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Full Paper

Atom Transfer Radical Polymerization Functionalization on Polypropylene Films for Immobilizing Active Compounds

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This work proposes the surface chemical modification of polypropylene films (PP) by atom transfer radical polymerization (ATRP) using glycidyl methacrylate (GMA) as the graft monomer. At a later stage, the epoxy groups of PP-*g*-PGMA were used for covalent binding of glucose oxidase (GOD) to obtain an active material (PP-*g*-PGMA-GOD) with 9.38 ± 0.06 mg cm^{-2} of enzyme bonded on the surface of PP. Preliminary microbiological studies have shown that this methodology of covalent binding of the enzyme onto the PP surface allowed its activity to be maintained. Therefore, this advantage would give to PP-g-PGMA-GOD films a potential use as an active packaging material if further specific studies on their antimicrobial properties can be verified.

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Introduction

For many applications, controlling the chemical and physical nature of an interface is of primary importance. In particular, there has been a great deal of interest in the development of molecularly ordered surface architectures that play a key role in several applications, including protein adsorption, clinical diagnostics, and cellular adhesion. Since unwanted steric hindrance from neighbouring molecules can be problematic, various extended approaches have been examined to avoid congested functional surfaces.[1]

Thus, it has been shown that the presence of well defined polymer brushes on the surface of various types of substrates with a predictable morphology, improves the performance of the immobilized active agent.[2,3] In most of these cases, atom transfer radical polymerization (ATRP), a 'controlled' radical polymerization, was the method chosen for the preparation of the brushes.[4–6]

Food-borne microbes are driving a search for innovative ways to inhibit microbial growth in foods while preserving quality, freshness, and safety,[7–11] giving rise to food industry research focusing on the development of 'active packaging' with antimicrobial properties.[12]

Over the last decade, numerous publications and patents have documented the successful generation of innovative food packaging with antimicrobial properties.[13–18] In active packaging, the active components can be physical (by entrapment), or chemical (included in the package material).[19] However, the covalent immobilization of active agents onto the surface of food-contact packaging films is gaining the attention of the industry, since it is unlikely that migration occurs from the package into the food. Active packaging technologies based on the concept of non-migration in the manufacturing process therefore opens the possibility of producing food without additives and better guarantees of safety for increasingly demanding consumers.[13,20–22] Various immobilization methods on polymeric surfaces have been described, such as ionic and covalent immobilization by cross-linking, graft co-polymerization, and chemical functionalization of the surface. These methodologies require the presence of suitable functional groups on both the active substance and the packaging surface.

In our previous work, a non-migratory active packaging was successfully developed by photografting through a radical non-controlled polymerization of poly(acrylic acid) (PAA) or poly (hydroxyethyl methacrylate) (PHEMA) on PP film surfaces.[21,23] In the first case, natamicine was electrostatically attached to the carboxy groups of the PP surface, and in the second case, caffeic acid was covalently immobilized on the hydroxy groups. The antimicrobial and antioxidant capacities of these surfaces were demonstrated. However, more recent studies demonstrated the heterogeneity of chemical modified surfaces through this uncontrolled radical polymerization process.[24] Therefore, the ATRP methodology could be a better option to correct these problems.

On the other hand, due to consumer demand for chemical-preservative-free foods, food manufacturers are now using naturally occurring antimicrobials to sterilize and/or extend the shelf life of foods. Some of the naturally occurring

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antimicrobial agents include herb extracts, spices, enzymes, and bacteriocins. The specificity of enzymes should be considered carefully, since antimicrobial activity is very sensitive to environment and substrate.[25] Glucose oxidase (GOD) is an enzyme used in antimicrobial active packaging.[26] This enzyme is a typical example of an oxidoreductase system that does not itself possess antimicrobial activity. However, the reaction products from reactions catalyzed by a given antimicrobial oxidoreductase system exhibit antimicrobial activity.[2]

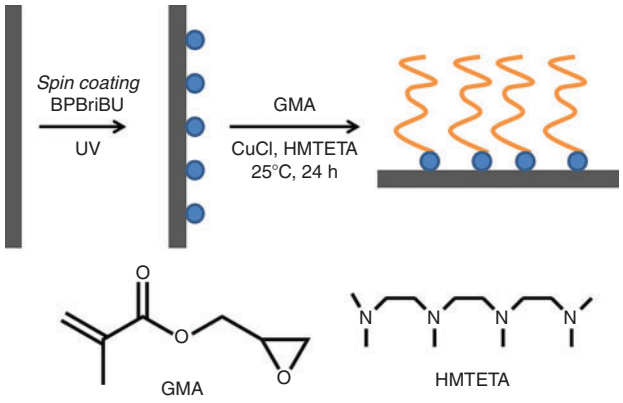
There are several reports on chemical or physical immobilization of GOD with applications in food packaging.[27] For example, Ge et al. immobilized GOD non-covalently into poly (vinyl alcohol)/chitosan/tea extract nanofibres by electrospinning, in order to obtain membranes for food preservation.[28] Vartianen et al. report covalently immobilized GOD on amino- and carboxy-plasma-activated bi-orientated polypropylene films (BOPP) via glutaraldehyde and carbodiimide chemistries.[2] However, glutaraldehyde is known to be toxic when released into food.[29–31]

Nevertheless, few studies about GOD immobilization via ATRP have been reported in the preparation of active packaging. Here we report the optimization and development of an active packaging by grafting poly(glycidyl methacrylate) (PGMA) onto a PP surface through a controlled radical polymerization like ATRP. The purpose of using PGMA as a grafting agent is to fulfil functions of a linker and spacer, providing anchoring sites for GOD immobilization. The epoxide groups of the grafted PGMA chains were used for direct covalent immobilization of GOD through the ring-opening reaction with the amine groups of GOD. In a further step, the microbiological properties will be studied for further application in active packaging of our modified films.

