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ARTICLE

2-Methoxy Aniline Grafted Poly(maleic anhydride-*alt*-butyl vinyl ether) Hemiester: A New Biocompatible Polymeric Free Radical Scavenger

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ABSTRACT: Free radical scavenger biocompatible polymers are being used increasingly in biomedical applications due to their antioxidant activity. In this study, the biocompatible polymer poly(maleic anhydride-*alt*-butyl vinyl ether) 5% grafted with monomethoxy poly(ethylene glycol) 2000 MW (PEG) and 95% grafted with 2-methoxyethanol (VAM41-PEG) was modified by inserting a conductive moiety represented by 2-methoxy aniline (mAn) in order to attribute to the polymer radical scavenger properties. Different reaction schemes were tested in order to maximize the reaction yield. The increase in the scavenger activity of the modified polymer was demonstrated by means of the DPPH$^{\bullet}$ radical assay. Cytotoxicity tests, performed on the mAn modified polymeric matrix, were carried out using the mouse embryo fibroblast cell line balb/3T3 Clone A31. Results have shown that the introduction of mAn into VAM41-PEG polymeric structure does not alter its cytocompatibility.

Introduction

Nontertiary aromatic amines and phenols are highly effective radical-trapping antioxidants which retard the rates of oxidative processes.[1]

In recent years aromatic amines have been used for various applications, and their relevant polymers have been synthesized and studied thanks to their conductive properties.[2]

Aromatic amine polymers such as polyaniline derivatives (PANI), due to their conductivity, environmental stability and fairly inexpensive production process, are some of the most extensively studied conductive polymers for electrochemical applications.[3−5] They are commonly employed for the production of batteries, supercapacitors, electrochromic devices, conductive supports of fuel cell electrodes, wiring of biosensors, desalination systems, drug release devices, ion sensitive electrodes, and electrochemomechanical actuators.[6]

A range of biomedical applications for conducting polymers are currently being considered. These include polypyrrole actuators in which the forces, created through doping and undoping the conducting polymers, are used to produce movements and create artificial muscles.[7] Other potential areas of biomedical engineering include the controlled release of drugs, using a change in conducting polymer redox state to increase the permeation of the drug.[8] The employment of aromatic amines in producing biomedical devices can also be promising in terms of minimizing oxidative processes which often lead to inactivation or to degradation phenomena. Although it is well-known that polyanilines oxidize at potentials much lower than their respective monomers (aniline displays an oxidation potential quoted at 1.07 V while polyaniline value is estimated to be around 0.41 V[9]), anilines were shown to be efficient also in slowing down oxidation processes, particularly when a methoxy-substituted aniline is used.[10] Poly(maleic anhydride-*alt*-butyl vinyl ether) 5% grafted with PEG and 95% grafted with 2-methoxyethanol (VAM41-PEG) polymer has been widely used in the formulation of bioerodible polymeric nanostructured systems for the controlled release of high and low molecular weight active agents;[11,12] although a low scavenger activity was previously observed for VAM41-PEG, due to the presence of residual electron accepting maleic anhydride groups,[13] the introduction of 2-methoxy aniline (mAn) moieties into VAM41-PEG polymeric backbone has been investigated with the aim of additional scavenging features. Scavenging of free radicals is in fact a property that is widely considered as beneficial for compounds designed to get in contact with biological tissues and for active moieties or materials sensitive to radical oxidation up on storage.

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Experimental Section

Materials. 2-Methoxyaniline (mAn), monomethoxy poly(ethylene glycol) MW 2000 (PEG), *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysulfosuccinimide (Sulfo-NHS), *N*-hydroxysuccinimide (NHS), 2-(*N*-morpholino)-ethanesulfonic acid (MES), 4-(dimethylamino)pyridine (DMAP), and α,α-diphenyl-β-picrylhydraziyl radical (DPPH) were purchased by Sigma-Aldrich. 2-Methoxyethanol (Sigma-Aldrich) was refluxed for 4 h over sodium under dry nitrogen atmosphere and then distilled, collecting the fraction having boiling point of 124 °C. Amberlyst 15 Styrene−divinyl benzenesulfonic resin (4.6 mequiv/g exchange capacity) was purchased by Fluka. Poly [maleic anhydride-*alt*-butyl vinyl ether] (VAM40) was kindly provided by Polymer Laboratories Ltd., Church Stretton, UK. *O*-Glycidyl-*O*-isopropylidenglycerol grafted β−cyclodextrin (GIG−βCD) and poly [maleic anhydride-*alt*-butyl vinyl ether] 5% grafted with PEG and 95% grafted with 2-methoxyethanol (VAM41-PEG) were synthesized at Biolab (University of Pisa, Department of Chemistry and Industrial Chemistry) as previously reported.[11] VAM40 and VAM41-PEG structures are shown in Scheme 1.

Solvents were obtained from Carlo Erba. Tetrahydrofuran (THF) was refluxed for 4 h over calcium hydride under a dry

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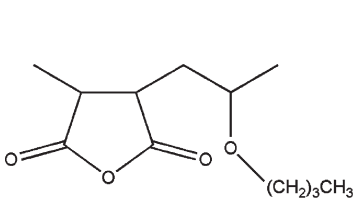
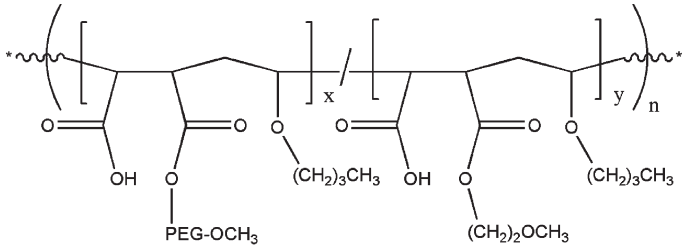
Scheme 1. VAM40 and VAM41-PEG Chemical Structures



VAM40



VAM41-PEG

nitrogen atmosphere and then distilled collecting the fraction having boiling point of 66 °C. Cell line BALB/3T3 Clone A31 mouse embryo fibroblasts (CCL163) was obtained from American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM), 0.01 M pH 7.4 phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS), calf serum, trypsine/EDTA, glutamine, and antibiotics (penicillin/streptomycin) were purchased from GIBCO Brl. PhalloidinAlexa488 and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (NewYork, NY). Dialysis tubes in cellulose esters (MWCO 3500 da) were purchased by Spectrum Lab. The commercial products were used without any preliminary purification if not otherwise stated

VAM41-PEG-mAn_1 Synthesis. A 200 mg sample of VAM41-PEG (corresponding to 0.33 mmol of repeating units) was dissolved in 8 mL of a 0.1 M MES and 0.5 M NaCl aqueous solution. After polymer dissolution, 210 mg of EDC (corresponding to 1.11 mmol) and 240 mg of NHS (corresponding to1.11 mmol) were added to the solution under magnetic stirring, at 25 °C for 60 min; 33 mg of mAn (corresponding to 0.27 mmol) were added to the solution and kept in the dark for 20 h under magnetic stirring. After the reaction took place, NaOH 10 N was added to the solution up to pH 8 in order to remove NHS esters that have not reacted with mAn. The resulting solution was then put in a dialysis tube (MWCO 3500 Da) to remove EDC, NHS and MES salts and then dried under high vacuum until constant weight. The obtained polymer was finally characterized by means of FT-IR and ^{1}H NMR.

