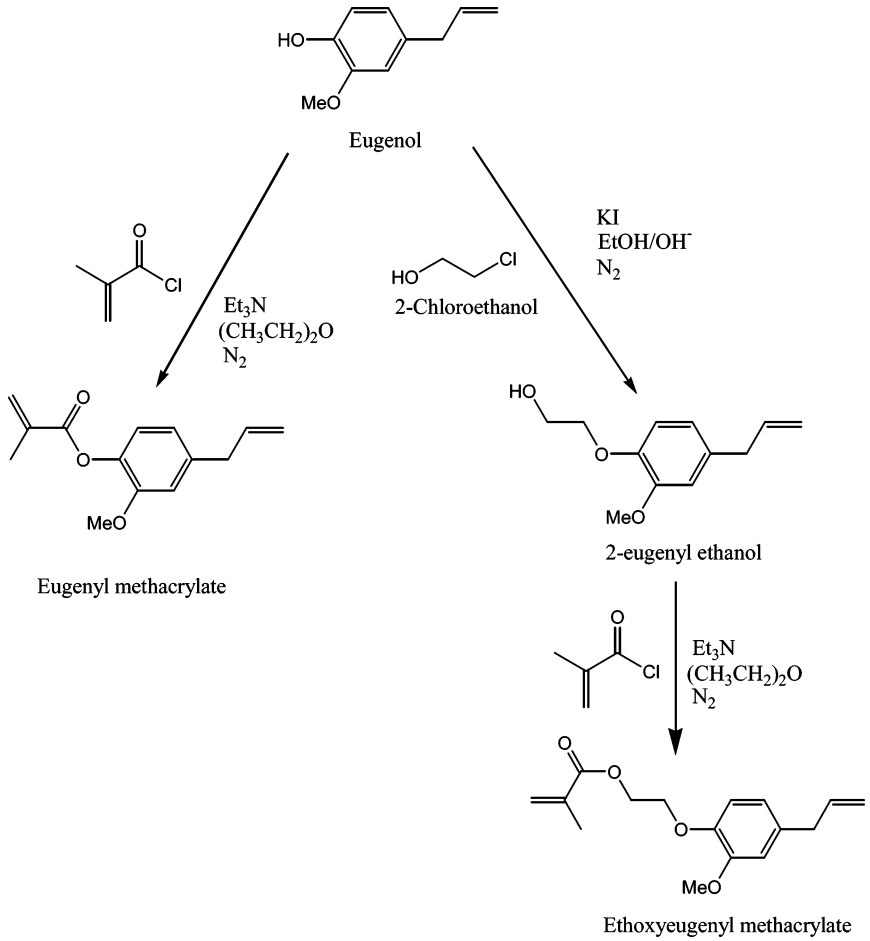
Scheme 1. Synthesis Mechanism Applied in the Preparation of Eugenyl Methacrylate and Ethoxyeugenyl Methacrylate



In this paper we report on the synthesis of two eugenol acrylic derivatives, which differ in the spacer group between both acrylic and eugenol moieties, their homopolymerization and copolymerization with ethyl methacrylate at both low (<10 wt %) and high conversions, and the characterization by ^{1}H and ^{13}C NMR and ATR-FTIR spectroscopy, size exclusion chromatography (SEC), and thermal analysis techniques. The copolymerization parameters determined from the low-conversion polymers by application of linearization and nonlinearization methods are also reported, along with the swelling behavior of high-conversion polymers selected to mimic the in vivo situation. Furthermore, a screening on the bactericide activity of the new derivatives against *Streptococcus mutans* is reported, as well as the in vitro biocompatibility of monomers and polymers using cell culture techniques.

Experimental Procedures

Materials. Eugenol (Acros) was used as received without further purification. Methacryloyl chloride (Aldrich), triethylamine (Scharlau), and ethyl methacrylate (EMA) (Aldrich) were purified by distillation under reduced pressure. 2,2′-Azobisisobutyronitrile (AIBN) (Merck) was recrystallized from methanol (mp 104 °C). The solvents diethyl ether (SDS), toluene (Merck), hexane (SDS), and ethyl acetate (Fluka) were purified by standard procedures. Phosphate-buffered solution (PBS) of pH = 7.4 (Sigma) was used as received. Thermanox (TMX) control disks were supplied by Labclinics S. L., and aqueous solutions of Triton X-100 were supplied by Aldrich. Tissue culture media, additives, trypsin, and 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from Sigma. The fetal bovine serum was obtained from Gibco and the alamar blue reagent from Serotec.

Characterization Techniques. ^{1}H and ^{13}C NMR spectra were recorded on an INOVA-300 spectrophotometer, operating at 300 and 75.5 MHz, respectively. The spectra were recorded by dissolving the corresponding sample (10 wt/v) in deuterated chloroform and using tetramethylsilane (TMS) as internal standard at 25 °C. An indirect detection experiment, heteronuclear multiple quantum coherence (HMQC), was performed to observe correlation between carbon-13 nuclei while detecting high-sensitivity protons. A pulse delay of 3 s between 90° pulses (13.5 μs), 32 scans, and 1024 data points for t1 increment, of 256 increments, sweep width of 2500 Hz in the F2 dimension and 13 000 Hz in the F1 dimension were used in the experiment with gap decoupling of carbon-13. ATR-FTIR spectra were recorded on a Perkin-Elmer-Spectrum One spectrophotometer, with an ATR attachment. Number and weight average molecular weights were determined by size exclusion chromatography (SEC) (Waters Alliance GPCV 2000 with a refractive index detector). Two columns of PL-gel 5 μm mixed bed were conditioned at 35 °C and used to elute the samples of 1 mg/mL concentration at 1 mL/min HPLC-grade chloroform flow rate. Calibration of SEC was carried out with monodisperse standard polystyrene samples obtained from Polymer Laboratories. Glass transition temperatures (T_g) were measured by differential scanning calorimetry (DSC) with a Perkin-Elmer DSC7 interfaced to a thermal analysis data system TAC 7/DX. The dry samples were prepared in the form of thin films (15−20 mg) placed in aluminum pans and heated from −20 to 150 °C at a constant rate of 10 °C/min. T_g was taken as the midpoint of the heat capacity transition. Thermogravimetric diagrams were obtained in a thermogravimetric analyzer using a TGA Q500 (TA instruments) apparatus, under dynamic nitrogen at a heating rate of 5 °C/min in a range of 40−600 °C.

Synthesis of Eugenyl Methacrylate. Stoichiometric amounts of eugenol and triethylamine were dissolved in diethyl ether (1 M) and introduced into a three-necked flask. An excess of methacryloyl chloride dissolved in 10 mL of diethyl ether was added dropwise with constant