Results and Discussion

Immobilization of PGMA on PP via ATRP

The grafting of PGMA on a PP film surface by ATRP was performed using the methodology set forth in Scheme 1.



Scheme 1. PGMA grafting methodology on PP surface.

PP surface modification involved two steps: first, synthesis and immobilization of initiator (BPBriBu), and second, grafting of GMA on the PP surface by ATRP.

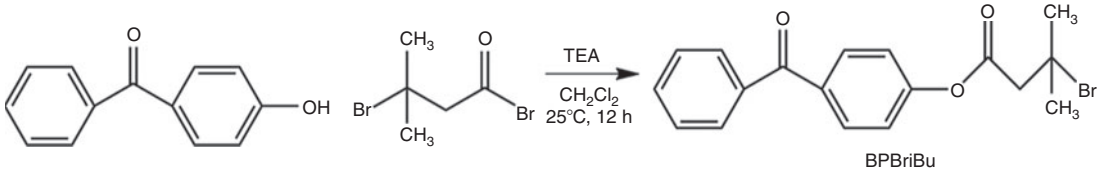
In the first step, the BPBriBu initiator was obtained according to Huang et al.[4] and synthesized with a yield of 90 % (Scheme 2). The structure of the final product was confirmed by ^{1}H NMR spectroscopy (Fig. 1) and the presence of BPBriBu on the PP surface was observed by ATR FT-IR spectroscopy (Fig. 2).

In the second step, ATRP of GMA was carried out in the presence of CuCl/hexamethyltriethylenetetramine (HMTETA) as a catalytic system. Three different films were prepared and characterized. Each film was labelled as PP-*g*-PGMA$^a{}_b$, where *a* symbolizes the BPBriBu concentration used, and *b* indicates the reaction time: PP-*g*-PGMA$^{0.1}{}_{24}$, PP-*g*-PGMA$^{10}{}_{24}$, and PP-*g*-PGMA$^{50}{}_{24}$. The presence of PGMA on the PP surface was confirmed by the gravimetric technique (where the percentage of PGMA grafted (%G), or grafting degree, on the PP surface was calculated based on the difference in mass of the film before and after grafting), water contact angle (WCA) measurements, ATR FT-IR spectroscopy, and microscopy. Table 1 shows the different modified surfaces obtained.

The different modified surfaces showed successful PGMA grafting at a higher reaction time (24 h). An increase in the degree of grafting (%G) was observed when the BPBriBu concentration was increased in reactions carried out during 24 h. Furthermore, the WCA values showed a slight decrease with an increase in the degree of PGMA grafting, which is consistent with the hydrophilic nature of the grafted polymer.

PP-*g*-PGMA$^{0.1}{}_{24}$, PP-*g*-PGMA$^{10}{}_{24}$, and PP-*g*-PGMA$^{50}{}_{24}$ samples were studied by ATR FT-IR spectroscopy (Fig. 3). The presence of PGMA grafting was confirmed by the following bands attributed to GMA functional groups: 1726 cm^{-1}, which corresponds to C=O stretching vibration; 1147 cm^{-1}, assigned to the C–O stretching vibration; and 1230 and 900 cm^{-1}, corresponding to the C–O–C (oxirane ring) symmetric and asymmetric vibrations, respectively.

In order to study the morphology of surface roughness, atomic force microscopy (AFM) images were recorded. AFM images obtained for the different surfaces are shown in Fig. 4, where high areas are shown in bright/white colour, while low areas are shown in dark colour. For unmodified PP a uniform surface was observed, with dark areas and a few white points which could be due to film impurities such as dust. In comparison, PP-*g*-PGMA surface images revealed non-uniform surfaces and the difference between bright/white (high) and dark (low) areas was noticeable, the bright/white areas were attributed to the zone where PGMA was grafted. In addition, the white areas presented as a striped pattern in the PP-*g*-PGMA images, which could be attributed to the immobilization of the initiator since it was performed using spin coating. Furthermore, an increase in the number of stripes was observed with increasing BPBriBu concentration, which is in agreement with the degree of grafting.



Scheme 2. Synthesis of BPBriBu (ATRP initiator).

