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Facile synthesis and promising antibacterial properties of a new guaiacol-based polymer

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ABSTRACT

A new acrylamide-type monomer (*N*-(4-hydroxy-3-methoxy-benzyl)-acrylamide) derived from guaiacol was successfully synthesized. Polymers containing guaiacol moiety were obtained *via* conventional radical polymerization of this monomer with AIBN as initiator. The influence of reaction time, initiator concentration and temperature on polymers characteristics was studied. Evaluation of the termination mode in free-radical polymerization was performed by MALDI-TOF mass spectrometry. Termination occurs mainly by disproportionation reaction. Additional peaks in the spectrum were attributed to side chain reactions implying phenoxy radicals. This new polymer exhibits a potential antibacterial activity against *Bacillus subtilis* by using anti-adhesion and anti-biofilm tests. After an adhesion time of 3 h, compared to a non-coated glass slide, there was a decrease of bacteria of 99% on the polymer coated glass slide. After three days of culture in a bacterial suspension, no biofilm was observed on the polymer coated surface.

1. Introduction

Microorganisms can colonize a wide variety of surfaces including food packaging and medical devices, this colonization leading to biofilm formation. Biofilms result from the accumulation of organic molecules, microorganisms and metabolites and are ubiquitous on surfaces submerged in aqueous environments. A reduction of surfaces contamination could be obtained through a well-adapted choice in the materials to use or through modifying them by way of adapted surface treatments [1–5].

In the literature, antimicrobial agents are numerous and are large-scale used. For example, quaternary ammonium salts, phosphonium salts, N-halamine, metal ions are more commonly used as antibacterial compounds [2]. Antibacterial agents of low molar mass can have the limitation of low durability, of free diffusion and of residual toxicity [6]. In recent years, an approach to prevent surface contamination by providing materials with antibacterial properties by functionalizing them with bacteriostatic groups appears [7]. This kind of materials can be prepared by incorporating antibacterial agents into them. For example, immersion of a tissue into an antibacterial solution can give the tissue antibacterial properties, but these properties could be lost after several washing and there is a risk of residual toxicity. An area of polymer research that presents great current interest is the development of polymers with antibacterial activities, the antibacterial agent being covalently bound to the polymer [6–9].

Phenolic compounds (as catechol, eugenol and others) represent another group of antimicrobial phytochemicals and are less studied and used [10–12]. Some recent studies associate phenolic compounds and polymer in order to prepare antimicrobial materials. Kenawy et al. reported the preparation of some antimicrobial polymers derived from polyacrylamide by reaction with aromatic aldehydes and phenolic esters [13]. Polymers and copolymers with eugenol moieties were synthesized using different eugenol monomeric derivatives. Microbiological studies have shown their capacity to reduce or inhibit colony formation by different bacteria species [14].

The aim of this work was to develop a new generation of antimicrobial polymers based on a natural biocide molecule named guaiacol (2-methoxyphenol), obtained from beechwood. Guaiacol

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is isolated from the guaiac resin. It is the main constituent of creosote obtained from wood tar (beech). Guaiacol and derivatives have been known and used for more than a century. Their main uses are antiseptics, gastric sedatives, flavorings, deodorants, fungicides and parasiticides [15,16].

To prepare this polymer, a new monomer was synthesized, its radical polymerization was studied, and preliminary antibacterial tests of polymers were carried out using *Bacillus subtilis* as Gram-positive bacteria.

2. Experimental part

2.1. Chemical materials

Guaiacol (99 + %), Dowex® 50WX8, 200–400 mesh and H_2SO_4 (P.A.) were obtained from Acros, acryloyl chloride was obtained from Fluka, vanillin (99%) was obtained from Alfa Aesar, Pd/C palladium (10 wt.-% on activated carbon) was obtained from Aldrich, hydroxylamine hydrochloride 97% was obtained from Janssen Chimica, absolute ethanol, min 99.5%, dimethylformamide (DMF), toluene and HCl 37% were obtained from VWR, all these products were used without purification. *N*-hydroxymethylacrylamide (48 wt.-% solution in water) was obtained from Aldrich and dried by freeze-drying. Dimethylsulfoxyde (DMSO) was dried on molecular sieve. Tetrahydrofuran (THF) was distilled from sodium and benzophenone. Triethylamine and diethyl ether were distilled on calcium hydride. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was recrystallized from ethanol.