FT-IR (film): 1735 (ν C═O), 1715 (ν C═O) and 1095 (ν C−O−C), 1600 (ν C═O), 1526 (ν N−H), 750 (ν =C−H) cm^{-1} .^{1}H NMR (acetone-d_6): δ= 12.2 (xH; COOH), 4.2−3.8 (2xH; CH_2OCO), 3.8−3.4 ((3 + 2x)H; $CHOCH_2$ + CH_2OCH_3), 3.4−3.2 (3xH; CH_3O), 3.2−2.4 (2H; OCCHCHCO), 2.4−1.6 (2H; CH_2CH), 1.6−1.0 (4H; $CH_2CH_2CH_3$) and 1.0−0.6 ppm (3H; CH_2CH_3), 7.5−7.2 (4H; $NHCCOCH_3CH_4$); x = 1

VAM41-PEG-mAn_2a Synthesis. A 186 mg sample of VAM41-PEG (corresponding to 0.31 mmol of repeating units) was dissolved in 15 mL of THF under nitrogen atmosphere; after the polymer dissolution, 57 mg of DCC (corresponding to 0.27 mmol) and 32 mg of mAn (corresponding to 0.26 mmol) were added to the solution and maintained in the dark under magnetic stirring overnight. Formed *N*,*N*′-dicyclohexylurea was removed by sedimentation. The solution was then added to a mixture of 50:50 vol/vol of diethyl ether and petroleum ether (volume ratio 1/10), under vigorous stirring. The obtained pink precipitate was dried under high vacuum until constant weight and characterized by means of FT-IR and ^{1}H NMR.

FT-IR (film): 1735 (ν C═O), 1715 (ν C═O) and 1095 (ν C−O−C), 1600 (ν C═O), 1526 (ν N−H), 750 (ν =C−H) cm^{-1} . ^{1}H NMR (acetone-d_6): δ = 12.2 (xH; COOH), 4.2−3.8 (2xH; CH_2OCO), 3.8−3.4 ((3 + 2x)H; $CHOCH_2$ + CH_2OCH_3), 3.4−3.2 (3xH; CH_3O), 3.2−2.4 (2H; OCCHCHCO), 2.4−1.6 (2H; CH_2CH), 1.6−1.0 (4H; $CH_2CH_2CH_3$), and 1.0−0.6 ppm (3H; CH_2CH_3), 7.5−7.2 (4H; $NHCCOCH_3CH_4$); x = 1.

VAM41-PEG-mAn_2b Synthesis. A 500 mg sample of VAM41-PEG (corresponding to 0.83 mmol of repeating units) was dissolved in 40 mL of THF under nitrogen atmosphere; after the polymer dissolution 1 g of DCC (corresponding to 4.81 mmol) and 564 mg of mAn (corresponding to 4.62 mmol) were added to the solution and maintained in the dark under magnetic stirring overnight. Formed *N*,*N*′-dicyclohexylurea was removed by sedimentation. The solution was then added to a mixture of 50:50 vol/vol of diethyl ether and petroleum ether (volume ratio 1/10), under vigorous stirring. The obtained pink precipitate was dried under high vacuum until constant weight and characterized by means of FT-IR and ^{1}H NMR.

FT-IR (film): 1735 (ν C═O), 1715 (ν C═O) and 1095 (ν C−O−C), 1600 (ν C═O), 1526 (ν N−H), 750 (ν =C−H) cm^{-1} . ^{1}H NMR (acetone-d_6): δ= 12.2 (xH; COOH), 4.2−3.8 (2xH; CH_2OCO), 3.8−3.4 ((3 + 2x)H; $CHOCH_2$ + CH_2OCH_3), 3.4−3.2 (3xH; CH_3O), 3.2−2.4 (2H; OCCHCHCO), 2.4−1.6 (2H; CH_2CH), 1.6−1.0 (4H; $CH_2CH_2CH_3$) and 1.0−0.6 ppm (3H; CH_2CH_3), 7.5−7.2 (4H; $NHCCOCH_3CH_4$) ; x = 1

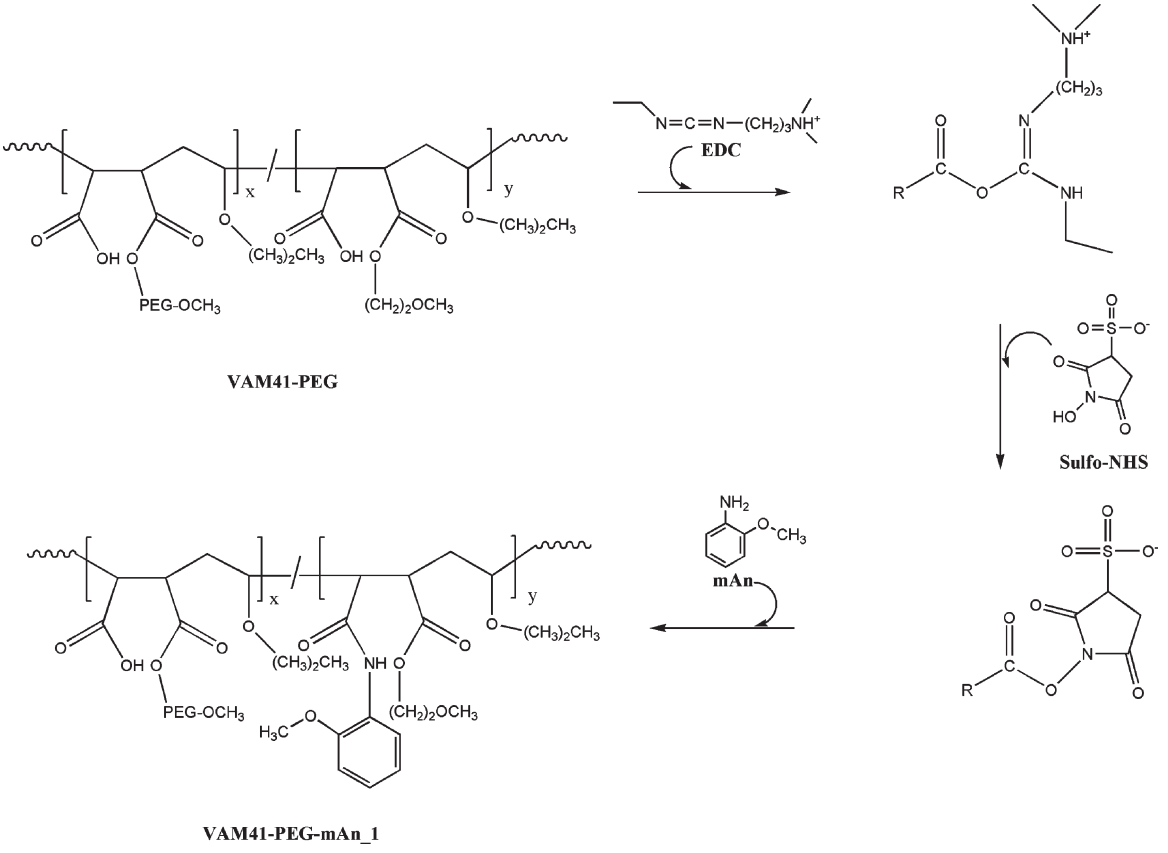
VAM41-PEG-mAn_3 Synthesis. A 500 mg sample of VAM40 (corresponding to 0.83 mmol of repeating units) was dissolved in 25 mL of THF under nitrogen atmosphere; 88 mg of mAn (corresponding to 0.71 mmol) were added to the polymeric solution and kept in the dark for 6 h under magnetic stirring. 0.25 g of PEG (corresponding to 0.12 mmol) and 14.5 mg of DMAP (corresponding to 0.12 mmol) were added to the polymeric solution and maintained at 65 °C for 24 h under magnetic stirring.