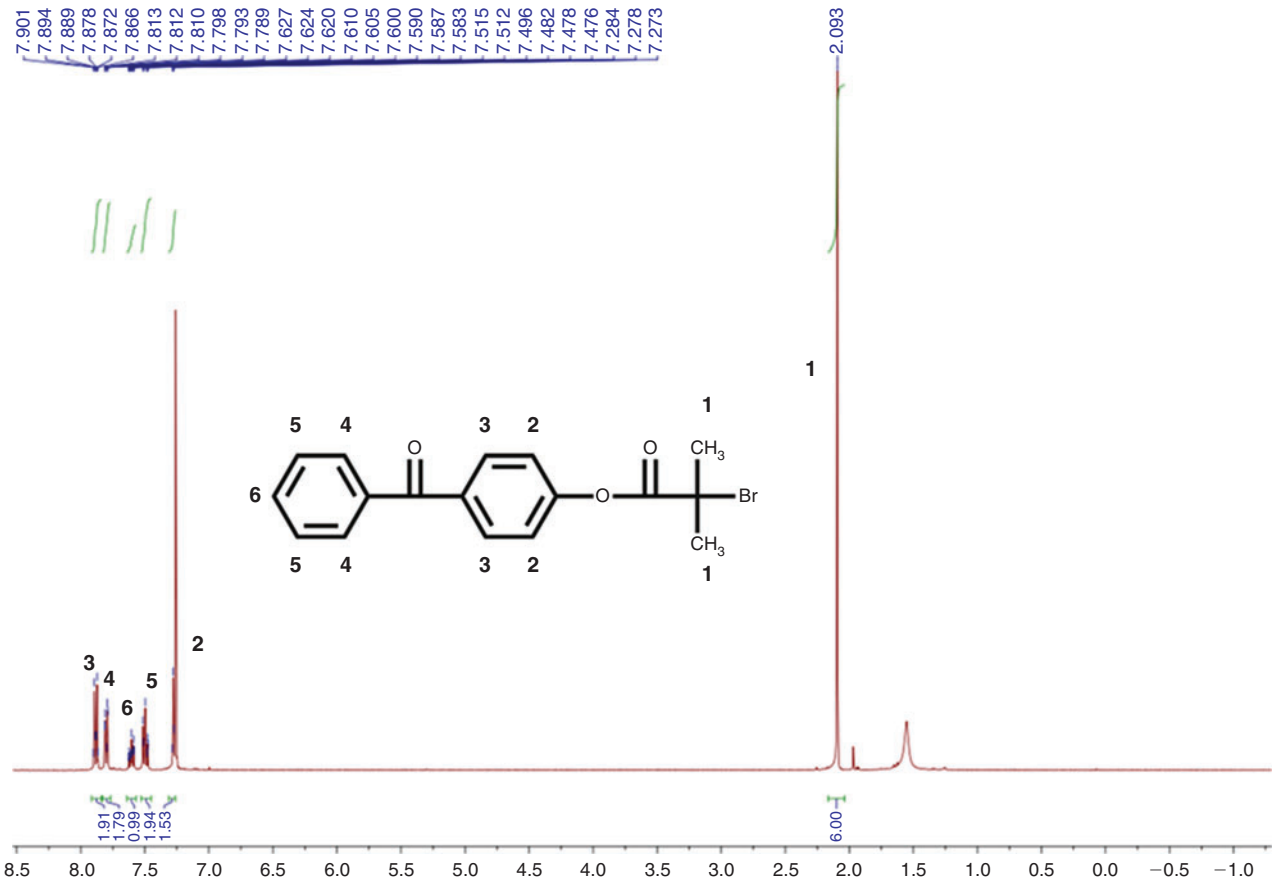


Fig. 1. ^{1}H NMR of BPBriBu (ATRP initiator).

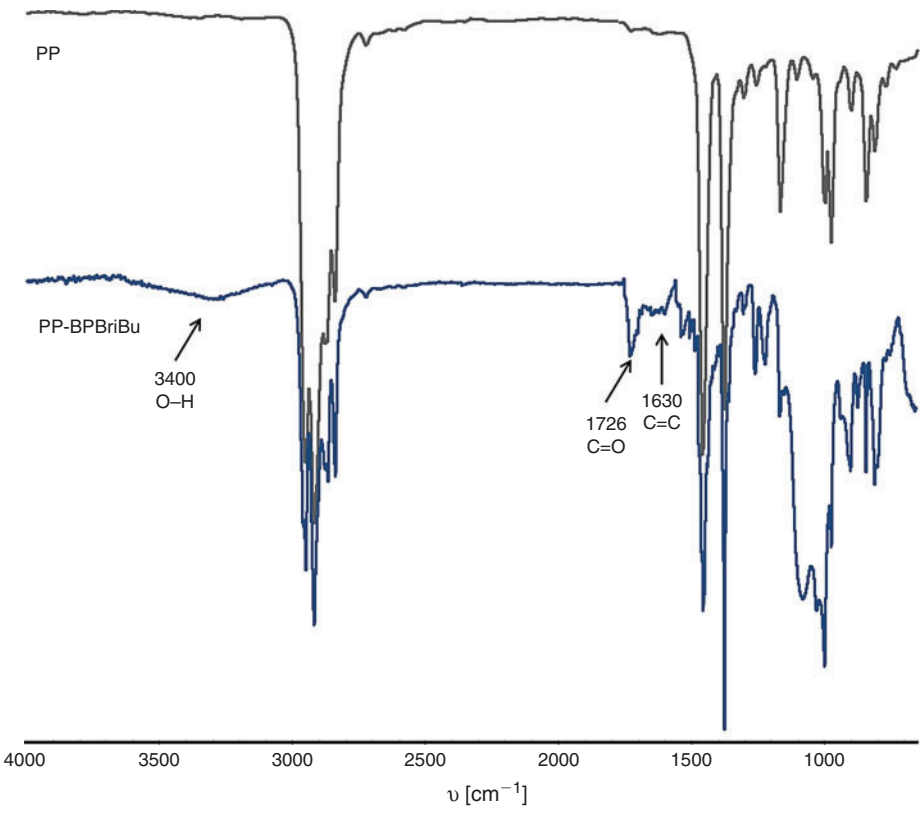


Fig. 2. ATR FT-IR spectra of the unmodified PP film and the PP-BPBriBu film.