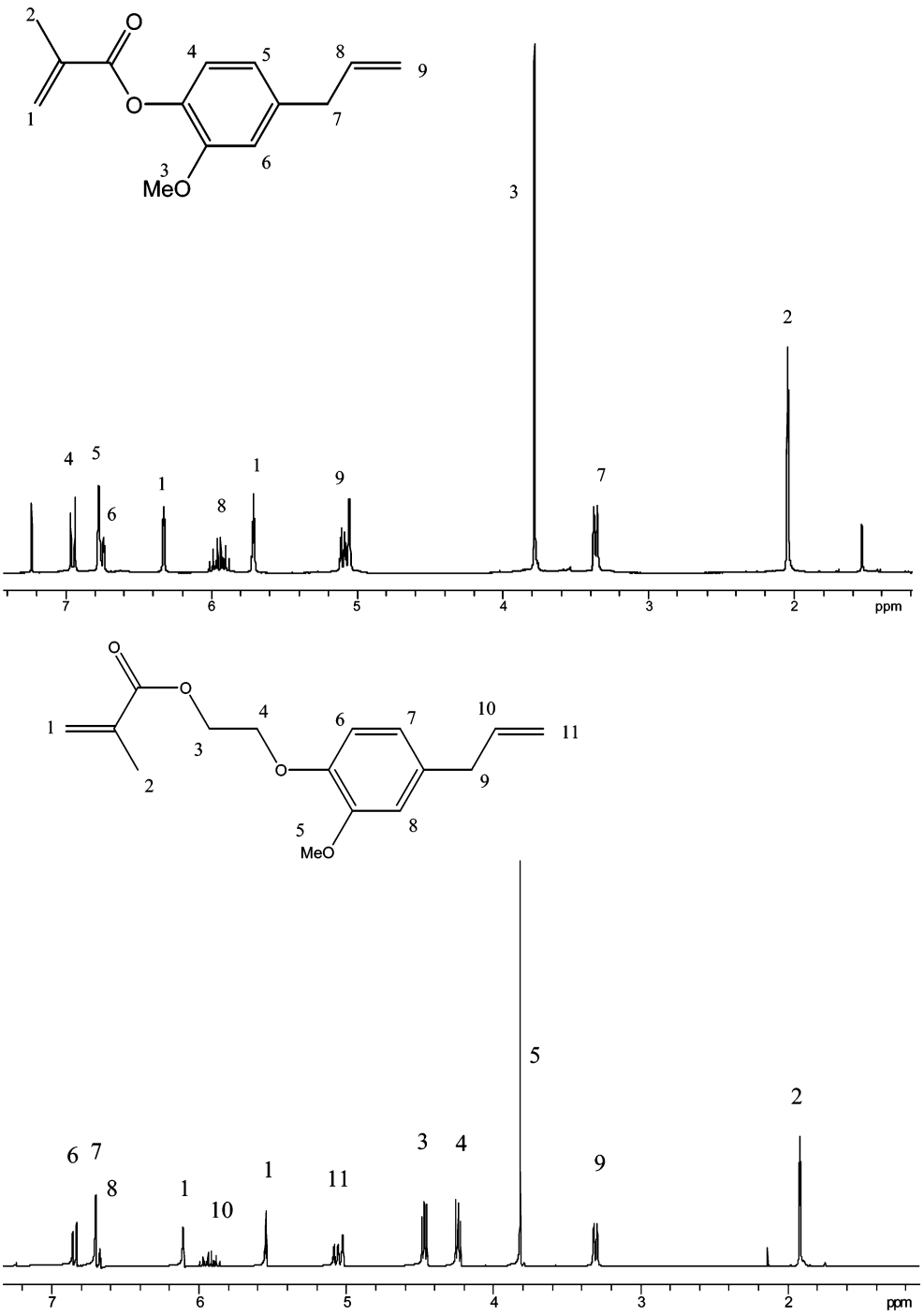


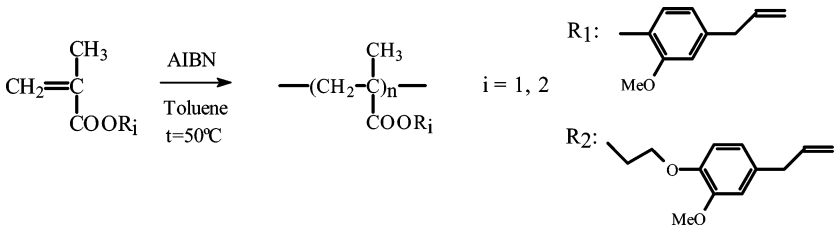
Figure 1. ^{1}H spectra of the acrylic monomers EgMA and EEgMA in $CDCl_3$ at 25 °C.

stirring at room temperature and under nitrogen atmosphere, and the reaction mixture was then kept stirred for 48 h at room temperature. The reaction medium was filtered to remove the triethylamine chlorhydrate, and any unreacted reagents were removed by successive extraction with 5% NaOH solution and distilled water. After drying over anhydrous Na_2SO_4, the solvent was removed by flash distillation and then under reduced pressure until constant weight. An ethyl acetate/hexane solution (10/90v/v) was used as an eluant to purify the product using silica gel column chromatography.

^{1}H NMR spectrum ($CDCl_3$): δ_H 7.0 (H_5−Ar), 6.8 ($H_{3,6}$−Ar), 6.4 and 5.7 (CH_2^{β}═C), 6.0−5.8 (C*H*═CH_2), 5.1 (CH═CH_2), 3.8 (CH_3-OPh), 3.4 (CH_2Ph), 2.1 (CH_3^{α}). ^{13}C NMR spectrum ($CDCl_3$): δ_C 165.7 (*C*O), 151.3 (C_2−Ar), 139.0 (C_1−Ar), 138.1 (C_4−Ar), 137.2 (*C*H═CH_2), 135.7 (CH_2═C^{α}), 126.9 ($C^{\beta}H_2$═C), 122.4 (C_5−Ar), 120.6 (C_6−Ar), 116,1 (CH═*C*H_2), 112.8 (C_3−Ar), 55.7 (*C*H_3OPh), 40.1 (*C*H_2Ph), 18.4 (*C*H_3^{α}). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1725 (C═O stretching), 1640 (C═C in the acrylic and vinyl groups), 1600 (C═C stretching in the aromatic ring).

Synthesis of Ethoxyeugenyl Methacrylate. Ethoxyeugenyl methacrylate was synthesized in two steps. First, 2-eugenyl ethanol was obtained through a typical Williamson reaction[17] by using the following procedure. A solution of eugenol in 2-chloroethanol was mixed with a 50% excess of aqueous KOH solution containing 0.5% KI as cocatalyst, under nitrogen atmosphere. The reaction medium was refluxed for 24 h, and the reaction product purified by silica gel column chromatography using ethyl acetate/hexane 30/70 volume ratio, as an eluant. The second step involved the esterification of 2-eugenyl ethanol and was carried out by dissolving stoichiometric amounts of it with triethylamine in diethyl ether (1 M). Subsequently, a 25% excess of methacryloyl chloride diethyl ether solution was introduced dropwise under nitrogen atmosphere with constant stirring. The reaction was then allowed to proceed for 48 h at room temperature. The isolation and purification

Scheme 2. Free-Radical Polymerization Reaction for the New Eugenol Derivatives



of the ethoxyeugenyl methacrylate (EEgMA) was performed in the same way as described previously for EgMA.

^{1}H NMR spectrum ($CDCl_3$): δ_H 6.8 (H_5−Ar), 6.6 ($H_{3,6-}$Ar), 6.1 and 5.6 (CH_2^β═C), 6.0−5.8 (C*H*═CH_2), 5.1 (CH═CH_2), 4.5 (OCH_2-CH_2OPh), 4.2 (OCH_2CH_2OPh), 3.8 (CH_3OPh), 3.3 (CH_2Ph), 1.9 (CH_3^α). ^{13}C NMR spectrum ($CDCl_3$): δ_C 167.1 (*C*O), 149.8 (C_2−Ar), 146.3 (C_1−Ar), 137.4 (*C*H═CH_2), 135.8 (CH_2═C^α), 133.9 (C_4−Ar), 126.0 ($C^\beta H_2$═C), 120.4 (C_6−Ar), 115.6 (CH═CH_2), 114.9 (C_5−Ar), 112.6 (C_3−Ar), 67.5 (OCH_2CH_2OPh), 63.2 (OCH_2CH_2OPh), 55.7 (*C*H_3O−Ph), 39.8 (*C*H_2Ph), 18.0 (CH_3^α). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1720 (C═O stretching), 1640 (C═C in the acrylic and vinyl groups), and 1590 (C═C stretching in the aromatic ring).

Synthesis of Poly(eugenyl methacrylate) and Poly(ethoxyeugenyl methacrylate). The corresponding monomer was dissolved ([M] = 1 mol/L) in toluene, and the mixture was deoxygenated with N_2 for 15 min. Afterward, the radical initiator azobisisobutyronitrile (AIBN) (1 wt % with respect to monomer) was added to the solution, and the reaction medium was transferred to an oven at 50 °C. The reaction time was maintained at 24 h to obtain high-conversion polymers, or it was adjusted to reach conversions lower than 10 wt %. The reaction product was precipitated in hexane, filtered off, washed, and dried under vacuum to constant weight.

Poly(eugenyl methacrylate). ^{1}H NMR spectrum ($CDCl_3$): δ_H 7.0 (H_5−Ar), 6.6 ($H_{3,6-}$Ar), 5.9 (C*H*═CH_2), 5.1 (CH═CH_2), 3.6 (CH_3OPh), 3.3 (CH_2Ph), 2.8−2.0 (CH_2^β), 1.8−1.4 (CH_3^α). ^{13}C NMR spectrum ($CDCl_3$): δ_C 175.6 (*C*OO), 151.0 (C_2−Ar), 138.2 (C_1−Ar), 137.8 (C_4−Ar), 137.1 (*C*H═CH_2), 122.5 (C_5−Ar), 120.2 (C_6−Ar), 116.0 (CH═*C*H_2), 112.3 (C_3−Ar), 55.2 (*C*H_3OPh), 54.0 ($C^\beta H_2$), 46.0 (C^α), 40.0 (*C*H_2Ph), 17.6 (CH_3^α). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1745 (C═O stretching), 1640 (C═C in the vinyl group), 1603 (C═C stretching in the aromatic ring).