2.2. Monomers synthesis

2.2.1. Synthesis of N-(4-hydroxy-3-methoxy-benzyl)-acrylamide (NA) by Friedel–Craft reaction (catalyzed by cationic exchange resin)

4.05 g (40 mmol) of *N*-hydroxymethylacrylamide, 4.5 ml (40.4 mmol) of guaiacol were dissolved in 40 ml of absolute ethanol in a 100 ml flask, and 28 g of cationic exchange resin was added. The reaction mixture was kept stirring for 6 days at 35 °C. Then 20 ml of absolute ethanol was added to the mixture under stirring. The cationic exchange resin was filtered off and the filtrate was concentrated to give a yellow liquid. The yellow liquid was poured into 20 ml of diethyl ether. The obtained solution was kept in refrigerator and white crystal of NA formed. The crystals were filtered and washed with diethyl ether. A yield of 25% was obtained.

2.2.2. Synthesis of N-(4-hydroxy-3-methoxy-benzyl)-acrylamide (NA) by Friedel–Craft reaction (catalyzed by H_2SO_4)

2.24 g (22 mmol) of *N*-hydroxymethylacrylamide and 2.7 ml (24 mmol) of guaiacol (2-methoxyphenol) were dissolved in 12 ml of absolute ethanol. The mixture was cooled with an ice-bath, and 1.8 ml of H_2SO_4 was added under stirring. The reaction mixture was kept stirring for 4 days at 35 °C. Then the product was extracted by CH_2Cl_2 and washed by H_2O. The organic phase was evaporated under reduced pressure to give a yellow liquid. The yellow liquid was poured into diethyl ether and white precipitate of NA formed. The crude product was recrystallized from ethanol, a yield of 31% was obtained.

2.2.3. Synthesis of N-(4-hydroxy-3-methoxy-benzyl)-acrylamide (NA) in three steps

2.0 g (13.2 mmol) of vanillin and 15 ml of acetic acid were added into a flask. 0.86 ml of a solution of NaOH (50 wt.-% in water) was added. Then 1.08 g (15.5 mmol) of hydroxylamine hydrochloride was added. The reaction mixture was kept under stirring at room temperature for 5 h. The mixture was extracted by diethyl ether and washed with water, the organic phase was washed by $NaHCO_3$ (10 wt.-% in water), then washed with saturated NaCl solution. The organic phase was dried over $MgSO_4$ and evaporated. The 4-hydroxy-3-methoxy-benzaldehyde oxime (white crystalline solid) was obtained with a yield of 67%.

0.62 g (3.71 mmol) of 4-hydroxy-3-methoxy-benzaldehyde oxime, 120 mg of 10% Pd/C, 2.4 ml of HCl (37%), 60 ml of absolute ethanol were added in a flask, the flask was firstly kept under vacuum and then under H_2 flow. The mixture was stirred at room temperature for 3 h. The obtained mixture was filtered through celite, the filtrate was evapored and a light yellow powder of 4-hydroxy-3-methoxy-benzylamine hydrochloride was obtained with a quantitative yield.

Before the synthesis of NA, 200 mg (1.06 mmol) of 4-hydroxy-3-methoxy-benzylamine hydrochloride was dissolved in 2 ml of water. 2 ml of NaOH solution (0.5 N in water, 1 mmol) was added under stirring, a white precipitate formed. The product was filtered and dried at room temperature. 4-hydroxy-3-methoxy-benzylamine was obtained.

100 mg (0.65 mmol) of 4-hydroxy-3-methoxy-benzylamine, 0.18 ml (1.29 mmol) of triethylamine, 10 ml of anhydrous THF and 47 µl (0.59 mmol) of acryloyl chloride were added into a flask cooled with an ice-bath under argon. The mixture was kept stirring at room temperature for 3 h. Then, the product was extracted by CH_2Cl_2, the organic phase was washed by a HCl solution (0.01 N), then washed with a saturated NaCl solution, dried over $MgSO_4$ and then evaporated. A yield of 41% was obtained.

^{1}H NMR (MeOD) δ (ppm) = 3.70 (s, 3H, O-C*H*$_3$), 4.21 (d, 2H, C*H*$_2$–NH), 5.55 (t, 1H, C*H*=), 6.12 (d, 2H, C*H*$_2$=), 6.60 (d, 2H, –*CH*=*CH*– aromatic), 6.74 (d, 1H, –*CH*– aromatic).