Table 1. PP-*g*-PGMA$^{a}_{b}$ film chemistry characterization
s.d., standard deviation; $n = 5$

Surface	Conc. BPBriBu [mg mL^{-1}]	Reaction time [h]	Grafting degree (%G)	WCA (mean ± s.d.) [°]
Unmodified PP	—	—	—	107 ± 2
PP-*g*-PGMA$^{10}_{5}$	10	5	—	105 ± 3
PP-*g*-PGMA$^{10}_{10}$	10	10	—	106 ± 4
PP-*g*-PGMA$^{0.1}_{24}$	0.1	24	3.1	107 ± 4
PP-*g*-PGMA$^{10}_{24}$	10	24	7.5	102 ± 5
PP-*g*-PGMA$^{50}_{24}$	50	24	11.8	101 ± 3

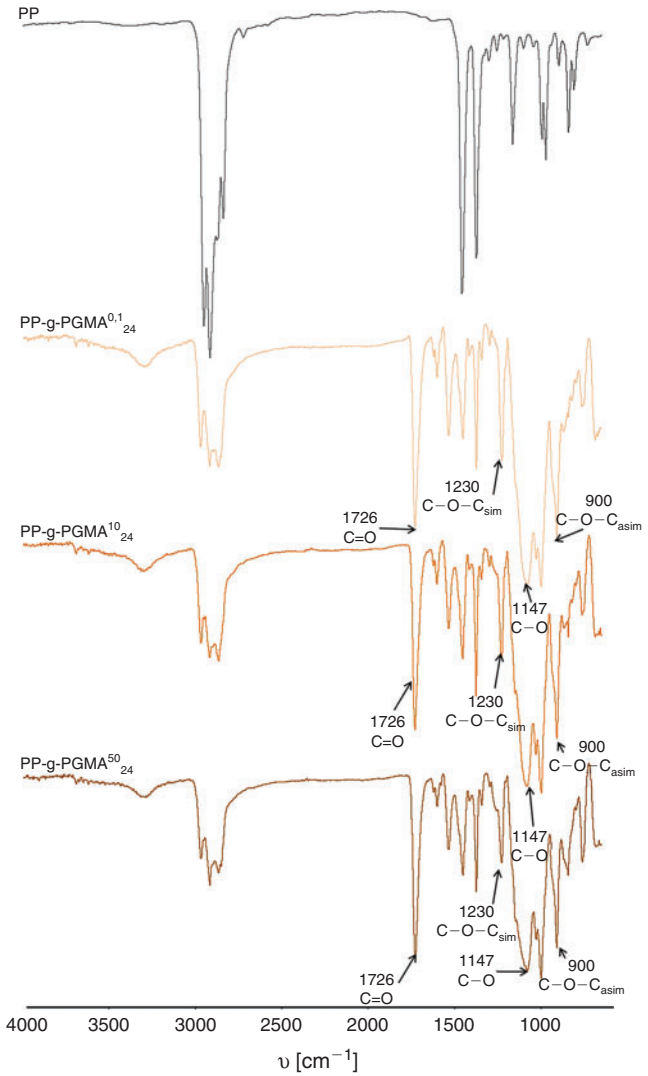


Fig. 3. ATR FT-IR spectra of the unmodified PP film and the PP-*g*-PGMA$^{a}_{b}$ films obtained by ATRP (PP-*g*-PGMA$^{0.1}_{24}$, PP-*g*-PGMA$^{10}_{24}$, and PP-*g*-PGMA$^{50}_{24}$).

The root mean square roughness (RMS) values for the different modified surfaces obtained from AFM images are shown in Table 2. The increase in RMS when surfaces are grafted, as opposed to unmodified PP (RMS 12.4 ± 0.1 nm), was clearly observed, the roughness increasing up to saturation with increasing initiator concentration. The growth of polymer chains with a smaller distance between grafting points produced a more homogeneous roughness.

It was observed that RMS roughness values are consistent with the degree of grafting and WCA values, showing that although a hydrophilic monomer is grafted, the WCA of the modified surfaces does not markedly decrease with respect to the unmodified PP film. This is due to the high roughness of these surfaces, since WCA values depend on the chemical structure of a surface and on its roughness.[32,33]

In summary, the above chemical and morphological characterization of surfaces indicates that GMA was successfully graft-polymerized onto PP via ATRP, with an increase in grafting degree and roughness as a result of an increase in BPBriBu concentration.

Immobilization of GOD on PP-g-PGMA

As we previously mentioned, PGMA is known as a grafting agent and has promising applications in advanced biotechnologies.[34–44] In this work, PGMA provides functional groups for covalent attachment of GOD and acts as a spacer arm between the surface and the biomolecule. It is worth mentioning that the ATRP method was chosen for GMA grafting since various reports, as cited above, show that the microstructure generated by this methodology is advantageous for retaining enzyme activity. It was previously demonstrated that the covalent binding of GOD often exhibits the highest stabilization of enzyme activities, because the active conformation of the immobilized enzyme is steadied.[45,46] The epoxide groups of the PGMA grafted from PP-*g*-PGMA were used for the direct coupling of GOD, with the concomitant formation of hydroxy groups (Scheme 3). The reaction involves the nucleophile attack of $-NH_2$ groups from GOD to epoxy groups on the PP-*g*-PGMA surface. Furthermore, the simultaneous presence of the surface spacer and the neighbouring hydroxy groups from the ring-opening coupling reaction means that GOD can assume a higher degree of conformational freedom in a more hydrophilic environment.[46]

GOD was immobilized under 0.1 M phosphate buffer (PBS) at pH 7.4, a pH found to produce better enzymatic activities than other pHs.[2]

In order to find out whether the enzymatic activity was retained, the film was exposed to a glucose solution. The reaction shown in Scheme 4 was used to recognise the appearance of H_2O_2 with the presence of blue colour by reaction with potassium iodine.[47–49]

The glucose solution that was exposed to a PP-*g*-PGMA$^{10}_{24}$-GOD film presents a blue coloration, which indicates that the covalently immobilized enzyme is active (Fig. 5). When glucose solution was exposed to unmodified PP, previously exposed to the GOD immobilization procedure, the blue coloration was not observed. The same result was observed 7 and 14 days after GOD immobilization, thus demonstrating the stability of the immobilized enzyme.