Poly(ethoxyeugenyl methacrylate). ^{1}H NMR spectrum ($CDCl_3$): δ_H 6.8 (H_5−Ar), 6.6 ($H_{3,6-}$Ar), 5.9 (C*H*═CH_2), 5.1 (CH═CH_2), 4.4−3.9 (OCH_2CH_2OPh), 3.6 (CH_3OPh), 3.3 (CH_2Ph), 2.1−1.6 (CH_2^β), 1.1−0.8 (CH_3^α). ^{13}C NMR spectrum ($CDCl_3$): δ_C 176.7 (*C*O), 149.8 (C_2−Ar), 146.3 (C_1−Ar), 137.6 (*C*H═CH_2), 134.0 (C_4−Ar), 120.5 (C_6−Ar), 115.3 (CH═CH_2), 114.7 (C_5−Ar), 112.7 (C_3−Ar), 67.0 (OCH_2*C*H_2-OPh), 63.2 (OCH_2CH_2OPh), 55.8 (*C*H_3O−Ph), 54.0 ($C^\beta H_2$), 45.3 (C^α), 40.0 (*C*H_2Ph), 16.0−18.6 (CH_3^α). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1722 (C═O stretching), 1635 (C═C in the vinyl group), 1592 (C═C stretching in the aromatic ring).

Synthesis of EMA-*co*-EgMA and EMA-*co*-EEgMA Copolymers. Low-conversion copolymers were obtained from molar ratio feeds of EMA/eugenyl monomer ranging from 20:80 to 80:20. The comonomers were dissolved in toluene ([M] = 1 mol/L), and the mixture was deoxygenated with N_2 for 15 min. Then, AIBN (1 wt % with respect to monomers) was added to the solution, and the reaction medium transferred to an oven at 50 °C. The reaction time was adjusted to reach conversions lower than 10 wt %, when the copolymer was precipitated in hexane, filtered off, washed, and dried under vacuum to constant weight. High-conversion copolymers were obtained by bulk copolymerization reaction of the corresponding comonomers in Teflon molds, using AIBN as a radical initiator (1 wt % with respect to monomers) at 60 °C for 48 h. The copolymer films were thoroughly washed with hexane to remove residual monomers and dried under vacuum until constant weight.

EMA-co-EgMA 50/50 Copolymer. ^{1}H NMR spectrum ($CDCl_3$): δ_H 7.0 (H_5−Ar, EgMA), 6.7 ($H_{3,6-}$Ar, EgMA), 5.9 (C*H*═CH_2, EgMA), 5.0 (CH═CH_2, EgMA), 4.0 (OCH_2, EMA), 3.7 (CH_3OPh, EgMA), 3.3 (CH_2Ph, EgMA), 2.8−1.8 (CH_2^β, EgMA, EMA), and 1.8−0.9 (CH_3^α, EgMA, EMA, and CH_3, EMA). ^{13}C NMR spectrum ($CDCl_3$): δ_C 178.0 (*C*O, EMA), 175.8 (*C*O, EgMA), 151.5 (C_2−Ar, EgMA), 138.8 (C_1−Ar, EEgMA), 138.4 (C_4−Ar, EgMA), 137.5 (*C*H═CH_2, EgMA), 122.9 (C_5−Ar, EgMA), 120.7 (C_6−Ar), 116.4 (CH═*C*H_2, EgMA), 113.0 (C_3−Ar, EgMA), 61.2 (OCH_2, EMA), 55.8 (*C*H_3OPh, EgMA), 54.4−51.5 ($C^\beta H_2$, EMA and EgMA), 46.3 (C^α, EgMA), 45.4−46.0 (C^α, EMA), 40.5 (*C*H_2Ph, EgMA), 17−19 (CH_3^α, EMA, EgMA), 14.2 (CH_3, EMA). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1752 and 1720 (shoulder) (C═O stretching, EgMA and EMA), 1640 (C═C in the vinyl group, EgMA), 1600 (C═C stretching in the aromatic ring, EgMA).

EMA-co-EEgMA 50/50 Copolymer. ^{1}H NMR spectrum ($CDCl_3$): δ_H 6.8 (H_5−Ar, EEgMA), 6.6 ($H_{3,6-}$Ar, EEgMA), 5.8 (C*H*═CH_2, EEgMA), 5.0 (CH═CH_2, EEgMA), 4.3−3.9 (OCH_2CH_2OPh, EEgMA and OCH_2, EMA), 3.8 (CH_3OPh, EEgMA), 3.3 (CH_2Ph, EEgMA), 2.0−1.7 (CH_2^β, EEgMA and EMA), 1.3−0.8 (CH_3, EMA and CH_3^α, EEgMA, EMA). ^{13}C NMR spectrum ($CDCl_3$): δ_C 177.7 (*C*O, EMA), 176.9 (*C*O, EEgMA), 150.3 (C_2−Ar, EEgMA), 146.6 (C_1−Ar, EEgMA), 138.0 (*C*H═CH_2, EEgMA), 134.3 (C_4−Ar, EEgMA), 121.0 (C_6−Ar, EEgMA), 116.1 (CH═*C*H_2, EEgMA), 115.4 (C_5−Ar, EEgMA), 113.2 (C_3−Ar, EEgMA),, 67.3 (OCH_2*C*H_2OPh, EEgMA), 63.7 (OCH_2CH_2OPh, EEgMA), 61.1 (OCH_2, EMA), 56.3 (*C*H_3O−Ph, EEgMA), 54.6 ($C^\beta H_2$, EMA, EEgMA), 45.4 (C^α, EMA, EEgMA), 40.2 (*C*H_2Ph, EEgMA), 17.1−19.8 (CH_3^α, EMA, EEgMA), 14.2 (CH_3, EMA). ATR-FTIR spectrum (cm^{-1}, the most characteristic bands): 1722 (C═O stretching, EEgMA and EMA), 1640 (C═C in the vinyl group, EEgMA), 1600 (C═C stretching in the aromatic ring, EEgMA).

Swelling Behavior. Disks of high-conversion copolymers, 10 mm in diameter and 1 mm thick, were accurately measured and weighed, introduced in phosphate-buffered solution (PBS), and kept until they reached equilibrium at 37 °C. The values of water sorption (WS) were determined gravimetrically using eq 1 where W_w is the weight of the wet sample and W_o is the weight of the initial dry sample:

$$\mathrm{WS}\ (\%) = 100 \times (W_w - W_o)/W_o \qquad (1)$$

Antibacterial Activity. The antibacterial activity was assessed by the agar disk method.[11] *S. mutans* CECT 479 was grown in 5 mL of BHI broth for 24 h at 37 °C. Approximately 1.5 mL of bacterial suspension was spread on a BHI agar plate. Eight wells were punched into the agar using a sterile cork borer, and the agar was removed aseptically. Solutions of the test compounds (1 M) were made in dimethyl sulfoxide (DMSO), and each solution (50 μL) was added to a well. The plate was incubated for 24 h at 37 °C. After incubation, the antibacterial effects of each compound were determined by measuring the diameter of zones of inhibition around the wells, in millimeters. The test compounds were eugenyl methacrylate (EgMA), ethoxyeugenyl methacrylate (EEgMA), and ethyl methacrylate (EMA). Eugenol was used as a reference compound.

Biocompatibility of Monomers, Polymers, and Copolymers. The negative control used was tissue culture plastic, Thermanox (TMX), and the positive control (toxic agent) was a 0.5% aqueous solution of Triton X-100. Disks of 10 mm diameter and 1 mm thickness of the high-conversion polymers, PEgMA, PEEgMA, and PEMA, eugenol-containing copolymers, and the controls were used for direct and indirect biocompatibility experiments. The monomers EgMA and EEgMA were