A 1.93 g sample of 2-methoxyethanol (corresponding to 25.41 mmol) was added to the polymeric solution and then maintained at 75 °C under magnetic stirring until the IR anhydride−CO-band disappeared (24 h). The solution was then cooled at room temperature and added to a mixture of 50:50 vol/vol of diethyl ether and petroleum ether (volume ratio 1/10), under vigorous stirring. The obtained pink precipitate was dried under high vacuum until constant weight. The complete removal of residual dimethylaminopyridine was achieved by further purification of the polymer by means of ion exchange chromatography. The polymer was dissolved in EtOH/H_2O 80:20 and purified by using Amberlyst 15 as exchange resin. The resin was first conditioned by gradually changing EtOH/H_2O gradient (H_2O, EtOH/H_2O 20:80, EtOH/H_2O 50:50, and EtOH/H_2O 80:20) then the polymer solution was added. The solution collected at the end of the column was dried under high vacuum until constant weight and the structure of the obtained polymer was assessed by FT-IR and ^{1}H NMR.

FT-IR (film): 1735 (ν C═O), 1715 (ν C═O) and 1095 (ν C−O−C), 1600 (ν C═O), 1526 (ν N−H), 750 (ν =C−H) cm^{-1}. ^{1}H NMR (acetone-d_6): δ= 12.2 (xH; COOH), 4.2−3.8 (2xH; CH_2OCO), 3.8−3.4 ((3 + 2x)H; $CHOCH_2$ + CH_2OCH_3), 3.4−3.2 (3xH; CH_3O), 3.2−2.4 (2H; OCCHCHCO), 2.4−1.6 (2H; CH_2CH), 1.6−1.0 (4H; $CH_2CH_2CH_3$) and 1.0−0.6 ppm (3H; CH_2CH_3), 7.5−7.2 (4H; $NHCCOCH_3CH_4$) ; x = 1

Functionalization Degree and Functionalization Efficiency. VAM41-PEG functionalization degree (F.D.) with mAn was calculated on ^{1}H NMR characteristic peaks at 7.5−7.2 ppm and

Scheme 2. VAM41-PEG-mAn_1 Reaction



at 1.0−0.6 ppm of mAn and polymer repeating unit, respectively. Functionalization efficiency (F.E.) was estimated as the ratio of the observed functionalization degree and the theoretical functionalization degree.

DPPH Assay. A 100 μL sample of an ethanolic solution of each test compound was added to 1.5 mL of 72 μM DPPH free radical in ethanol, vortexed, and kept in the dark for 30 min. The absorbance of the reaction mixture was then measured at 516 nm in order to determine the scavenger activity of the tested compound. The experiments were then repeated at different concentrations in order to calculate the amount required to scavenge 50% of DPPH radicals (IC_{50}). Infrared spectra of the reaction mixture were then recorded both after 30 min and at the steady state.

Cytotoxicity Tests. To investigate VAM41-PEG-mAn_3 cytotoxicity, cells were seeded onto glass coverslips in a 12 wells cell culture plate at an appropriate density (10^4 cells/mL) and incubated at 37 °C, 5% CO_2 for 24 h . When 60−70% cell confluence was reached, polymers samples were dissolved in cells growth medium at different concentrations (1, 2.5, 5, 10 mg/mL) and then added to cell cultures. Plates were then incubated at 37 °C, 5% CO_2 for 24 h and then fixed with 3.8% paraformaldehyde solution in PBS 0.01 M pH 7.4 for 1 h; cells permeabilization was performed by means of a 15 min incubation in Triton X-100 to enhance dyes binding to cellular structure. Triton X-100 samples were incubated with a PBS 0.01 M solution of DAPI and phalloidin-Alexa488 for 45 min at room temperature. After dyeing incubation, samples were washed with PBS and mounted on a glass slide for microscopic observation. A Nikon Eclipse TE2000 inverted confocal laser scanning microscope equipped with 405 nm laser diode (405 nm emission) and argon ion laser (488 nm emission) was used. Images were captured with Nikon EZ-C1 software with identical settings for each sample. Images were further processed with GIMP (GNU Free Software Foundation) image manipulation software and merged with Nikon ACT-2U software.

Instrumentation. IR spectra were recorded on KBr pellets by using Jasco FT−IR 410 spectrophotometer. NMR spectra were recorded on Varian Gemini 200 spectrometer using a Sparc 4 (Sun) console and VNMR 6.1B software. Spectra were processed by using MacFID 1D 5.3 (Tecmag Inc.) software. NMR spectra were recorded on 5−10% (w/v) solutions, in deuterated solvents, at 25 °C, with tetramethylsilane (TMS) as internal standard. ^{1}H−NMR spectra were recorded at 200 MHz, using the following spectral conditions: 3 kHz spectral width, 30° impulse, 2s acquisition time, at least 16 transients.

UV−vis absorption spectra measurements were performed by using Jasco V-530 spectrophotometer.

Results and Discussion

VAM41-PEG-mAn_1 Reaction. VAM41-PEG was functionalized with mAn using 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) as activator moieties. The reaction took place in aqueous environment using MES buffer. The EDC/NHS system is generally used in different applications such as forming amide bonds in peptide synthesis,[14,15] attaching haptens to carrier proteins to form immunogens,[16] labeling nucleic acids through 5′ phosphate groups[17] and creating amine-reactive NHS-esters.

In the reaction scheme (Scheme 2) EDC reacts with the polymer carboxylic moieties to form an amine-reactive *O*-acylisourea intermediate.

If this intermediate does not encounter an amine, it will hydrolyze and regenerate the carboxyl group; in the presence of Sulfo-NHS, the reactive Sulfo-NHS esters is formed, which is considered a good leaving group for mAn.[18] After the amidic bond formation between mAn and VAM41-PEG carboxylic