^{13}C NMR (MeOD) δ (ppm) = 44.8 (–CH_2-), 57.2 (–CH_3), 112.9, 116.9, 122.2 and 131.1 (aromatic), 127.1 (CH_2=), 132.2 (CH=), 147.2 and 149.2 (=C–O), 168.0 (C=O).

Elemental analyses:

$C_{11}H_{13}NO_3$ 207 : Calcd. C 63.76, H 6.32, N 6.76, O 23.16; Found C 63.55, H 6.47, N 6.63, O 23.29.

Melting point: 145–147 °C.

IR (KBr): 3325 (OH), 3097 (NH), 1655 (C=O), 1596 cm^{-1} (C=C).

2.3. Radical polymerization

2.3.1. Synthesis of poly [N-(4-hydroxy-3-methoxy-benzyl)-acrylamide] (PNA)

400 mg (1.93 mmol) of NA, 4 mg (0.024 mmol) of AIBN, 2 ml of toluene and 2 ml of DMF were added into a flask under an argon flow for 10 min. Then the mixture was kept under stirring at 70 °C for 1, 2, 4, 6, 18 or 24 h. After the polymerization, the polymer was precipitated in 40 ml of cold Et_2O. The polymer (white solid) was filtered and dried in a desiccator.

2.4. Protection of –OH groups

50 mg (0.242 mmol of –OH) of PNA, 1 ml of acetic anhydride, 1 ml of pyridine were added in a flask. The mixture was stirred at 100 °C for 5 h. The product was extracted with dichloromethane and washed with water. The organic phase was washed with $NaHCO_3$, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to obtain the product.

2.5. Monomers and polymers characterization

NMR spectra were recorded at room temperature with Brüker DPX-250 spectrometers. Chemical shifts (in ppm) were determined relative to residual undeuterated solvents as an internal reference.

IR-FT spectra were recorded on a Brüker IFS 66 spectrometer.

Elemental analyses were performed at the Service Central de Microanalyses du CNRS (Gif-sur-Yvette, France).

Monomer melting point were determined using BUCHI Melting Point B-545 with heating rate equal to 0.5 °C/min.

Glass transition temperature was obtained using a differential scanning calorimeter (DSC) from TA Instruments (Q100-0729 apparatus) working in the temperature range from 25 °C to 180 °C at a heating rate of 10 °C min^{-1} under a continuous nitrogen flow. The data were collected during the second heating process after cooling at 25 °C. The sample mass was about 7 mg.

Size exclusion chromatography (SEC) was employed to obtain the mean number-average molar mass (M_n), mean weight average molar mass (M_w), and polydispersity index (PDI = M_w/M_n) of the PNA. The polymer samples for SEC were prepared after protection of the alcohol groups and dissolved in THF to a final concentration of 1 mg ml^{-1}. SEC was carried out in two series connected VISCOTEK ViscoGEL columns 17360 GMHHR-H. The chromatographic system consisted of an HPLC pump (Waters-600-Pump-and-Controller) operating at 1 ml min^{-1}. THF was used as the mobile phase. Samples of 100 μl were injected with a syringe. The RI Detector was also from Waters. SEC was calibrated with polystyrene (PS) standards. The hardware and software for data acquisition and treatment were supplied from hs GmbH (Germany).

A Perseptive Voyager DE-STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA, USA), equipped with a 337-nm pulsed nitrogen laser (20 Hz) and a Acqiris® 2 GHz digitizer board, was used for all experiments. Mass spectra were obtained in reflectron positive ion mode with the following settings: accelerating voltage 20 kV, grid voltage 62% of accelerating voltage, extraction delay time of 100 ns. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. The mass spectrometer was externally calibrated using PEG1500. All data were processed using the Data Explorer software package (Applied Biosystems).

2,5-Dihydroxybenzoic acid (DHB, used as the matrix for MALDI-TOF experiments, was of the highest grade available and used without further purification) and sodium iodide (NaI, used as the cationizing agent) were purchased from Sigma Aldrich Co.

Polymer sample for MALDI analysis was prepared at a concentration of 0.6 mM in MeOH/$CHCl_3$ (50/50 vol.-%). The matrix solution was prepared at a concentration of 6 mM in MeOH/$CHCl_3$ (50/50 vol.-%). The cationizing agent, sodium iodide (NaI) was prepared at 0.7 mM in MeOH. The sample was prepared by mixing the polymer solution with matrix solution and cationizing agent solution at a volume ratio of 1:9:1.