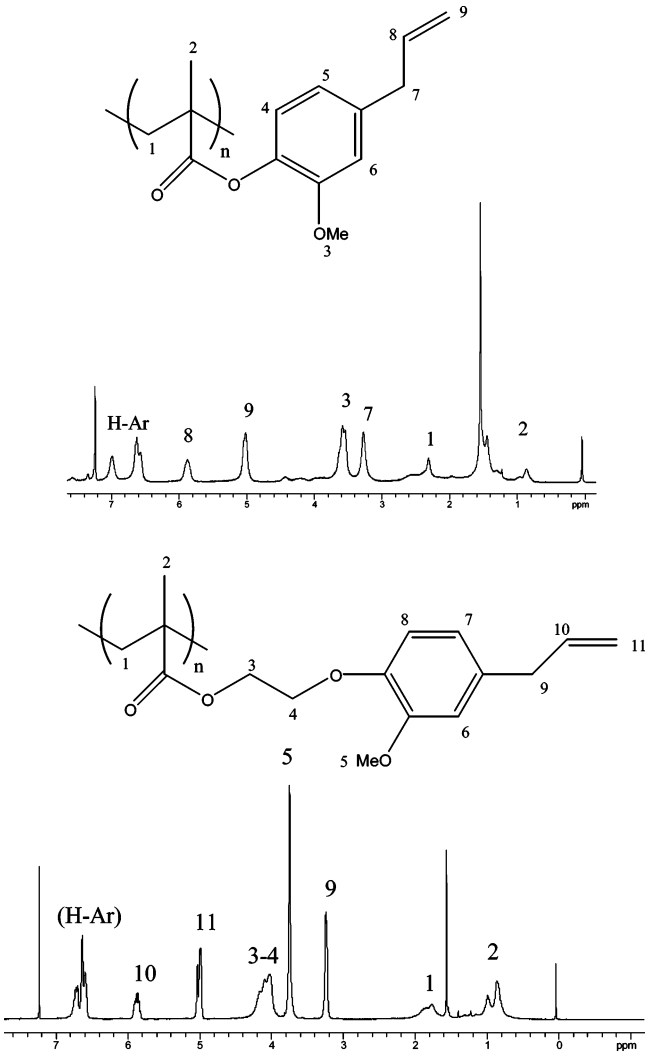


Figure 2. ^{1}H NMR spectra of the acrylic polymers PEgMA and PEEgMA obtained at low conversion (<10 wt %) in $CDCl_3$ at 25 °C.

tested in cytotoxicity assay. All specimens were sterilized with ethylene oxide. The cells used in the primary cell culture were human fibroblasts and were cultured at 37 °C. The culture medium was minimal essential medium eagle (MEM), modified with HEPES (Sigma) and supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The culture medium was changed at selected time intervals with care to cause little disturbance to culture conditions.

Environmental Scanning Electron Microscopy (ESEM). The materials were placed in a 24-well plate (in duplicate) and seeded with human fibroblast at a density of 14×10^5 cells/mL. These were incubated at 37 °C. The cells were fixed with 1.5% glutaraldehyde buffered in 0.1 M phosphate buffer after a 24 h incubation period. The dried samples were sputter-coated with gold before examination under an ESEM apparatus (Philips XL 30) at an accelerating voltage of 15 keV.

MTT Assay for Monomers. The corresponding monomer was mixed with the surfactant Tween 80 in a monomer/surfactant weight ratio of 3:1. The mixture was dispersed in the serum-free medium in order to obtain 0.1 wt % mixture solution containing 0.075 wt % of monomer and 0.025 wt % of surfactant. This solution was successively diluted with serum-free medium. Human fibroblasts were seeded at a density of 11×10^4 cells/mL in complete medium in a sterile 96-well culture plate and incubated to confluency. After 24 h of incubation the medium was replaced with the corresponding dilution and incubated at 37 °C in humidified air with 5% CO_2 for 24 h. A solution of MTT was prepared in warm PBS (0.5 mg/mL), and the plates were incubated at 37 °C for 4 h. Excess medium and MTT were removed, and

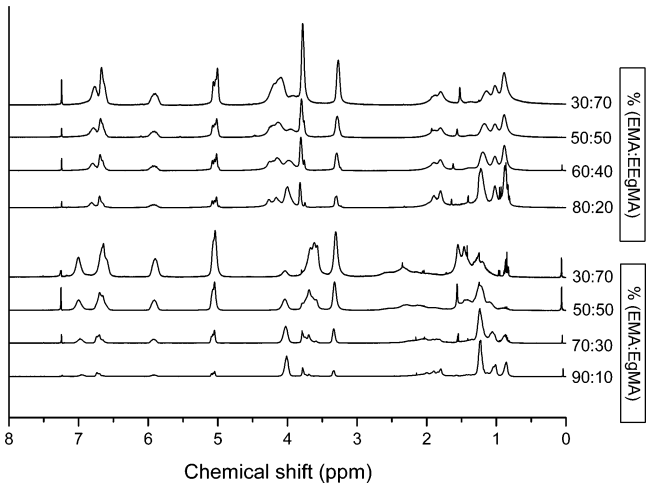


Figure 3. ^{1}H NMR spectra of several EMA-*co*-EgMA and EMA-*co*-EEgMA copolymers of different composition in $CDCl_3$ at 25 °C.

dimethylsulfoxide (DMSO) was added to all wells in order to dissolve the MTT taken up by the cells. This was mixed for 10 min, and the absorbance was measured with a Biotek ELX808IU detector using a test wavelength of 570 nm and a reference wavelength of 630 nm. The cell viability was calculated from eq 2:

$$\text{Relative cell viability} = 100 \times (\text{ODS} - \text{OD}_\text{B})/\text{OD}_\text{C} \quad (2)$$

where OD_S, OD_B, and OD_C are the optical density of formazan production for the sample, blank (MEM without cells), and control (Tween solution in free serum supplemented MEM), respectively. A dose−response curve of relative cell viability was plotted to delineate the concentrations of the monomer that depressed MTT−formazan production by 50% (IC_{50} value).

MTT Assay for Polymers and Copolymers. TMX, Triton, and disks of copolymers were set up in 5 mL of MEM, FCS-free. They were placed on a roller mixer at 37 °C, and the medium was removed at different time periods (1, 2, and 7 days) and replaced with another 5 mL of fresh medium. All the extracts were obtained under sterile conditions. Human fibroblasts were seeded at a density of 11×10^4 cells/mL in complete medium in a sterile 96-well culture plate and incubated to confluency. Then, the medium was replaced with the corresponding eluted extract and incubated at 37 °C in a humidified air with 5% CO_2 for 24 h. A solution of MTT was prepared in warm PBS and filtered before use. MTT, 10 μL, was added to all wells to give a final concentration of 0.5 mg/mL, and the plates were incubated at 37 °C, 5% CO_2 for 4 h. Excess medium and MTT were removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to all wells in order to dissolve the MTT taken up by the cells. This was mixed for 10 min, and the absorbance was measured with a Biotek ELX808 IU plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. Results were normalized with respect to a negative control (TMX = 100%) and statistically tested with ANOVA ($p < 0.05$).

Alamar Blue Assay. Human fibroblasts were seeded at a density of 14×10^4 cell/mL for 24 h over the testing dry specimens placed in 24-well culture plate. After that, 2 mL of alamar blue dye (10% alamar blue solution in phenol red free DMEM medium) was added to each specimen. After 4 h of incubation 100 μL ($n = 4$) of culture medium for each test sample was transferred to a 96-well plate, and the absorbance was measured at 490 nm on a Biotek ELX808IU. The specimens were washed with PBS twice to remove the rest of the reagent, and 1 mL of culture medium was added to monitor the cells over the materials. This step was done at 2, 7, 14, and 21 days. Analysis of variance (ANOVA) of the results for copolymers was carried out with respect to PEMA at $p < 0.05$, $p < 0.01$, and $p < 0.001$ of significant levels.

Table 1. Reactivity Ratios of the Copolymers EMA-*co*-EgMA and EMA-*co*-EEgMA

system	*r*	Fineman−Ross method	Kelen−Tudos method	Tidwell−Mortimer method	Levenberg−Marquardt method
EMA-*co*-EgMA	r_{EgMA}	1.23 ± 0.04	1.38 ± 0.25	1.48	1.48
	r_{EMA}	0.38 ± 0.10	0.51 ± 0.06	0.55	0.55
	r_1r_2	0.47	0.70	0.81	0.81
EMA-*co*-EEgMA	r_{EEgMA}	0.98 ± 0.07	1.09 ± 0.36	1.22	1.22
	r_{EMA}	0.27 ± 0.1	0.36 ± 0.09	0.42	0.42
	r_1r_2	0.26	0.39	0.51	0.51