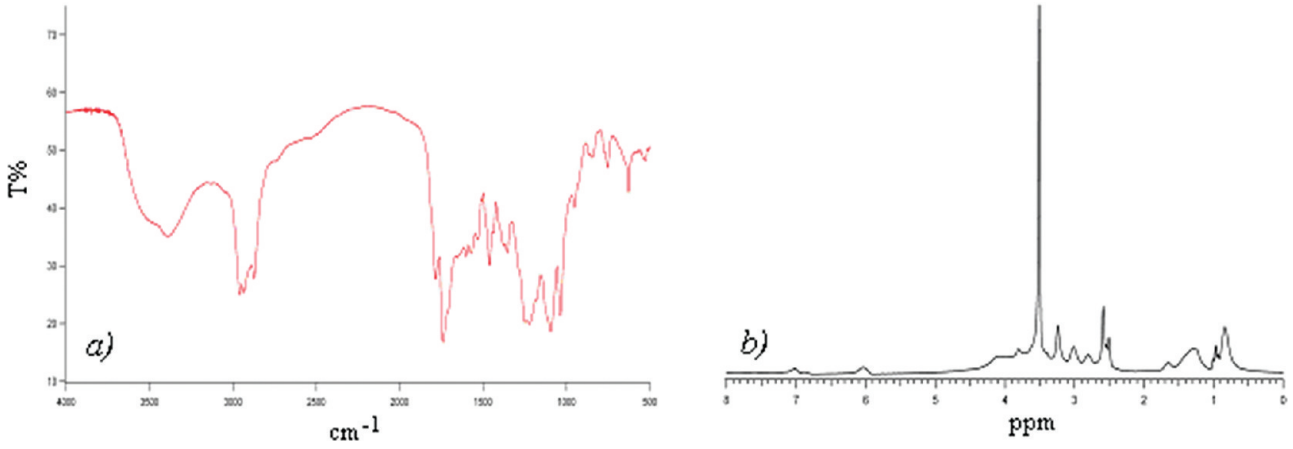
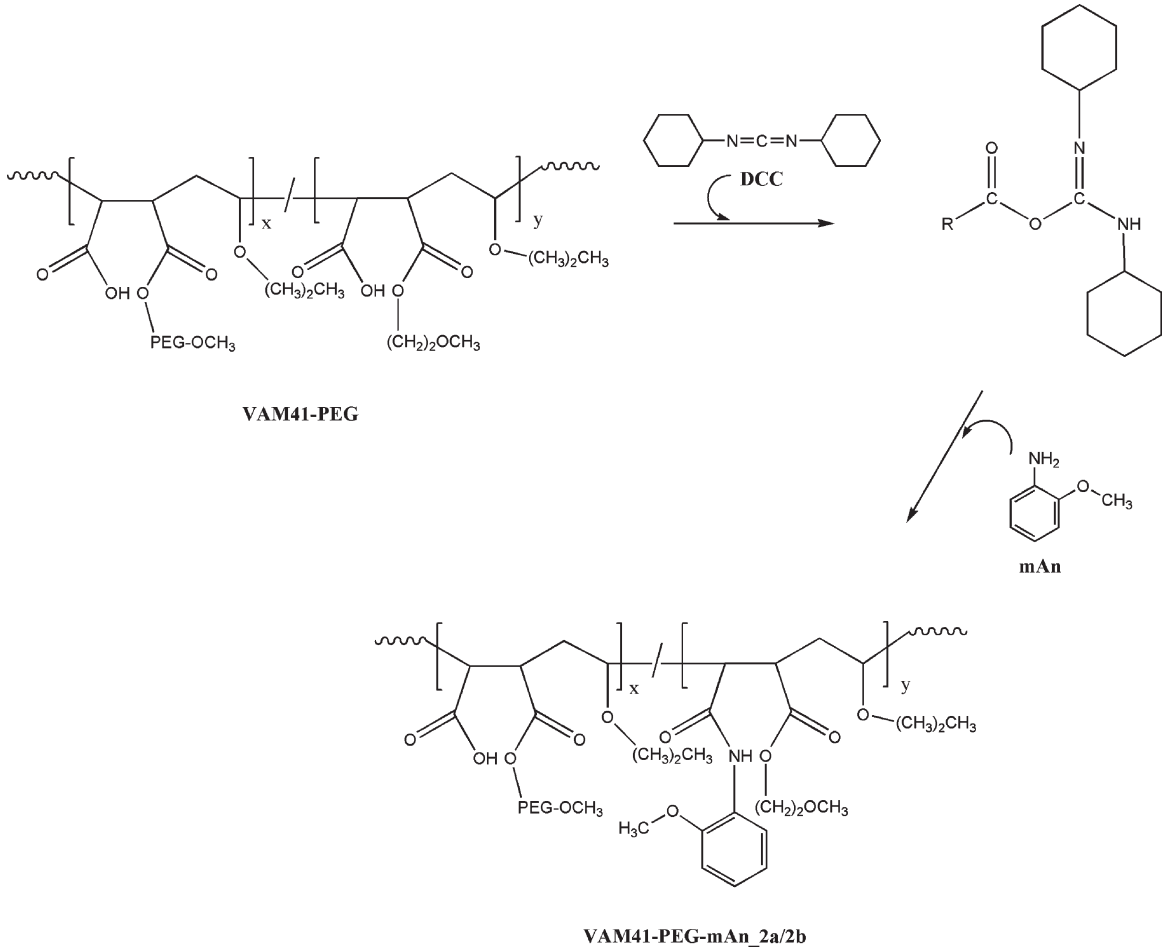


Figure 1. FT-IR (a) and ^{1}H NMR (b) spectra of VAM41-PEG-mAn_1.

Scheme 3. VAM41-PEG-mAn_2a/2b Reaction



moiety, the polymer was purified by means of a dialysis process in order to remove both EDC and Sulfo-NHS. Polymer precipitation was then performed by means of acid precipitation. The structure of the obtained polymer was assessed by FT-IR and ^{1}H NMR (Figure 1); the presence of mAn on the polymeric structure was ascertained and quantified.

For VAM41-PEG-mAn_1 F.D., the result was 16%, while the F.E. was about 32%.

VAM41-PEG-mAn_2a/2b Reaction. In order to increase the F.E., the reaction was performed in tetrahydrofuran (THF) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) was used to promote the amidic bond formation. In this reaction scheme, VAM41-PEG acidic moiety reacts with DCC to produce the *O*-acylisourea[19−21] characterized by an activated leaving group. The addition of amines determines the formation of the desired amide and of *N*,*N*′-dicyclohexylurea (DCU) which is insoluble in most organic solvents and therefore can be removed by sedimentation[22] (Scheme 3). Anyhow this synthesis strategy presents some drawbacks related to the side reactions of O-acylisourea which produce both desired and undesired products. The *O*-acylisourea can react with an additional carboxylic acid to give an acid anhydride which