The amount of immobilized GOD did not depend on the degree of grafting, all samples immobilizing around 9.38 ± 0.06 mg cm^{-2} (Table 3). This concentration was higher than that reported in previous works.[46] Although increasing the degree of grafting makes more functional groups available for the binding of the enzyme, the large size of GOD prevents a higher concentration of the enzyme from being immobilized due to steric hindrance problems. Based on these results, the PP-*g*-PGMA$^{0.1}_{24}$-GOD film was selected for microbiological assays since as it is obtained with less initiator, it uses less reagents.

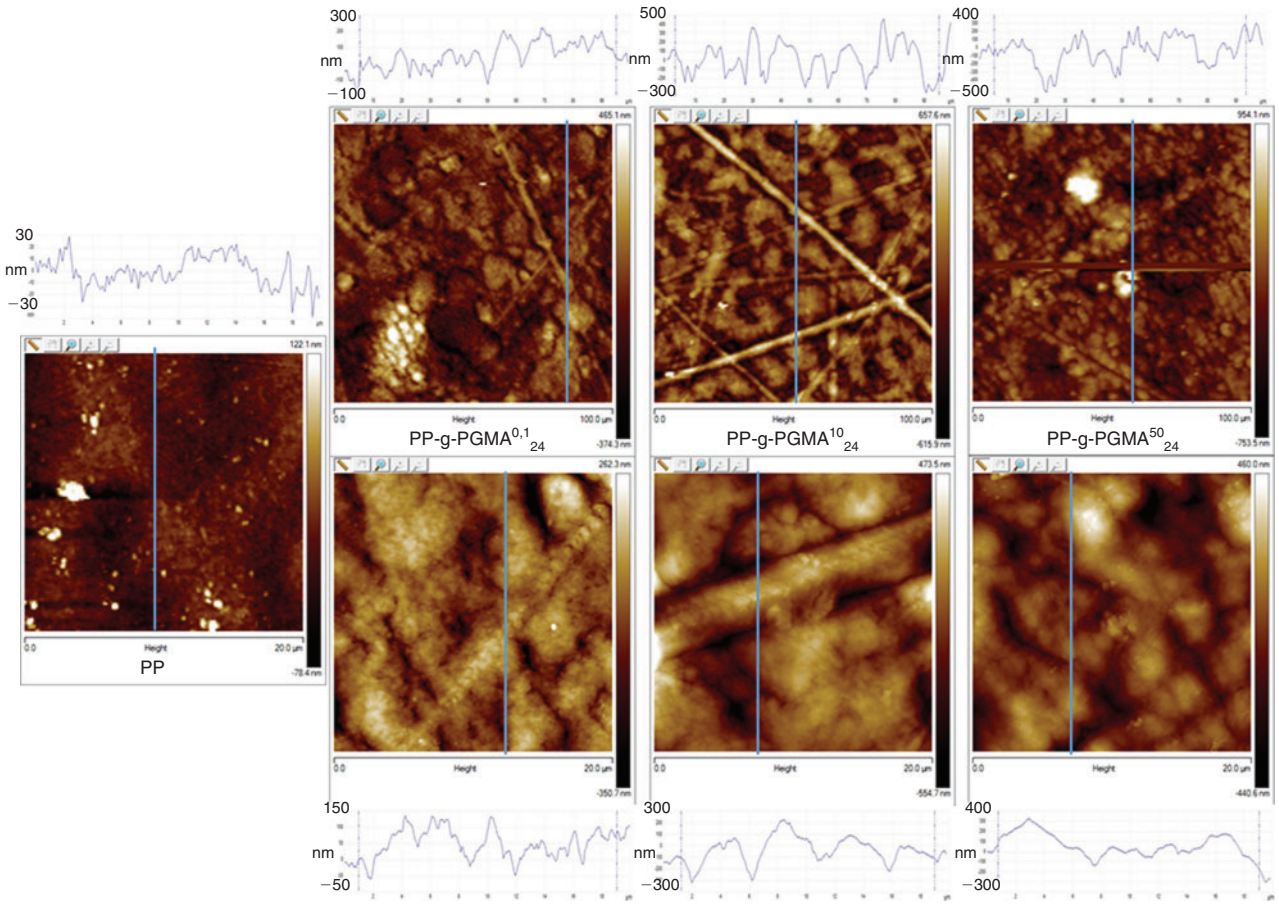
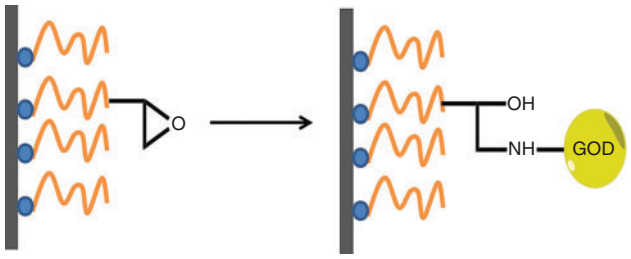


Fig. 4. Representative AFM images of unmodified PP and the surface-modified PP films with PGMA by ATRP (PP-*g*-PGMA$^{0.1}{}_{24}$, PP-*g*-PGMA$^{10}{}_{24}$, and PP-*g*-PGMA$^{50}{}_{24}$). Each image has a height histogram of the section indicated by the blue line. For each sample, 100 μm^2 images are presented in the first row; images in the second row correspond to a 20 μm^2 extension of the blue section above.