2.6. Microbial material

B. subtilis (ATCC 6633) were obtained from the American Type Culture Collection. Bacterial strain was stored at −80 °C in Tryptone Soy Broth (TSB, Biomerieux, France) supplemented with 20% (v/v) glycerol. Prior to each experiment, frozen cells were subcultured twice in TSB at 30 °C. All adhesion and biofilms tests were performed on cells in the early stationary growth phase. Thus the cells were harvested by centrifugation (7000 g, 4 °C, 10 min), then washed twice using 1.5×10^{-3} mol l^{-1} NaCl and resuspended in a 1.5×10^{-3} mol l^{-1} NaCl solution. The NaCl solution was used to favor the survival of microbial cells and prevent osmotic shock. The final bacterial suspension was diluted in 1.5×10^{-3} mol l^{-1} NaCl solution to reach 10^6 CFU ml^{-1}.

2.7. Antibacterial activity assessment

2.7.1. Planktonic cells

Antibacterial activity of chemical compound against *B. subtilis* in suspension was evaluated from the relation that turbidity presents bacterial growth. Bacteria were cultivated in liquid culture media with or without chemical compound. Different concentrations of chemical compound were involved. Turbidity of the suspension was estimated by optical density (OD). Then data of kinetical OD measurement were converted into growth curve.

Minimal Inhibitory Concentration (MIC) of guaiacol against *B. subtilis* was estimated from turbidity growth curves generated by an automatic spectrophotometer (Bioscreen C, Labsystems, Finland).

MIC means the lowest concentration of the compound that completely inhibits macroscopic growth. Bioscreen C is a microbiological incubator and a culture growth-monitoring device allowing the simultaneous analysis of 200 samples divided into two microplates of 10 × 10 wells. By measuring the turbidity of the bacterial suspension over time, an optical density (OD) curve can be generated. The curve presents the bacterial growth. In this case, the wavelength was set to be 600 nm.

Each well of the Bioscreen plate was inoculated with 30 μl of the bacterial suspension, 6 μl of a guaiacol solution in EtOH and 264 μl of TSB, to obtain a target value of bacterial concentration of 10^6 colony forming units per ml (CFU ml^{-1}) [17].

Each well of the Bioscreen plate was thoroughly mixed and the plate was run in the Bioscreen for 24 h at 30 °C. The Bioscreen was programmed to read the OD of each well every 30 min, shaking the plate with medium intensity for 30 s before each reading.

2.7.2. Bacterial adhesion tests

Glass slides (Erie Scientific, USA) of 25 mm × 13 mm, were used as support for adhesion tests. Before use and modifications, they were washed 20 min at 50 °C in a solution of RBS 35 (2 vol.-%, Société des Traitements Chimiques de Surfaces, France), then rinsed five times with 50 °C sterile distilled water and five times with sterile distilled water (Purelab, Veolia water, UK). They were then conditioned by physical adsorption by drop deposit (V = 200 μl) of polymer solution with a concentration of 5×10^{-2} mol l^{-1} in a mixture of MeOH/CH_2Cl_2. The samples were then dried at room temperature during 14 h, for evaporating solvents.

Bacterial adhesion tests were performed by sedimentation. So, solid surfaces were incubated in 30 ml of bacterial suspension in 1.5×10^{-3} mol l^{-1} NaCl solution in Petri dishes for 3 h, then rinsed five times to remove non-adherent bacteria, each time with 8 ml of distilled water (care was taken to prevent samples from drying between washes). The number of viable and cultivable adherent bacteria was determined using the colony forming units technique after detachment of adherent bacteria by ultrasonic treatment (Ultrasonik 6QT Ney, USA) for 2 min at 20 °C [5].

Prior to image acquisition, the solid surface was mounted on the motorized stage of Leica SP2 AOBS confocal laser scanning microscopy (CLSM, LEICA Microsystems, France) at the MIMA2 microscopy platform [18]. The observations were carried out by using the CLSM after a coloration with acridine orange (0.01wt.-% in distilled water).

2.7.3. Biofilm tests

After 3 h of bacterial adhesion, the solid surfaces were rinsed with TSB in order to eliminate any non-adherent bacteria before being recovered with 8 ml TSB and incubated for 72 h at 30 °C. The observations were carried out by using the CLSM after coloration with acridine orange (0.01 wt.-% in distilled water). All biofilms were scanned using a 63× with a 1.3 N.A. (Leica HCX Apo) oil immersion objective lens with a 488 nm argon laser. Emitted fluorescence was recorded within the range of 500–600 nm. Three stack of horizontal plane image (512 × 512 pixels) with a z-step of 1 μm were acquired for each contaminated solid surface at different areas in the solid surface.