Results and Discussion

Synthesis of Monomers. Naturally occurring eugenol has been widely studied due to its bactericide and pharmacological properties.[12] The modification of the chemical structure of eugenol has been attempted in order to improve its properties[13] or to obtain additional pharmacological activities, e.g., 2-acetyloxybenzoyl eugenol[14] and dimethyldehydrodieugenol.[15] In dental applications, one of the main drawbacks of eugenol-containing cements is derived from the antioxidant character[16] of the unreacted molecules of eugenol which severely inhibits the free-radical polymerization of the overlying permanent dental composite resin materials. Thus, in this work the acrylic functionalization of eugenol has been attempted, and two methacrylates differing from the spacer group between the methacrylic group and the eugenol moiety were synthesized: eugenyl methacrylate (EgMA), in which the acrylic residue was directly bonded to the aromatic ring of eugenol, and ethoxyeugenyl methacrylate (EEgMA), in which the acrylic and eugenol moieties were separated through an oxyethylene group. A typical Fisher esterification reaction was used for the synthesis of the eugenol derivatives.[17] EgMA was obtained directly from eugenol, by reaction with methacryloyl chloride, using triethylamine as a catalyst, under mild conditions, with a product yield of 80%. EEgMA was obtained from 2-eugenyl ethanol, previously prepared through a Williamson reaction (75% of yield), by reaction with methacryloyl chloride following the same procedure as that of EgMA. The reaction yielded 80% of purified monomer. The synthetic route followed to obtain both the chemical reactions is shown in Scheme 1. Both monomers were characterized by ^{1}H and ^{13}C NMR spectroscopy. The assignments of resonance signals in the NMR spectra were supported by the two-dimensional HMQC spectra obtained for both monomers and eugenol. Assignment of signals is given in the Experimental Section, and the ^{1}H NMR spectra of both monomers are displayed in Figure 1. In both cases the esterification reaction was confirmed by the disappearance of the resonance signal of the hydroxylic group of eugenol (δ = 5.5 ppm) or that of 2-eugenyl ethanol (δ = 2.4 ppm) and the presence of the bands corresponding to the methacrylic residue (see chemical shifts in the Experimental Section). The monomers were also characterized by ATR-FTIR spectroscopy, the assignment of the most characteristic bands being in the Experimental Section.

Polymerization of EgMA and EEgMA. The free-radical polymerizations of EgMA and EEgMA were carried out following well-known polymerization procedures[18] (Scheme 2).

The low-conversion polymers (<10 wt %) were soluble in organic solvents and were characterized by ^{1}H and ^{13}C NMR spectroscopy. The assignments of the resonance signals in the spectra of both PEgMA and PEEgMA were made based on the corresponding two-dimensional HMQC spectra, and they are given in the Experimental Section. Figure 2 shows the ^{1}H NMR spectra of both homopolymers, in which the corresponding

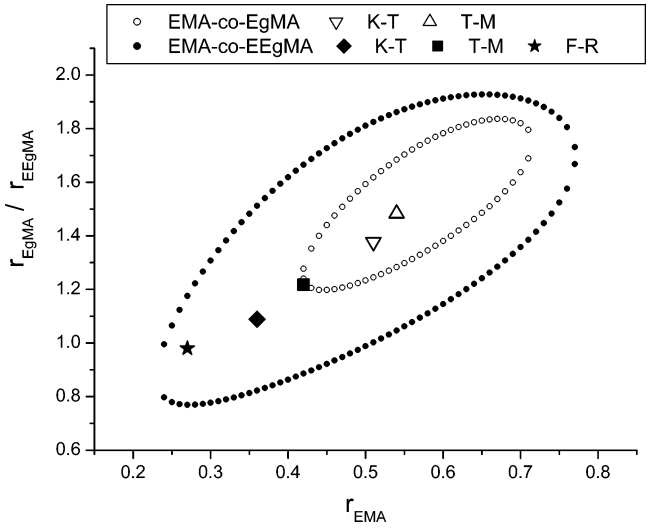


Figure 4. Plot of 95% confidence diagrams for the reactivity ratios of the pairs EMA-*co*-EgMA and EMA-*co*-EEgMA determined by the nonlinear least-squares method.

allylic protons, δ_H 5.8 ppm (C*H*═CH_2) and 5.0 ppm (CH═CH_2), can be observed. This fact indicates that the polymerization reaction proceeded via the methacrylic double bonds to give hydrocarbonated macromolecular chains with pendant eugenol moieties. This observation is consistent with previous studies carried out on the radical polymerization of allyl methacrylate.[19] However, when the reaction was allowed to proceed until 24 h, insoluble polymers with yields of 60% and 70% for PEgMA and PEEgMA, respectively, were obtained. This fact seems to indicate that, when the majority of the methacrylic double bonds have reacted, the allylic double bonds were involved in the radical polymerization. In the case of the high-conversion polymers, the viscosity of the medium increased as the polymerization proceeds, decreasing the mobility of the macroradicals; however, it is suspected that some of the pendant allylic groups may have contributed to the grafting and cross-linking[19] in the network. To know the soluble fraction of high-conversion polymers, the reaction products were subjected to Soxhlet extraction with toluene for 48 h, and the extracted weight percentages of soluble polymers were as little as 1.1 and 0.6 wt % for PEgMA and PEEgMA, respectively, which means the formation of a cross-linked network with dimensional stability.

Copolymerization of Eugenol Derivatives with Ethyl Methacrylate. Antecedents on the preparation of copolymers of methyl methacrylate and a derivative of eugenol having pendant urethane linkages are found in the literature.[20] In this work copolymers containing the acrylic derivatives of eugenol were obtained with ethyl methacrylate (EMA) as a comonomer. Ethyl methacrylate, being the higher homologue of methyl methacrylate, imparts a certain degree of ductility to the polymeric system, and its established beneficial mechanical[21] and biological[22] properties makes it a candidate of choice for

self-curing formulations. Low-conversion copolymers (<10 wt %) of EMA-*co*-EgMA and EMA-*co*-EEgMA were white powders and soluble in common organic solvents. They were characterized by ^{1}H and ^{13}C NMR spectroscopy, and the NMR spectra of both systems showed the resonance signals of both comonomeric units. The assignment of NMR signals was made according to the HMQC spectra (see the Experimental Section). Copolymer composition was determined from ^{1}H NMR spectra of several copolymer samples with different composition, for both systems (Figure 3). The mole fraction of ethyl methacrylate in the copolymer was calculated by considering the resonance signal of the OCH_{2-} protons at 4.2−3.8 ppm. The eugenol derivatives mole fractions were calculated using the integral of the allylic protons $=CH_2$ at 5.3−4.7 ppm, and those of the aromatic ring protons at 6.5−7.2 ppm. The differences obtained considering these two groups of signals was less than 1 mol %, which confirms that at low conversion the participation of the allylic group in the free-radical polymerization was very low and not detected by the usual NMR technique. This observation was confirmed by comparison of the ratio of the integrated intensities of the aromatic and allylic protons for the free eugenol (1.50 ± 0.1) with that obtained for the same protons in copolymers of the acrylic derivatives of eugenol with ethyl methacrylate (1.48 ± 0.1). Copolymer composition values showed that in both systems the copolymers were enriched in the eugenol derivative monomer. Copolymerization parameters were studied assuming that the copolymerization reaction follows the terminal model,[18] and that, at low conversions, only the reactive methacrylate groups participate in the polymerization.[23] Thus, reactivity ratios were calculated by application of Fineman−Ross[24] and Kelen−Tudos[25] linearization methods as well as the nonlinear least-squares analysis suggested by Tidwell and Mortimer,[26] and the algorithmic method of Levenberg−Marquardt.[27] The values of reactivity ratios determined by the four methods are given in Table 1.

The product r_1r_2 remained less than unity indicating that both types of copolymers present a predominantly statistical distribution of monomeric units. Thus, the reactivity of the eugenol derivative was higher toward both types of growing macroradicals, those ending in its own monomer or those ending in EMA. To know the experimental error and the goodness of the experimental conditions used to calculate the composition data, the 95% confidence limits defined by the area of an elliptical diagram were obtained by the mathematical treatment suggested by Behnken[28] and Tidwell and Mortimer.[26] The limits for each system drawn in Figure 4 show that the reactivity ratios determined by the nonlinearization methods are located in the central sector of the ellipses, indicating that these are the most reliable values for these parameters.

By application of these values to the Lewis−Mayo equation[29] the composition diagrams were obtained, which are plotted in Figure 5 and correlated well with the experimental composition data represented by points in the diagrams.

On the basis of the reactivity ratios determined from the Tidwell and Mortimer method, statistical sequences analysis was performed. Figure 6 shows the statistical monomeric distribution in terms of triad centered in EgMA or EEgMA as a function of the molar fraction of the corresponding monomer in the feed. The mole fraction of homotriads increased with the eugenol derivative content in the feed, that of heterotriads presented a wide maximum centered in 0.6 molar fraction in the feed for both systems, and that of alternating triads was maximum for a 0.2 molar fraction of EgMA or EEgMA in the feed.