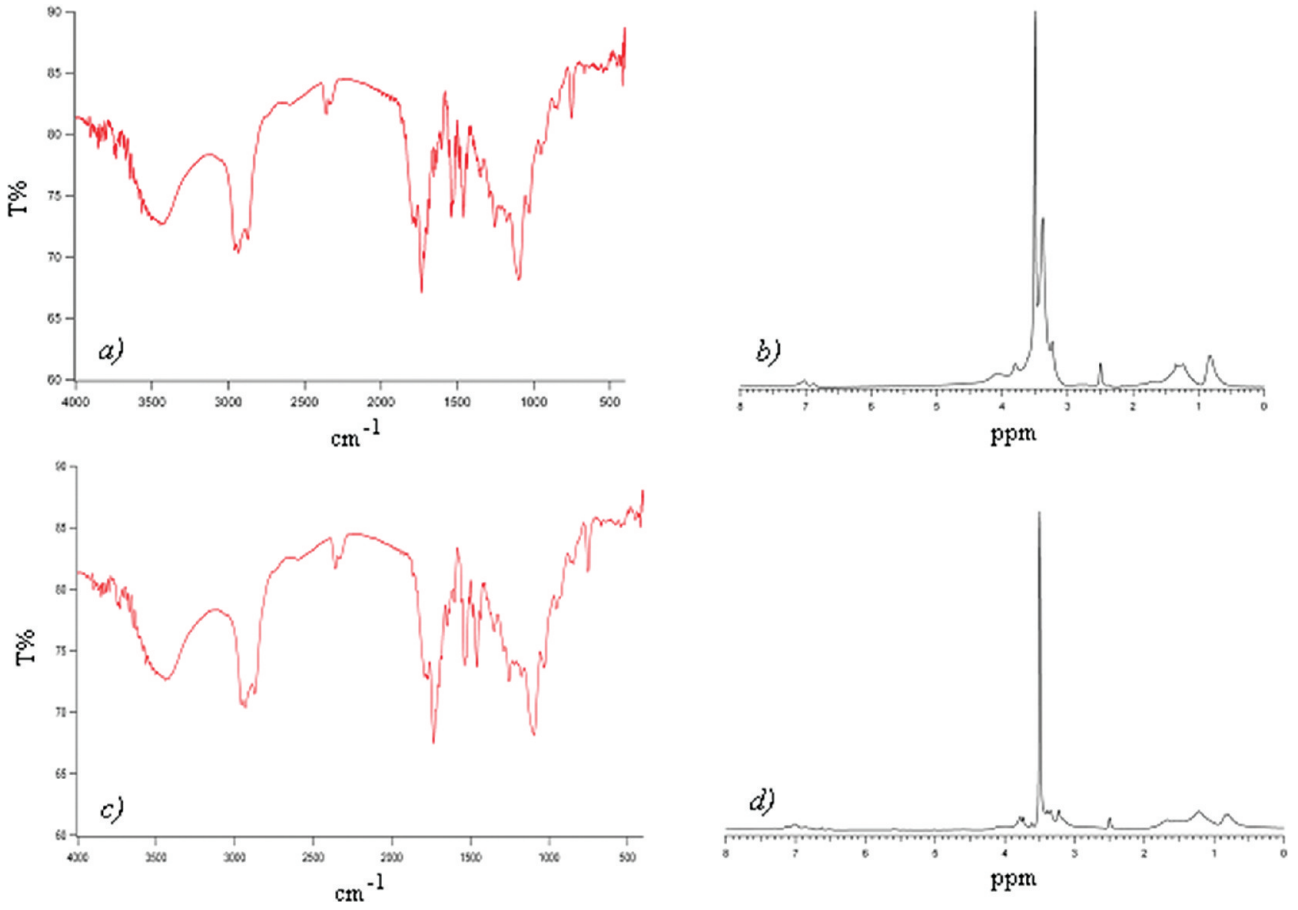


Figure 2. FT-IR and ^{1}H NMR spectra of VAM41-PEG-mAn_2a (a, b) and VAM41-PEG-mAn_2b (c, d).

can react further to give the desired amide. The main undesired reaction pathway involves the rearrangement of the *O*-acylisourea to the stable and unreactive *N*-acylurea.[23] The use of solvents with low-dielectric constants such as dichloromethane chloroform or tetrahydrofuran can minimize this side reaction.[24]

Two different reaction feedings were tested. In VAM41-PEG-mAn_2a and VAM41-PEG-mAn_2b, the amount (moles) of mAn corresponded to 55% and 100% of carboxylic moieties of the starting polymer, respectively. The structures of the obtained polymers were assessed by FT-IR and ^{1}H NMR (Figure 2); the presence of mAn on the polymeric structure was ascertained and quantified.

The F.D. result was 15% for VAM41-PEG-mAn_2a and 26% for VAM41-PEG-mAn_2b, while the F.E. was about 27% and 26% for VAM41-PEG-mAn_2a and VAM41-PEG-mAn_2b, respectively.

VAM41-PEG-mAn_3 Reaction. To increase the F.E., a third strategy was tested. For the preparation of VAM41-PEG-mAn_3, the modification of the polymer with mAn started with the reaction of poly(maleic anhydride-*alt*-butyl vinyl ether) (VAM40) with mAn. Amines can react with maleic anhydride to give the corresponding half a mide derivative. The nitrogen of the amine attacks an anhydride carbonyl resulting in the ring-opening and the formation of an amic acid.[25] After the production of the half amide derivative VAM40_mAn, the hemiesterification with PEG and 2-methoxyethanol was performed (Scheme 4).[11] VAM40 modification with mAn is obtained by adding 30% in moles of mAn with respect to maleic anhydride monomeric units.

The reaction was followed by means of FT-IR spectra analysis at different steps, in order to check the formation of the amidic bond and the hemiesterification levels by PEG and by 2MeOEtOH.

At the end of the first step, the aromatic C−H band at 750 cm^{-1} and the amidic C═O at 1600 cm^{-1} are present, while the anhydride-CO-band at 1786 cm^{-1} fully disappeared after the third step at completed hemiesterification with 2-methoxyethanol.

Polymer sample isolations were performed by precipitation in a mixture 50:50 vol/vol of diethyl ether and petroleum ether (ratio 1/10 in vol). The obtained precipitate was dried under high vacuum until constant weight. The polymers were then purified by ion exchange chromatography using Amberlyst 15 as exchange resin.

The structure of the obtained polymer was assessed by FT-IR and ^{1}H NMR presence of mAn was ascertained (Figure 3).

For VAM41-PEG-mAn_3 the F.D. result was 26%, while the F.E. was about 83%. As reported in Table 1 F.E. was increased by using the reaction 3 scheme.

Thanks to a high functionalization efficiency obtained for VAM41-PEG-mAn_3 this polymer was selected for further characterizations.

DPPH Assay. In order to evaluate the effectiveness of a scavenger activity of the modified polymer, the ability of the compound to scavenge the stable α,α-diphenyl-β-picrylhydraziyl (DPPH$^•$) free radical was tested.[27] In its radical form DPPH$^•$ shows an absorbance peak at 516 nm, which progressively disappears as a function of DPPH$^•$ reduction. It is thus possible to correlate the ability of reducing the DPPH$^•$ 516 nm absorbance level with the scavenger activity of the tested compound. VAM41-PEG-mAn_3, VAM41-PEG, and mAn were applied in the DPPH$^•$ assay in order to evaluate the increased scavenger activity of the modified polymer. At fixed DPPH$^•$ quantity, different polymers concentrations were