Three-dimensional projections of biofilms structure were reconstructed using the Easy 3D function of the IMARIS 7.0 software (Bitplane, Switzerland). Quantitative structural parameters of the biofilms, such as substratum coverage and thickness, were determined directly from the confocal stack images. Substratum coverage (%) and thickness reflected the efficiency of substratum colonization by bacteria.

3. Results and discussion

3.1. Synthesis of a new monomer derived from guaiacol

A new monomer named *N*-(4-hydroxy-3-methoxy-benzyl)-acrylamide (NA) bearing an acrylamide polymerizable fragment was synthesized using guaiacol as starting material. The chemical structures of guaiacol and monomer are presented below (Fig. 1).

Two methods were studied for synthesizing the *N*-(4-Hydroxy-3-methoxy-benzyl)-acrylamide (NA). It can be synthesized in one step *via* a Friedel–Crafts alkylation reaction of *N*-hydroxymethylacrylamide with guaiacol (Scheme 1) [19]. When cationic exchange resin (strongly acidic) was used as a catalyst, a 25% yield was obtained after 4 days, purification treatments are easy, and the resin can be used many times after regenerations. Sulfuric acid (H_2SO_4) was tested as catalyst, the yield was 31% after 4 days reaction. The yield did not increase for longer reaction time, with both catalysts. Because of the adsorption of the NA on the resin, the yield was slightly lower with cationic exchange resin than with H_2SO_4.

NA was also synthesized from vanillin (4-hydroxy-3-methoxy-benzaldehyde) using a three steps reaction path (Scheme 2).

First, the oxime was prepared from vanillin by reaction with hydroxylamine. Then, reduction with Pd/C yielded the 4-hydroxy-3-methoxy-benzylamine hydrochloride [20]. NA was obtained by reaction with acryloyl chloride in anhydrous conditions. The total yield of the three steps reaction was 27%.

The Friedel–Crafts reaction is a one step process, easy to carry out, but the reaction time is long. On the other hand the synthesis from vanillin requires three steps, but the reactions are faster. With both methods, similar yields were obtained.

For both procedures, chemical structure of the monomer was confirmed by ^{1}H NMR, ^{13}C NMR and elemental analysis. All integrations of NMR spectra matched very well. Using Friedel–Crafts alkylation reaction, the monomer was obtained in a very simple one step procedure with a high purity; this method was chosen for large-scale preparation of NA (10–30 g).

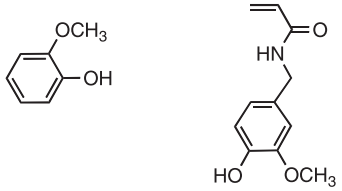
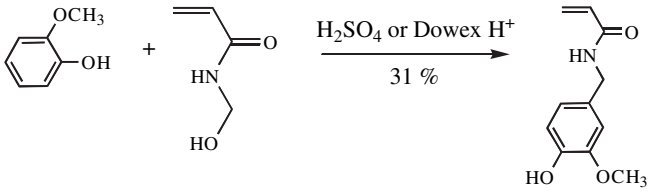


Fig. 1. Chemical structures of guaiacol and NA.



Scheme 1. Synthesis of NA in one step *via* Friedel–Crafts reaction.

3.2. Polymerization of N-(4-hydroxy-3-methoxy-benzyl)-acrylamide (NA)

Conventional radical polymerization of the monomer was carried out in solution using AIBN as thermal initiator (Scheme 3). Different parameters were studied such as reaction time, temperature and initiator concentration. Their influence on average molar masses, polydispersity and monomer conversion was also determined. Due to the particular solubility of NA monomer, solution polymerizations were performed in a mixture of DMF and toluene (50/50 vol.-%).

Molar masses and polydispersity were determined by size exclusion chromatography (SEC). In order to improve the solubility of poly(*N*-(4-hydroxy-3-methoxy-benzyl)-acrylamide) (PNA) in THF, a common mobile phase for SEC, protection of phenol functionality by acylation reaction with anhydride acetic was carried out (Scheme 3) [21]. The yield of acylation reaction was quantitative and the acetylated polymer (PNA-Ac) has a good solubility in THF. For all polymerizations, conversions were calculated based on the ^{1}H NMR spectrum by integration comparison of monomer and polymer.