Table 2. RMS roughness values obtained from the AFM images
s.d., standard deviation; $n = 5$

Surface	RMS (mean ± s.d.) [nm]
Unmodified PP	12.4 ± 0.1
PP-*g*-PGMA$^{0.1}{}_{24}$	62.9 ± 0.6
PP-*g*-PGMA$^{10}{}_{24}$	97.6 ± 0.8
PP-*g*-PGMA$^{50}{}_{24}$	91.5 ± 0.8



Scheme 3. Immobilization of GOD on PP-*g*-PGMA films.

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{glucolactone} + H_2O_2$$

Scheme 4. GOD reaction.

Preliminary Microbiological Assay

The values for microbiological load obtained from control suspensions are shown in Table 4. As can be seen, both *S. aureus* and *E. coli* suspensions were stable throughout the test period. The microbial load of the *S. aureus* inoculum suspension remained around 794 ± 60 colony forming units (CFU) mL^{-1}, while *E. coli* remained around 416 ± 18 CFU mL^{-1} within 6 h of performing the test.

The microbial load of the *S. aureus* inoculum suspension exposed to PP and PP-*g*-PGMA films remained at 729 ± 31 and 771 ± 57 CFU mL^{-1}, respectively, while the load of the *E. coli* inoculum suspension exposed to PP and PP-*g*-PGMA films remained at 412 ± 26 and 411 ± 22 CFU mL^{-1}, respectively. In brief, growth inhibition by unmodified PP and PP-*g*-PGMA films was not observed, at least during the experimental time used in the test.

Microbiological assays were performed using the PP-*g*-PGMA$^{0.1}{}_{24}$-GOD film in the selected procedure in order to avoid bacterial growth inhibition by physical impediment caused by the film on an agar surface. The selected bacterial strains, *S. aureus* and *E. coli*, commonly transmitted in food manipulation and found on food surfaces, are indicators of sanitation in food manipulation.[50]

The results of the preliminary microbiological assays on the PP-*g*-PGMA$^{0.1}{}_{24}$-GOD film are shown in Table 4. Activities on films immobilized with GOD were in good accordance with the

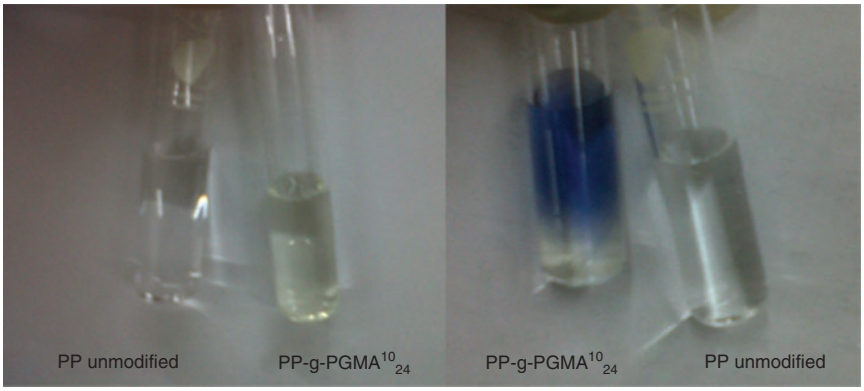


Fig. 5. The photographs represent the solution of the redox reaction after unmodified PP and PP-*g*-PGMA$^{10}_{24}$-GOD were exposed to a glucose solution. The photographs on the right were captured without starch and the ones on the left in the presence of starch.

Table 3. Quantification of immobilized GOD
s.d., standard deviation; $n = 3$

Surface	GOD (mean ± s.d.) [mg cm^{-2}]
PP-*g*-PGMA$^{0.1}_{24}$	9.32 ± 0.02
PP-*g*-PGMA$^{10}_{24}$	9.40 ± 0.02
PP-*g*-PGMA$^{50}_{24}$	9.43 ± 0.02

above-mentioned enzymatic activities, in both cases the PP-*g*-PGMA$^{0.1}_{24}$-GOD film produced an inhibitory effect. In the case of *S. aureus* the PP-*g*-PGMA$^{0.1}_{24}$-GOD film yielded an inhibitory effect of around 54.6 %, 6 h after the test was performed. On the other hand, the PP-*g*-PGMA$^{0.1}_{24}$-GOD film showed an inhibitory effect of around 46.3 % in *E. coli*, 6 h after the test was performed. Although the reduction in logarithmic units was not noticeable, the results were promising because they showed a growth inhibitory effect of the material within a short time after performing the assay (6 h). As seen in the previous section, the GOD-immobilized enzyme exhibits enzymatic activity for 14 days, and the growth inhibitory effect is expected to be prolonged with time. The very mild antimicrobial property of GOD is considered to be mainly due to the production of hydrogen peroxide.