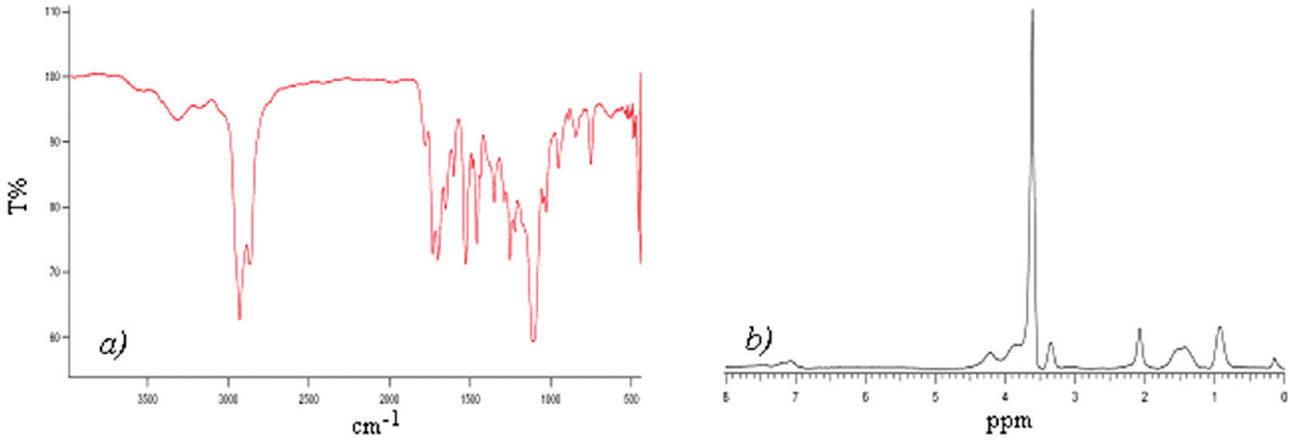
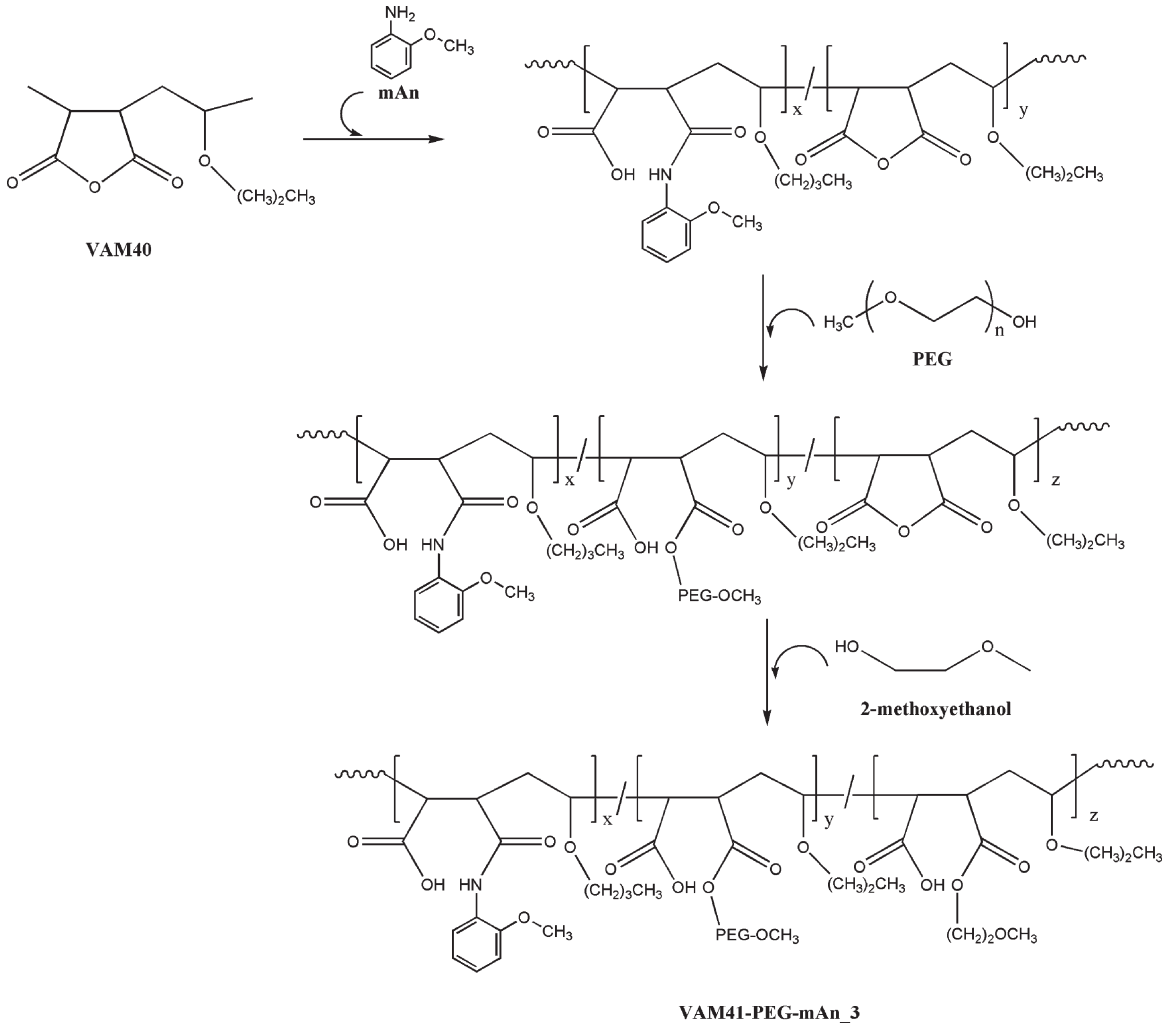


Figure 3. FT-IR (a) and ^{1}H NMR (b) spectra of VAM41-PEG-mAn_3.

Scheme 4. VAM41-PEG-mAn_3 Reaction



tested in order to estimate the 50% inactivation concentration (IC_{50}) (Figure 4).

By using a 5five-parameter logistic equation the IC_{50} values were estimated to be 0,97 μM for VAM41-PEG-mAn_3 and 2,1 μM for VAM41-PEG revealing a reduction of 0,034 mols of DPPH$^\bullet$ per VAM41-PEG-mAn_3 mol and 0,015 mols of DPPH$^\bullet$ per VAM41-PEG mole. Although some compounds react very quickly with DPPH$^\bullet$, and it can be assumed that after 30 min the reaction can reach the steady state,[26] macromolecular compounds displaying a complex structure such as VAM41-PEG and VAM41-PEG-mAn_3

Table 1. VAM41-PEG-mAn_1/2*a*/2b/3 F.D. and F.E.

polymer	solvent	F.D., %	F.E., %
VAM41-PEG-mAn_1	water	16	32
VAM41-PEG-mAn_2a	THF	15	27
VAM41-PEG-mAn_2b	THF	26	26
VAM41-PEG-mAn_3	THF	25	83

can be characterized by a lower reactivity. With the purpose of monitoring the reaction kinetic of both VAM41-PEG-mAn_3 and VAM41-PEG with DPPH$^{\bullet}$, the absorbance values at 516 nm were monitored at different times, for solutions containing 1.2×10^{-6} mol of the polymers and 10^{-7} mol of DPPH$^{\bullet}$ (Figure 5).

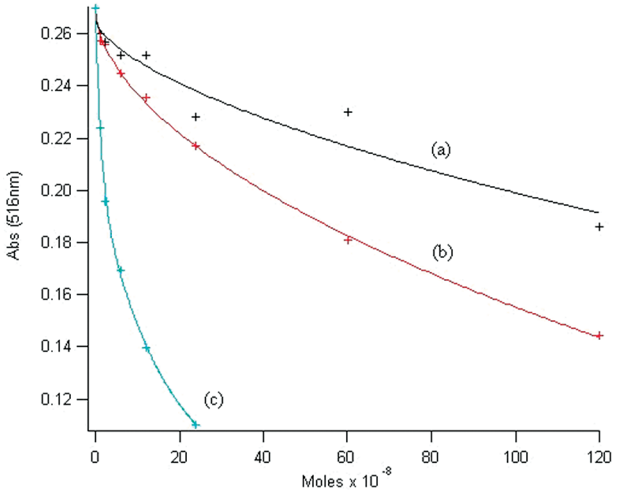


Figure 4. 516 nm absorbance value of a DPPH solution after 30 min exposure to increasing quantities of VAM41-PEG (a), VAM41-PEG-mAn_3 (b), and mAn (c).