Low-conversion copolymers were characterized by SEC for determination of average molecular weight and by DCS and TGA for evaluation of their thermal properties. Results of these experiments are shown in Table 2 and Figure 7. All the polymers prepared in diluted solution and at low conversion (<10 wt %) were soluble in chloroform, and the number average molecular weight values determined by SEC were relatively high, which demonstrate that the monomers EgMA and EEgMA do not present any inhibitory effect in the free-radical reaction process. It is clear that when the eugenyl methacrylate was copolymerized with ethyl methacrylate a decrease in the molecular weight

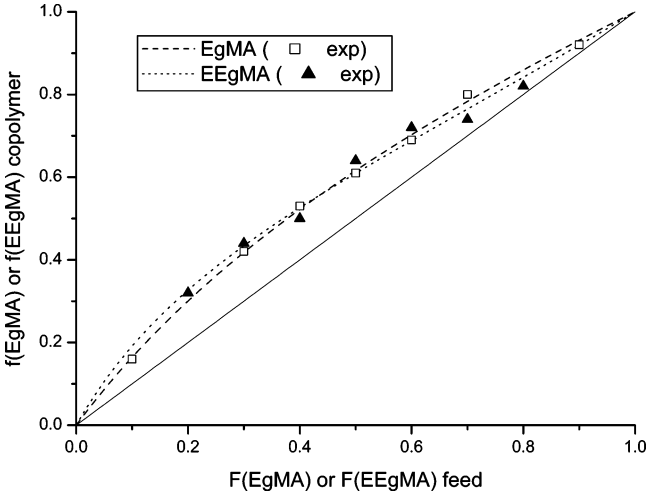


Figure 5. Composition diagrams for the copolymerization of ethyl methacrylate with eugenyl methacrylate and ethoxyeugenyl methacrylate in solution initiated with AIBN at 50 °C.

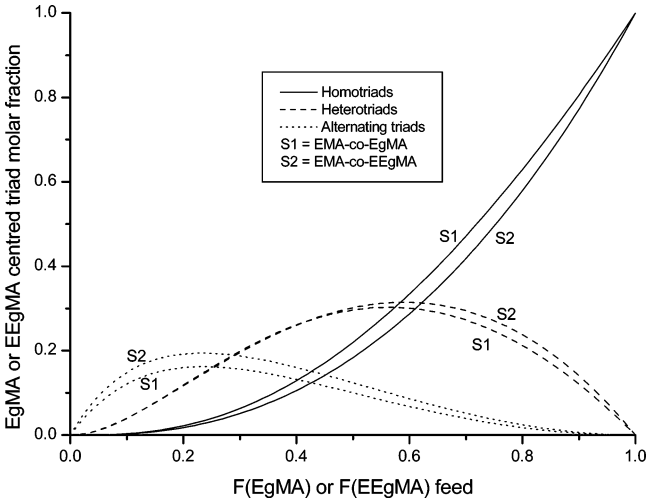


Figure 6. Variation of the theoretical molar fraction of triads centered in EgMA or EEgMA with the content of the eugenol derivative in the feed for both copolymeric systems.

Table 2. Number Average Molecular Weight (M_n), Glass Transition Temperature (T_g), and TGA Data for Degradation of Low-Conversion Poly(ethyl methacrylate) and Eugenol-Containing Polymers and Copolymers[a]

sample	M_n (g/mol)	T_g (°C)	T_{max} (°C)[b]	$T_{50\%}$ (°C)[c]
PEgMA	81 000	95	432	416
EgMA/EMA 50:50	82 000	90	425	397
EEgMA/EMA 50:50	275 000	31	425	397
PEEgMA	640 000	20	425	397
PEMA	158 000	71	350	336

[a] Concentration of monomers in the solution [M] = 1 mol/L, conversion <10 wt %, in all cases. [b] Temperature for maximum rate of decomposition. [c] Temperature for 50% weight loss.

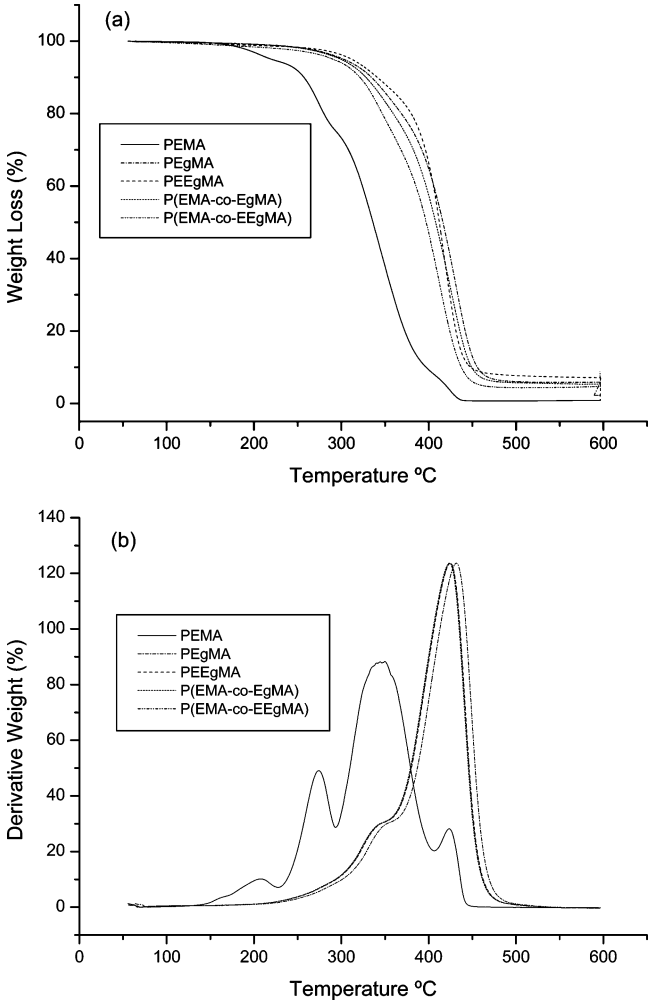


Figure 7. (a) TGA and (b) DTG curves of PEMA and eugenol-containing polymers and 50:50 copolymers.

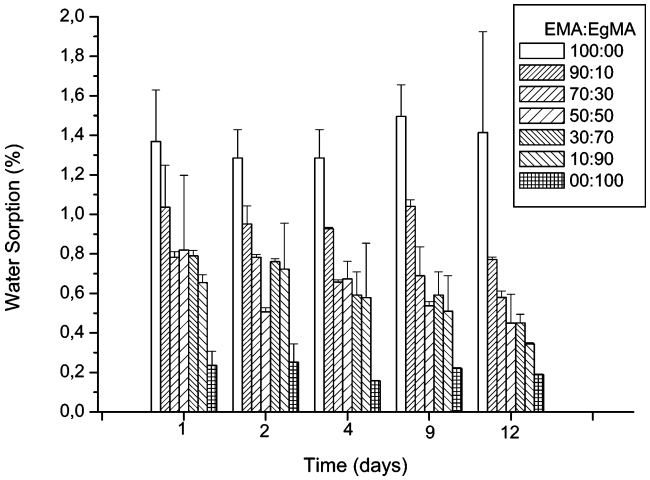


Figure 8. Evolution of water content with time of immersion in PBS and at 37 °C for EMA-*co*-EgMA copolymers.

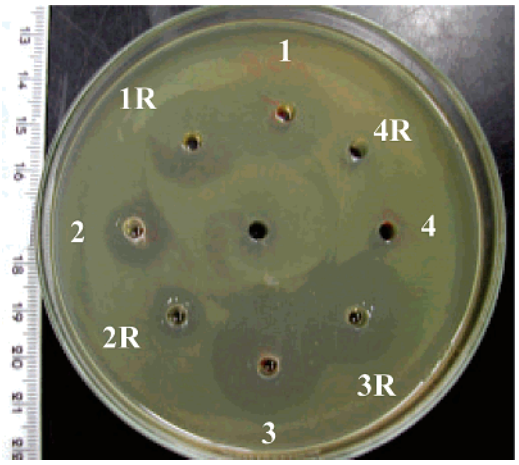


Figure 9. Results of agar disc diffusion test of eugenol (1 and 1R), eugenyl methacrylate, EgMA, (2 and 2R), ethoxyeugenyl methacrylate, EEgMA, (3 and 3R), and ethyl methacrylate, EMA, (4 and 4R) against *S. mutans* CECT 479 after 24 h of incubation at 37 °C.

of the corresponding copolymer with respect to PEMA was produced. However, the value obtained indicated that the M_n is high enough to be considered for self-curing composites in dental and bone applications. It is necessary to take into consideration that the self-curing systems result in cross-linked networks by the participation of the allylic groups of the eugenol moieties. This participation is clear in the homopolymerization of EEgMA, which gave a soluble polymer at low conversion but with a M_n higher than the corresponding copolymer or even PEMA. This is a result of the branching structures formed by the participation of a small faction of the allylic groups in the polymerization process.