3.3. Study of reaction time

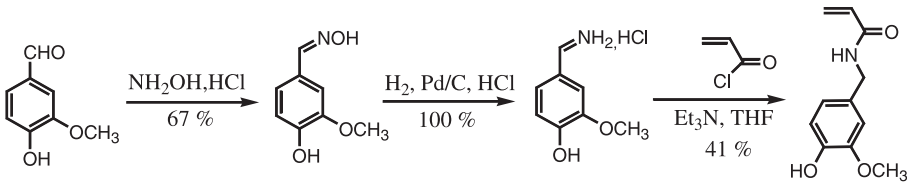
The polymerizations were carried out with 1 wt.-% of AIBN relative to monomer. The reactions were stopped after 1, 2, 4, 6, 18, or 24 h. Results are summarized in Table 1.

During the polymerization, an increase of the reaction time from 1 h to 24 h led to an increase of the conversion from 7% to 78%. Polymers with average molar masses in the range of 5200–6600 g mol^{-1} were obtained. The polydispersity index (equal to M_w/M_n) was between 1.2 and 1.6.

3.4. Study of the initiator concentration

To study the influence of initiator concentration on polymerization features, polymerizations with different amounts of AIBN were carried out at 70 °C during 24 h. Results are presented in Table 2.

Polymerizations were carried out with initiator concentration between 0.25 and 4 wt.- %. Monomer conversions increase from 10% to 96% by increasing the initiator concentration. Number-average molar masses were in the range 3600–4600 g mol^{-1}. Polydispersity indexes were in the range 1.4–1.7. Few evolution of molar masses versus initiator concentration was observed in opposition with a classical radical polymerization (decrease of molar masses with increasing initiator concentration). A slight increase of polydispersity index with initiator concentration is observed.



Scheme 2. Synthesis of NA in three steps from vanillin.

3.5. Influence of the temperature

The influence of temperature was studied with 1 wt.-% of AIBN concentration and a reaction time of 24 h. Results are presented in Table 3.

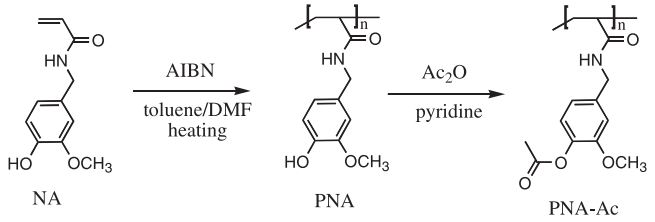
These results show a small decrease of molar masses with increasing temperature in agreement with a classical radical polymerization.

Whatever the conditions used (reaction time, initiator concentration and temperature) average molar masses are relatively low (≤11 000 g/mol) and very few evolutions are observed when changing those parameters. It is well known that phenolic compounds (as *p*-methoxyphenol, hydroquinone and 3,5-di-*t*-butylcatechol) are polymerization inhibitors, they are added to many commercial monomers to prevent polymerization during transport and storage. Carbon-centered radicals react with phenol by abstracting a phenolic hydrogen. The phenoxy radicals may then scavenge a further radical [22]. NA monomer bearing a phenolic group could act as polymerization inhibitor.

The reactivity of phenolic group could also explain the decrease in number-average molar masses of 10–20% observed after 4 h reaction time. For higher reaction time number-average molar masses level off. That decrease with time could be explained by the reactivity of phenolic substituents of an oligomer chain formed after a sufficient reaction time providing phenoxy radicals which could react by an inter-chain coupling reaction with other propagating radicals. This mechanism could explain the synthesis of branched structures with a broader molar mass distribution occurring mainly at higher conversion rates. It has to be mentioned here that we used a calibration curve to calculate a relative molar mass by SEC. So a molar mass decrease could also be related to a more branched polymer. The increase in heterogeneity in molar mass is confirmed by the increase in PDI from 1.2 to 1.6.

3.6. MALDI-TOF characterization of PNA

For a better understanding of the mechanisms involved in the polymerization process, we decided to use MALDI-TOF mass spectrometry to highlight eventual side reactions (such as transfer reaction) and to measure the ratio of disproportionation to combination in the free-radical polymerization of NA [23,24]. The polymer sample obtained at 70 °C with 1 wt.-% of AIBN for a reaction time of 24 h (Table 1 entry 6) was subjected to MALDI-TOF mass spectroscopy. 2,5-Dihydroxybenzoic acid was used as the matrix and sodium iodide as the cationic agent.