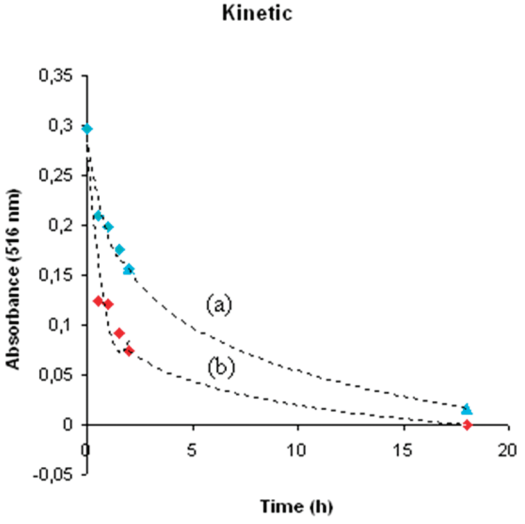


Figure 5. 516 nm DPPH$^{\bullet}$ absorbance values after different times of incubation with VAM41-PEG (a) and VAM41-PEG-mAn_3 (b).

As shown in Figure 5, the reaction was not completed after 30 min of incubation with DPPH$^{\bullet}$ both for VAM41-PEG-mAn_3 and VAM41-PEG. Moreover it can be noted that VAM41-PEG-mAn_3 in the reaction with DPPH$^{\bullet}$ reaction displays a relative higher reactivity when compared to VAM41-PEG. After an overnight incubation, which assured the achievement of the steady state for both of the reaction, the UV−vis complete spectra of VAM41-PEG-mAn_3/DPPH$^{\bullet}$ and VAM41-PEG/DPPH$^{\bullet}$ were analyzed (Figure 6).

At the steady state a progressive decrease of absorbance value at 516 nm is observed as a function of polymers moles. It is notable that the 516 nm absorbance value reduction is much higher in presence of VAM41-PEG-mAn_3 polymer confirming that the introduction of mAn moiety into the polymeric backbone increase the scavenger activity of the polymer also at the steady state. Moreover a progressive appearance of a 600 nm peak can be observed in the VAM41-PEG-mAn_3/DPPH$^{\bullet}$ spectra. The same peak can also be observed in the mAn reaction with DPPH$^{\bullet}$ which derives from the oxidation of mAn (Figure 6, parts f and g (black line). Thus, it is possible to assign a direct role in the increased scavenger activity of the modified polymer to the presence of mAn into the polymeric backbone. To confirm this hypothesis FT-IR spectroscopy was performed on VAM41-PEG-mAn_3/DPPH$^{\bullet}$ solutions containing 1.2×10^{-6} mol of the polymer and 10^{-7} mol of DPPH both after 30 min of incubation and after the achievement of the steady state (Figure 7).

As reported in Figure 8, it is possible to note a progressive shift of the 1461 cm^{-1} band, which is present in the VAM41-PEG-mAn_3 spectrum, to 1458 cm^{-1} after 30 min of incubation with DPPH$^{\bullet}$ and to 1457 cm^{-1} after the achievement of the steady state. Since the 1461 cm^{-1} band can be attributed to the C=C stretching of the aromatic ring, the shift to lower wavenumbers can be related to a progressive oxidation of double bonds of the aromatic rings, as also reported by Nikolaidis et al. in relation to polyaniline emeraldine base oxidation by DPPH radical.[10,28]

Moreover it is possible to note a decrease in the peak area at 1505 cm $^{-1}$ (N−H bending) proportional to the incubation time with DPPH$^{\bullet}$. In particular the ratio between 1505 cm^{-1} peak area and 1096 cm^{-1} area (which was used as a reference since it can be attributed to the ether C−O−C stretch) was recorded as 0,033 for VAM41-PEG-mAn_3 0,029 for VAM41-PEG-mAn_3/DPPH$^{\bullet}$ after 30 min of incubation and 0 for VAM41-PEG-mAn_3/DPPH$^{\bullet}$ at the steady state. In agreement with data reported by the literature[29] it is thus possible to hypothesize that during the reaction between VAM41-PEG-mAn_3 and DPPH$^{\bullet}$, the radical can oxidize mAn moieties in a one-to-one reaction (Scheme 5).

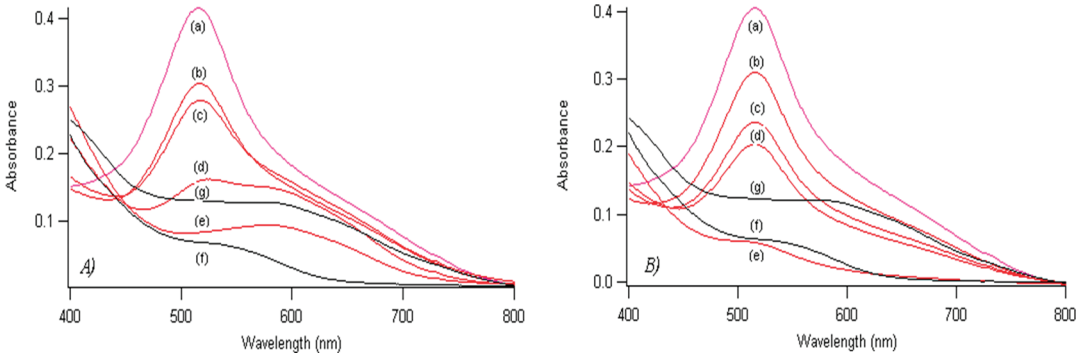


Figure 6. UV−vis (400−800 nm) absorbance spectra of VAM41-PEG-mAn_3/DPPH (A) and VAM41-PEG/DPPH (B) after the achievement of the steady state; Polymer moles: (a) = 0, (b) = 0.12×10^{-6} (c) = 0.24×10^{-6} (d) = 0.6×10^{-6} (e) = 1.2×10^{-6}. (f) = mAn UV−vis spectrum. (g) = mAn/DPPH UV−vis spectrum.