The glass transition temperature values of the eugenol-containing polymers were 95 °C for PEgMA and 20 °C for PEEgMA. The T_g for PEgMA is higher than that reported for other polymethacrylates containing an aromatic ring in the side chain,[30] which may indicate that some type of interactions involving the substituent groups of the aromatic ring could take place. The value of 20 °C for the T_g of PEEgMA, on the other hand, indicates that the oxyethylene group separating the acrylic and eugenol residues contributes to the flexibility of the side group. The values of T_g of the 50:50 molar ratio copolymers were closer to that of the respective eugenyl-containing homopolymer.

The thermal stabilities of polymers and copolymers were investigated with TGA in a nitrogen stream, and the thermogravimetric curves are compared in Figure 7. PEMA thermogram showed four degradation stages, and the derivative of the weight loss versus temperature showed four peaks centered at 207, 274, 350, and 423 °C (Figure 7b). The thermal degradation of PEMA has been reported to be very similar to that of PMMA.[31] According to this and based on the work reported by Kashiwagi et al.,[32] the first stage of degradation (around 207 °C) would originate from the sterically hindered linkages that result from head-to-head coupling during polymerization. The second stage of degradation (around 274 °C) is attributed to the decomposition of the chain end from vinylidene end formed by disproportionation, and the following stages (around 350 and 430 °C) are ascribed to the random chain scission of the main chain leading to depolymerization of the acrylic polymer. The eugenol-containing polymers and copolymers underwent practically single-step decomposition, with a clear increase of the stability with the composition of the eugenyl derivatives (Figure 7a). It is reasonable to ascribe the single peak to random scission of the main chain. It should be noted that for the 50:50 copolymers of either eugenyl monomer, the onset of the degradation remained unaffected despite the introduction of approximately 50% of ethyl methacrylate units. Accordingly, the values of the temperature at 50% weight loss were higher for the eugenyl-containing homopolymers and copolymers with respect to that of PEMA. All these results indicate that the eugenyl side residue is very active as scavenger of the free radicals produced during the thermal treatment at temperatures higher than 250 °C and makes it clear that this effect could be

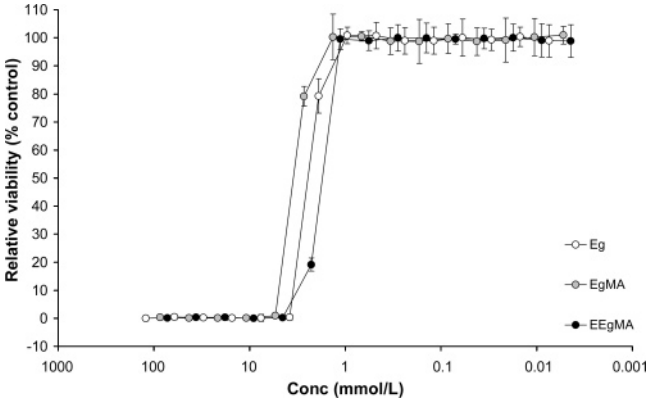


Figure 10. Comparison of dose−response curves (MTT assay) of eugenyl methacrylate (EgMA), ethoxyeugenyl methacrylate (EEgMA), and eugenol (Eg). Each point is the mean ± standard deviation; $n = 16$. From these diagrams the IC_{50} values were determined.

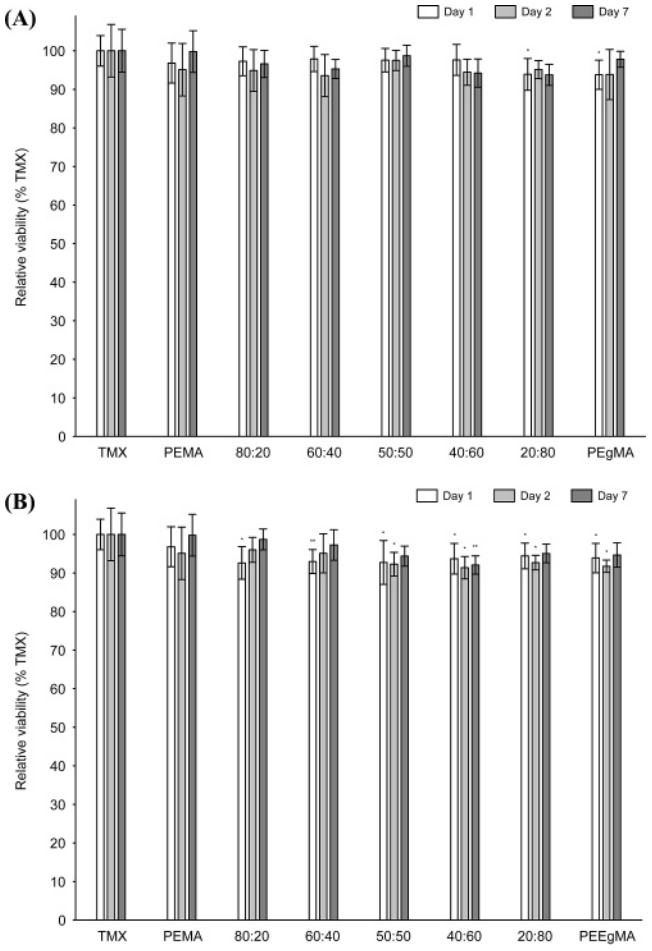


Figure 11. MTT cytotoxicity results for control TMX, PEMA, PEgMA, and EMA-*co*-EgMA copolymers (A) and PEMA, PEEgMA, and EMA-*co*-EEgMA copolymers (B). Results are the mean ± standard deviation. Statistical analysis ($n = 16$) of each polymer or copolymer was performed with respect to TMX at a significance level of ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$.

kept in a similar way for the stabilization and inhibition of the free radicals produced by the decomposition of hydroperoxide compounds associated to inflammatory processes in the physiological medium. A similar behavior was found for the polyacrylic derivative of vitamin E.[33]

Swelling Behavior of Copolymers. The acrylic copolymers bearing eugenol moieties have potential application as dental composites and orthopedic cements. Hence, an evaluation of

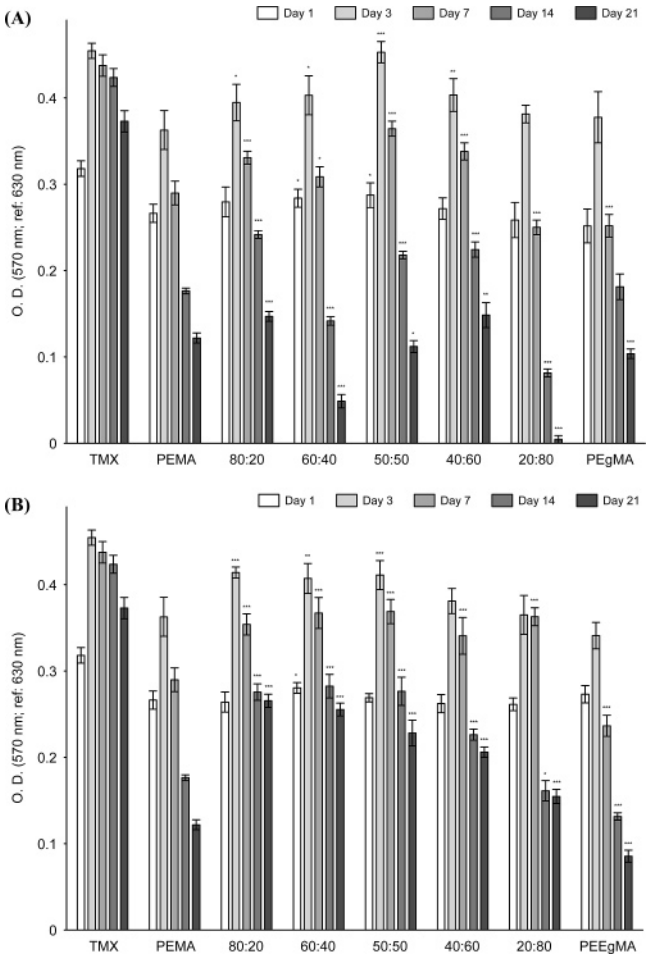


Figure 12. Direct alamar blue results for control TMX, PEMA, PEgMA, and EMA-*co*-EgMA copolymers (A) and PEMA, PEEgMA, and EMA-*co*-EEgMA copolymers (B) over a period of 21 days. Results are the mean ± standard deviation. Statistical analysis ($n = 16$) for each polymer or copolymer containing eugenol residues was performed with respect to PEMA at a significance level of ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$.