Scheme 3. Homopolymerization of NA followed by acetylation reaction.

The MALDI spectrum of PNA is presented in Fig. 2 and an expansion of the spectrum is presented in Fig. 3. The main peaks attribution is given in Table 4 (Fig. 4).

Compared with the number-average molar mass of 5500 g/mol obtained by SEC, molar masses of the different peaks obtained by MALDI-TOF are very low. It is well established that for PDI > 1.1 MALDI-TOF spectra fail to yield reliable molar mass values [25]. Therefore, great caution is needed in estimating average molar masses and molar mass distribution of polymers by MALDI-TOF. Therefore we used MALDI-TOF results only to evidence polymerization mechanisms distinguished by different end groups formation.

Several distributions with a peak-to-peak mass increment of 207 g mol^{-1}, corresponding to the mass of the NA unit are observed. The main distribution (series 1 in Fig. 2) could be assigned to the polymer with one AIBN fragment. This distribution was attributed to polymer chain obtained by radical termination by disproportionation reaction. In that case a good agreement between the experimental and calculated molar masses is obtained (Table 4). It has been shown that acrylamide polymerization termination occurs mainly by disproportionation, in agreement with our results [26]. A second distribution (series 2 in Fig. 2) with minor intensity could be assigned to the polymer chain with two AIBN fragments. This distribution could be attributed either to polymer chains obtained by recombination termination or to polymer chains obtained by disproportionation with one phenoxy radical having reacted with an activated monomer (RM°). A third distribution (series 3 in Fig. 2) could be assigned to a polymer chain possessing three AIBN fragments. The presence of three AIBN fragments could effectively occur in a polymer chain obtained by recombination with one phenoxy radical having reacted with an activated monomer (RM°). Another possibility is the synthesis of polymer chain obtained by disproportionation with two phenoxy radicals having reacted with two AIBN fragment radicals (R°). Finally the MALDI-TOF study of PNA confirmed our hypothesis on the inter-chain coupling reaction proposed above to explain the decrease in number-average molar mass with time.

The structures corresponding to the other minor series observed on the spectrum have not been assigned yet.

The thermal properties of PNA were studied by differential scanning calorimetry (DSC). The polymer exhibited typical thermoplastic behavior. The glass transition temperature (*Tg*) was around 101 °C, no melting was observed.

Table 1
Influence of reaction time on the polymerization of NA (1 wt.-% of AIBN, temperature 70 °C).

Reaction time (h)	M_n (g.mol^{-1})	M_w/M_n	Conversion
1	6600	1.31	7%
2	6480	1.22	27%
4	6170	1.60	38%
6	5510	1.49	58%
18	5210	1.52	60%
24	5460	1.55	78%

Table 2
Influence of initiator concentration on NA polymerization features (temperature 70 °C, 24 h).

AIBN concentration (wt.-% on monomer)	M_n (g.mol^{-1})	M_w/M_n	Conversion
0.25	4200	1.43	10%
0.5	4600	1.42	74%
1.0	4500	1.47	75%
2.0	3600	1.58	92%
4.0	3700	1.71	96%

3.7. Antibacterial properties assessment

Our initial concern was to study the antibacterial activity all along the chemical pathway. According to the solubility of the molecules (natural biocide and polymer), different microbiological tests were used.

3.8. Molecules and monomers in suspension (planktonic tests)

Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after overnight incubation [27].

By using the dilution method, the MIC of guaiacol was found to be equal to 35 mM (4.3 mg ml^{-1}) against 10^6 CFU ml^{-1} *B. subtilis* (Fig. 5). It was impossible to obtain the MIC of NA and PNA as the monomer was only slightly soluble and the polymer was not soluble in aqueous alcoholic solution. However, the antibacterial properties of guaiacol could give the NA and the PNA a potential inhibition against the bacterial growth.

3.9. Adhesion tests

Adhesion tests were performed after PNA solution deposit on glass slides followed by solvent evaporation. Polymer coating on glass slides is a method commonly used to study antibacterial property of non water-soluble polymers [28]. Weight measurements showed that PNA coating was stable against time in excess water during the experimental measurement (no weight loss due to polymer detachment or leaching). Results of the biocontamination of the antimicrobial solid surfaces are shown in Fig. 6.