In conclusion, these preliminary studies on the very mild antimicrobial property of GOD-modified PP showed that the synthetic methodology used for chemical surface modification was effective. These results suggest that PP-*g*-PGMA$^{0.1}_{24}$-GOD films may have potential for use in various active packaging film applications.

Therefore, the results demonstrate the importance of an adequate design and optimized methodology for surface modification of PP films, so as to have the functional groups necessary for linking GOD (active agent), as well as a surface microstructure capable of promoting enzymatic activity. The methodology optimized in this work demonstrates that a low degree of grafting of brush polymeric chains is sufficient for the covalent immobilization of the active compounds.

Conclusion

Surface chemical modification of PP films was successfully obtained by grafting, using ATRP, thus helping to understand how the concentration of initiator affects the process of chemical modification and surface morphology. Three different films were prepared and characterised: PP-*g*-PGMA$^{0.1}_{24}$, PP-*g*-PGMA$^{10}_{24}$, and PP-*g*-PGMA$^{50}_{24}$. Afterward, the PGMA chain with the hydroxy groups from the ring-opening coupling reaction with GOD serves as an effective spacer to provide the immobilized GOD with a higher degree of conformational freedom and a more hydrophilic environment. Therefore, a relatively high concentration of GOD was immobilized (9.38 ± 0.06 mg cm^{-2}). This concentration was higher than that reported in previous works.[46] The stability and enzymatic activity of GOD was maintained for 14 days.

The material developed has two main advantages. First, the modified surface is permanent with time and does not change the PP characteristics such as transparency and mechanical properties. Second, covalent binding prevents migration of the active agent to the food (non-migratory active packaging). Such packaging is likely to be acceptable to consumers since there would be no organoleptic properties that might alter the packaged food. Further studies with food specialists will be conducted to assess the possibility of promoting the technological development of these types of active packaging.

Materials and Methods

Materials

Biaxially oriented, 60 μm thick PP films, with a corona treatment on one side (42 dyn cm^{-2}) were supplied by Vitopel SA. GMA, 4-hydroxybenzophenone, 2-bromoisobutyryl bromide, HMTETA, GOD, and glucose were obtained from Sigma–Aldrich. Dichloromethane was purchased from Anedra. Brain-heart infusion agar (BHI, Britania), *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were obtained from the collection of microbiological cultures of the Center of Applied Chemistry (CEQUIMAP-UNC).

Surface Chemical Functionalization of PP Films with GMA via ATRP

The synthesis was divided into two stages: the first stage involved the synthesis and immobilization of the primer on the PP surface; and the second, the grafting of GMA via ATRP.

Synthesis and Immobilization of the Initiator

Synthesis. The initiator (BPBriBu) was obtained by reacting 2-bromoisobutyryl bromide with 4-hydroxybenzophenone in the presence of triethylamine. 4-Hydroxybenzophone (0.301 g), triethylamine (0.209 mL), and 1 mL of CH_2Cl_2 were added into a 25 mL round bottom flask. 2-Bromoisobutyryl

Table 4. Preliminary microbiological essay

Evaluation of *S. aureus* and *E. coli* inocula stability for 6 h and exposed to PP, PP-*g*-PGMA, and PP-*g*-PGMA$^{0.1}{}_{24}$-GOD. Average values CFU mL^{-1} were determined using the pour plate technique in brain-heart infusion agar. s.d., standard deviation; $n = 2$

Time [h]	*S. aureus* (mean ± s.d.) [CFU mL^{-1}]				*E. coli* (mean ± s.d.) [CFU mL^{-1}]			
	Control	PP	PP-*g*-PGMA	PP-*g*-PGMA$^{0.1}{}_{24}$-GOD	Control	PP	PP-*g*-PGMA	PP-*g*-PGMA$^{0.1}{}_{24}$-GOD
0	840 ± 10	751 ± 42	766 ± 51	108 ± 4	409 ± 10	440 ± 31	400 ± 30	441 ± 9
1	870 ± 20	710 ± 30	801 ± 32	115 ± 5	421 ± 20	400 ± 19	420 ± 15	435 ± 10
2	710 ± 31	745 ± 31	840 ± 55	111 ± 5	430 ± 25	420 ± 19	440 ± 30	419 ± 11
4	770 ± 12	732 ± 20	748 ± 30	54 ± 6	410 ± 19	399 ± 30	400 ± 20	350 ± 10
6	781 ± 30	705 ± 32	699 ± 88	49 ± 1	410 ± 17	400 ± 29	423 ± 15	225 ± 5

bromide (0.187 mL) in 1 mL of CH_2Cl_2 was slowly added to the flask at 0°C. After the addition of 2-bromoisbutyryl bromide, the reaction mixture was kept at 0°C for 1 h before increasing the temperature to 25°C. The white precipitate was filtered out after the reaction had proceeded overnight. The obtained solution was washed with saturated bicarbonate solution three times before drying with anhydrous magnesium sulfate overnight. The final product was obtained as a white powder after filtration and evaporation of the solvent.

Immobilization. Initiator immobilization on PP surfaces was achieved in two sequential steps: (1) deposition of BPBriBu on the PP surface via spin coating from a solution in toluene at a speed of 2000 rpm; and (2) UV irradiation (λ 365 nm, 2 min) of the initiator-deposited surface. A PP film with a circular diameter of 5 cm was used. The film was washed in a Soxhlet extractor with CH_2Cl_2 to remove the physically attached BPBriBu. Three concentrations of BPBriBu were studied: 0.10, 10, and 50 mg mL^{-1}.