water absorption characteristics is clearly important since the uptake of water can affect both biomechanical and biological properties. Swelling behavior was conducted on copolymer samples obtained at high conversion, to mimic self-curing formulations, which are applied as a prepolymerized paste and allowed to cure in vivo up to high conversions; thus, it is likely that some degree of cross-linking would occur as a result. The maximum water sorption in PEMA and PEgMA were 1.4% and 0.25%, respectively, as shown in Figure 8. The water content was observed to decrease with increasing EgMA content in the EMA-*co*-EgMA copolymer as expected due to the hydrophobicity of the eugenyl methacrylate unit and the increase in cross-linking. A clear trend of the decreasing degree of hydration was evident (Figure 8), and compositions with a 50:50 feed content showed a value of 0.8%, which was the midpoint of the uptake of the two homopolymers and was in the range of those of resin restorative materials.[34]

Antibacterial Assay. The antibacterial effects of the two monomers derived from eugenol and those of ethyl methacrylate were studied against *S. mutans*, a commonly facultative bacteria found in infected root canals. Results are shown in Figure 9 along with those of eugenol. It can be seen that both EgMA and EEgMA present antibacterial effects showing an inhibition halo of average diameters of the zone of inhibition of 7 and 21 mm, respectively. The antibacterial effect of EgMA was slightly

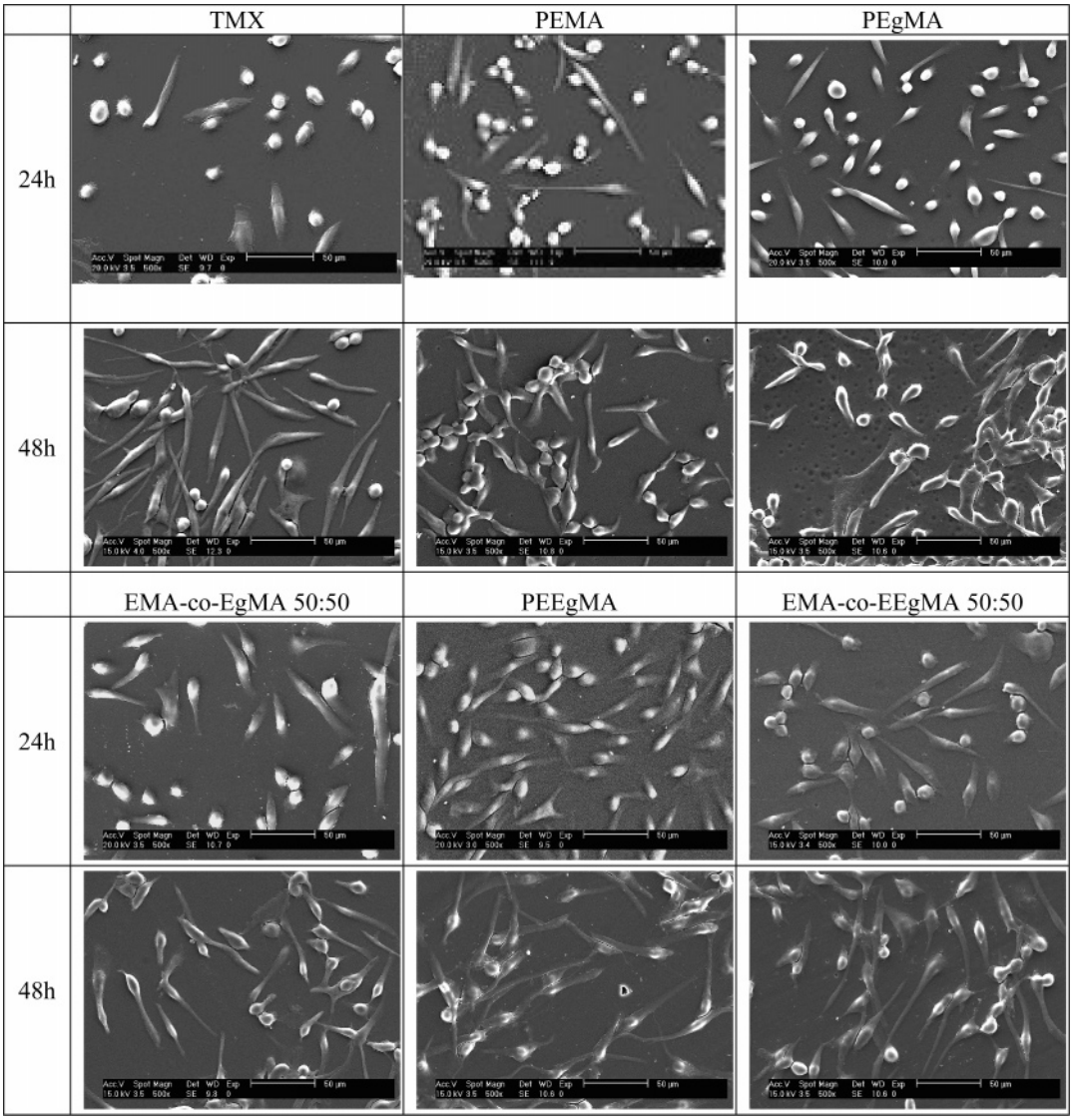


Figure 13. ESEM images of human fibroblasts colonization on control TMX, PEMA, and eugenol-containing polymers and copolymers after 24 and 48 h of seeding.

lower than that of eugenol (20 mm), but that of EEgMA was comparable, which indicates that the eugenyl residue in the novel monomers maintains the bactericide effect. This finding agrees with those reported by Yuwono et al.,[6] who state that both 4-allylic and 2-methoxy groups contribute to the aseptic and anesthetic activity of eugenol. The acrylic residue seems to have negligible contribution to this activity, at sight of the absence of inhibition halo in the presence of ethyl methacrylate. Based on these results, one can expect that the polymers bearing eugenyl residues will kept the bactericide activity, and in this sense new experiments are being conducted. In fact, preliminary results with the corresponding polymers indicate a similar effect. However, because of the cross-linked structure of the polymerized systems it is necessary to apply other kind of experiments for quantitative or semiquantitative comparison.

Biocompatibility. It is known that low concentrations of eugenol exert antiinflammatory and local anesthetic effects on the dental pulp; however, it has been demonstrated that high concentrations are cytotoxic.[35] Cytotoxic effects of EgMA, EEgMA, and eugenol on the relative cell viability of human fibroblasts are shown in Figure 10. All compounds exhibited a dose-dependent effect, and the IC_{50} values were 3.70, 1.83, and 2.60 for EgMA, EEgMA, and eugenol, respectively (Figure 10). The differences are not very significant, but it is important to understand that the eugenol derivatives would only be added in small amounts to high molecular weight polymers, with the derivatives being incorporated in a cross-linked network, and any unreacted monomer that may arise would be predominantly due to the major component, ethyl methacrylate.

Biocompatibility of the polymers PEgMA, PEEgMA, PEMA, and copolymers containing eugenol moieties was evaluated using the MTT assay for testing the toxicity of eluates and the alamar blue assay for cell proliferation. Results of both analyses are shown in Figures 11 and 12, respectively. Figure 11 shows that cell viability was not affected by the presence of extracts from any copolymer system within 7 days, reaching values higher than 90% of the control TMX in all copolymers.

The cell growth, proliferation and differentiation results are shown in Figure 12. They showed that in all cases, fibroblasts proliferated between day 1 and 3 in a fashion similar to that of the control, and after that time, proliferation started to decrease. The statistical analysis at day 3 showed that all EMA/EgMA copolymers exhibited significantly higher cell proliferation in comparison to that of PEMA, and the same was obtained for EMA/EEgMA copolymers of composition 80:20 to 50:50. At day 7, when a decrease in cell proliferation was generally observed even in the control systems, also a significantly higher cell proliferation in a majority of the eugenol derivatives

containing copolymers (see Figure 12) with respect to that of PEMA was measured, which seems to indicate that during the initial periods, the eugenol derivatives provide an improvement of cell growth with respect to that of PEMA. Finally, cell morphology and cell−material interaction of fibroblasts with the copolymeric systems was examined by ESEM, and the results are shown in Figure 13. Cells appeared with normal morphology well-spread and flattened over the materials at 24 h and remained at 48 h, indicating the formation of stable adhesive contacts and showing a good cytocompatibility for all copolymers.

Conclusions. The synthesis of novel eugenol derivatives bearing acrylic moieties provides a new approach to incorporate the benefits of eugenol in acrylic resins without the inhibitory effect characteristic of the phenol derivatives. This approach will exploit the applications of eugenol, which will clearly be an advance in the development of biomedical systems in the field of dentistry and orthopedic applications.

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