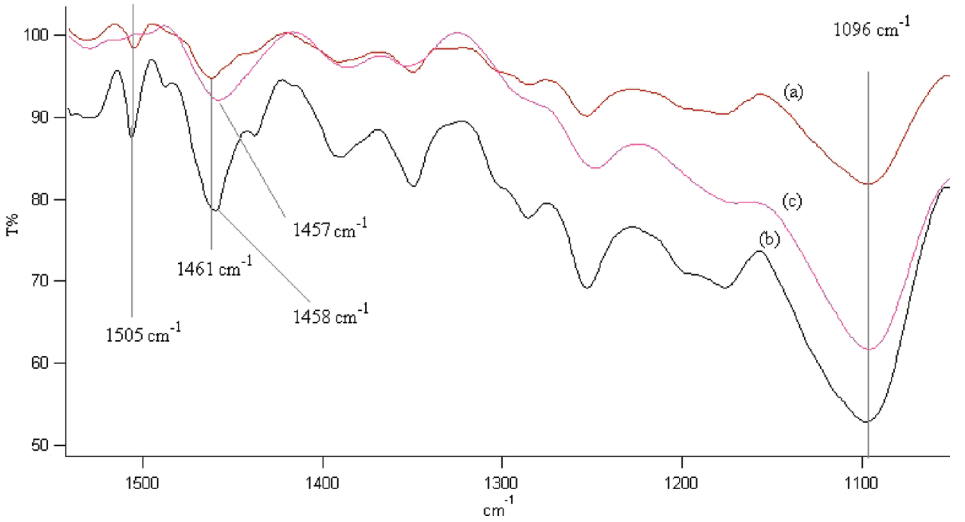


Figure 7. FTIR spectra of VAM41-PEG-mAn_3 (a), VAM41-PEG-mAn_3/DPPH after 30 min of incubation (b), and VAM41-PEG-mAn_3/DPPH at the steady state (c).

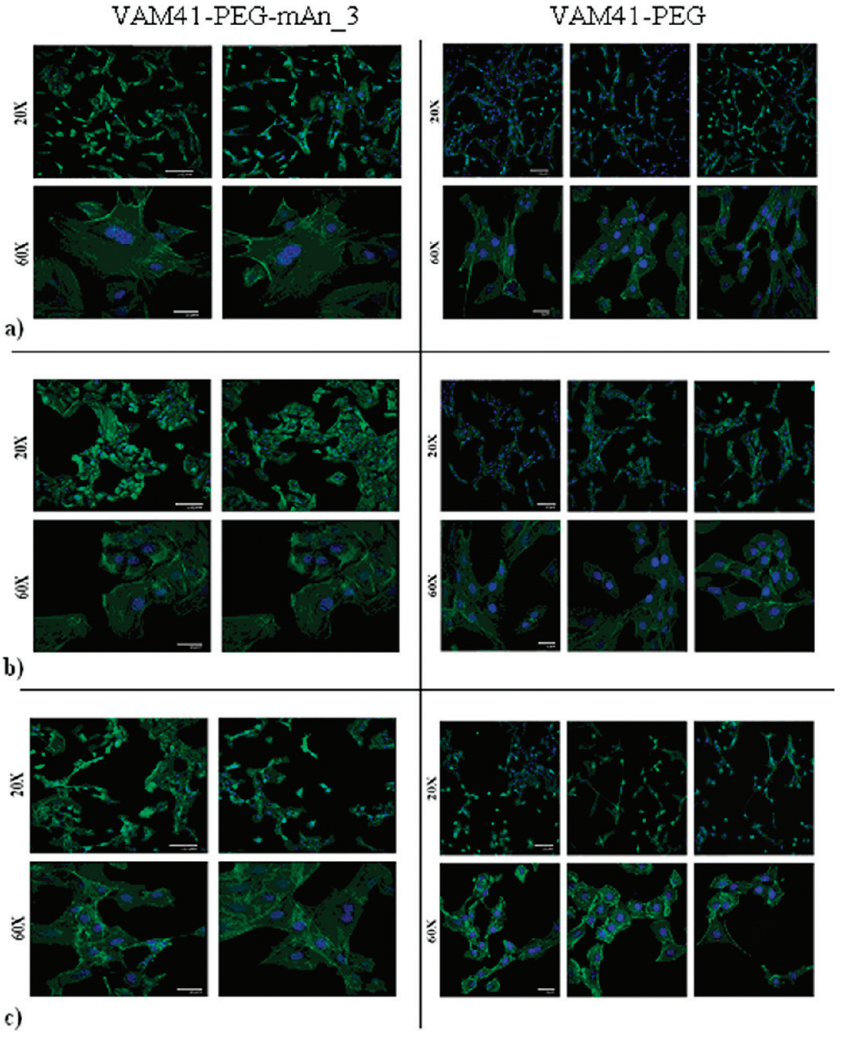
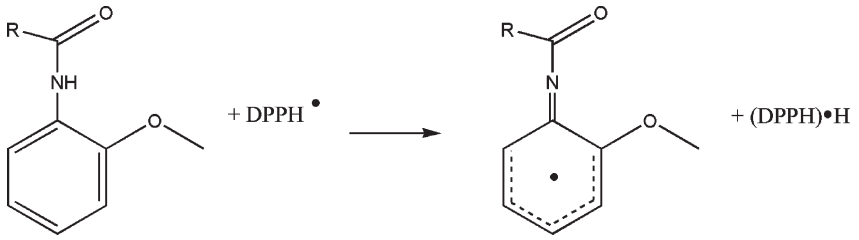


Figure 8. Cytotoxicity Tests Performed on VAM41-PEG-mAn_3 (left column) and VAM41-PEG (right column): (a) control, (b) 1 mg/mL, and (c) 2.5 mg/mL.

VAM41-PEG-mAn_3 Cytocompatibility Evaluation. According to ISO 10993 guidelines, regarding the biocompatibility evaluation of materials for biomedical applications, the presence of biotoxic compounds is commonly investigated by evaluating the cell response to the tested materials in terms of cell morphology and/or viability evaluations.

Scheme 5. DPPH Oxidation of mAn Moieties of VAM41-PEG-mAn_3 Polymer



Previous biological investigations of hemiesters of poly(maleic anhydride−*alt*−butyl vinylether) proved a high cytocompatibility for the VAM41-PEG polymer.[11] In order to check whether the introduction of mAn moieties into VAM41-PEG polymeric structure could affect its cytocompatibility, qualitative cytotoxicity tests were performed using balb/3T3 clone A31 mouse embryo fibroblast cell line. Experiments were carried out by incubation of the cells with polymeric solutions in complete growth medium at different concentrations. After 24 h, cells morphology was investigated using DAPI and Phalloidin-FITC as nuclear and cytoskeleton stains, respectively. Commonly used colorimetric quantitative enzymatic assay could not be employed in detecting VAM41-PEG-mAn_3 cytocompatibility due to a mAn colorimetric interference. Cell morphology was investigated by testing different VAM41-PEG-mAn_3 concentrations, using VAM41-PEG as a reference. As reported in Figure 8 cells incubated with VAM41-PEG-mAn_3 polymer display no alterations in terms of dimension, shape and cell spreading when compared with controls and VAM41-PEG incubated cells. Moreover nuclei morphology and actin filaments orientation highlighted cells healthy conditions, suggesting that the introduction of mAn does not seem to affect VAM41-PEG cytocompatibility.

Conclusions

Results reported in the present study highlighted the feasibility of chemically grafting 2-methoxy aniline (mAn) moiety to Poly(maleic anhydride-*alt*-butyl vinyl ether) 5% grafted with monomethoxy poly(ethylene glycol) 2000 MW and 95% grafted with 2-methoxyethanol (VAM41-PEG) sample with optimized levels of functionalizing efficiency. UV−vis and FT-IR spectra analysis revealed a substantial ability of the grafted polymer sample in free radical scavenging as detected by the reduction of α,α- diphenyl-β-picrylhydraziyl (DPPH)· radical. This property, as well as the maintenance of biocompatible features of the functionalized polymer system, suggests the biomedical area as one of the potential application fields of the synthesized polymer.

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