Preparation of PP-g-PGMA

Subsequently, grafting of GMA was performed. For this, the film with immobilized initiator, 15 mL (113.4 mmol) of GMA, and 20 µL (0.07 µmol) of HMTETA were placed in a reactor. Three cycles of freeze–vacuum were performed in order to degas the system. Afterwards, 6 mg (0.06 mmol) of CuCl were added under a nitrogen stream. The reaction was conducted with stirring at room temperature and under nitrogen atmosphere. The film was washed in a Soxhlet extractor using CH_2Cl_2, dried, and weighed. Reaction times of 5, 10, and 24 h were studied.

Characterization of Modified Surfaces

The percentage of PGMA grafted (%G), or grafting degree, on the PP surface was calculated based on the difference in mass of the film before and after grafting, according to Eqn 1.

$$\%G = \frac{M_f - M_i}{M_i} \times 100 \quad (1)$$

where M_i represents the film mass before grafting, and M_f indicates the film mass after GMA grafting.

ATR FT-IR spectroscopy was performed on a Nicolet Avatar 360 FT-IR spectrometer. A 45 ZnSe crystal (1.67 µm beam penetration) was used to monitor samples. All spectra were obtained as an average of 32 scans, at a 4.0 cm^{-1} resolution in a range between 4000 and 650 cm^{-1}. Infrared data were processed using the *Omnic 8.0* software.

Static WCAs were measured at ambient temperature by gently depositing a drop of deionized water (4 µL) on the substrate using a micro syringe. Images were captured with a digital video camera using suitable software in specially designed equipment, and then analyzed for contact angle measurements using the *Image j* software. All measurements were repeated at least five times at different positions for each sample.

The surface morphologies of the films were mainly observed by AFM. AFM experiments were conducted at the Centro de Metrología, CEMETRO, Universidad Tecnológica Nacional, Regional Córdoba. AFM images of modified PGMA surfaces were obtained using an Innova (Bruker) apparatus and the images were recorded in intermittent ('tapping') mode with commercial silicon nitride tips of 8 nm in diameter. AFM data were processed using the *NanoScopeAnalysis version 1.4* software.

Immobilization of GOD on PP-g-PGMA

Immobilization of GOD

A 1 cm^2 PP-*g*-PGMA film was used. The film was placed in a 3 mL of 0.1 M PBS (pH 7.4) containing GOD at a concentration of 4 mg mL^{-1}. Immobilization was brought to room temperature under constant stirring during 24 h. Finally, the surface was washed with PBS for 24 h at room temperature, the PBS was renewed every 8 h, the washing was performed until the concentration of extracted compounds was zero in the washings as determined by UV-vis analysis. Samples were stored in PBS at room temperature. The same procedure was performed with an unmodified PP film.

Determination of Immobilized GOD

D-(+)-Glucose solution (0.1 M in PBS) was used as the test medium. The PP-*g*-PGMA-GOD film was placed in 3 mL of the test medium and stirred at 37°C for 4 h. Subsequently, the presence of H_2O_2 was studied by a redox reaction. Ten drops of 0.1 M potassium iodide solution and 5 drops of 1 M sulfuric acid were placed in a Kanh tube to which the test medium was added drop by drop followed by 3 drops of starch. The same procedure was performed with an unmodified PP film previously exposed to the GOD immobilization procedure. This process was carried out after GOD immobilization, and repeated after 7 and 14 days.

GOD UV spectra were recorded using a Shimadzu MultiSpect 1800 240 v spectrometer, between 200 and 900 nm at 25°C. Subsequently, a calibration curve was performed to determine the molar absorption coefficient (ε) of GOD. A maximum GOD absorption at 451.5 nm, and a molar absorption coefficient (ε 0.1206 mg mL^{-1}) at said wavelength were determined. The amount of GOD immobilized was quantified spectrophotometrically using the corresponding calibration curve.

Preliminary Microbiological Assay of PP-g-PGMA-GOD

The preliminary microbiological assay of the PP-*g*-PGMA-GOD film was studied against a Gram-negative bacterium

(*E. coli* ATCC 25922) and a Gram-positive one (*S. aureus* ATCC 25923). Working cultures were obtained from stock cultures of both bacteria, stored at −80°C in brain-heart broth with 20 % glycerol, placed in tubes with brain-heart agar, and incubated at 36 ± 1 °C for 36 h, in order to obtain fresh cultures during the exponential growth phase of both microorganisms.

Inocula of both bacteria were prepared by suspension in dilute phosphate buffer (pH 6.8–7.2) under aseptic conditions. From the mother suspension, a 10-fold dilution was prepared to evaluate the microbiological activity of films against inocula with a lower microbial load of 500–800 CFU mL^{-1}. Subsequently, 1 cm^2 sections of the films were immersed in tubes containing 10 mL of microorganism suspensions. Changes in microbial loads were evaluated every 2 h from the initial contact time (0) and for a period of 6 h (0, 2, 4, and 6). The bacterial population at each control point was determined in duplicate, using the pour plate technique in brain–heart infusion agar. Counts were performed after 48 h of incubation at 36 ± 1°C.

In order to determine whether the microorganism suspension in the phosphate buffer dilution was stable during the test period, the bacterial load of the same microorganism suspension used in each case without having contact with the films was tested at equivalent time intervals and compared with suspensions in contact with PP-*g*-PGMA-GOD film, as a suspension control. In addition, the same procedure was undertaken with unmodified PP and PP-*g*-PGMA films.

Conflicts of Interest

The authors declare no conflicts of interest.

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