After an adhesion time of 3 h, the confocal laser scanning microscopy (CLSM) images (Fig. 6) showed that, on the blank glass slide there was a great number of bacteria arranged in a common necklace conformation. On the glass slide coated by PNA an absence of bacteria were observed.

The CLSM images are consistent with the results of colony forming units per cm^2 (CFU.cm^{-2}). On the blank glass slide, the bacterial concentration was equal to 3.5×10^3 CFU cm^{-2}. On the glass slide conditioned by PNA, the CFU cm^{-2} value decreased drastically down to 4.5×10^1, corresponding to a reduction of 99%.

3.10. Biofilm tests

After 3 days of culture, the glass slides were observed by CLSM. Biofilms thickness and surface coverage percentage were determined. Results are shown in Fig. 7.

Table 3
Influence of temperature on NA polymerization features (1 wt.-% of AIBN, 24 h).

Temperature	Conversion	M_n (g.mol^{-1})	M_w/M_n
50 °C	13%	11,000	1.61
60 °C	92%	7800	1.77
70 °C	62%	5800	1.50
80 °C	76%	4600	1.60

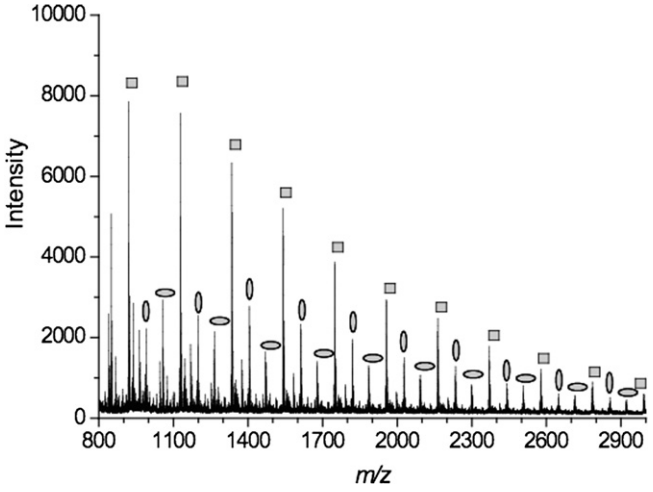


Fig. 2. MALDI-TOF mass spectrum of PNA (series 1: square, series 2: vertical oval-shaped and series 3: horizontal oval-shaped).

CLSM images (Fig. 7) showed that after 3 days of culture, blank glass slides were covered by *B. subtilis* biofilm with a surface coverage equal to 80% (thickness between 0 and 10 μm). On PNA coated glass, no biofilm formation was observed (surface coverage close to 1% without any thickness). So PNA clearly exhibits an anti-biofilm activity against *B. subtilis*.

3.11. Mode of antibacterial action

Due to a methoxy substituent difference with phenol it seems reasonable to think that the antibacterial mechanism of action of a free molecule of guaiacol would be similar to other phenolics. Their mechanism of action is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents. Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to cell death [29].

Antibacterial materials with immobilized molecules showed their activity only against bacteria which come into contact with

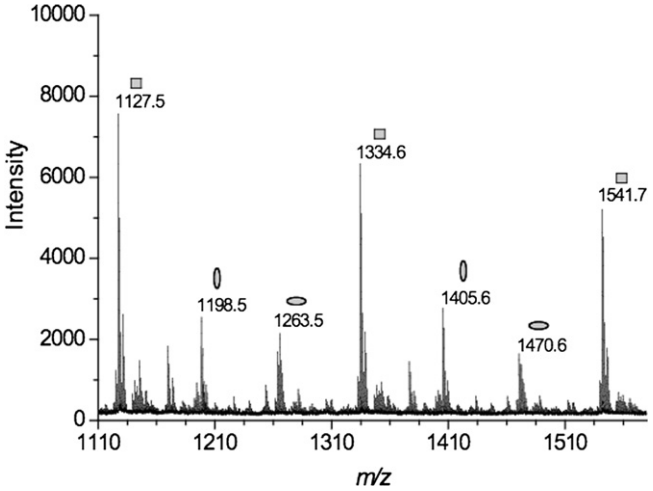


Fig. 3. Expansion of the MALDI-TOF mass spectrum of PNA (series 1: square, series 2: vertical oval-shaped and series 3: horizontal oval-shaped).

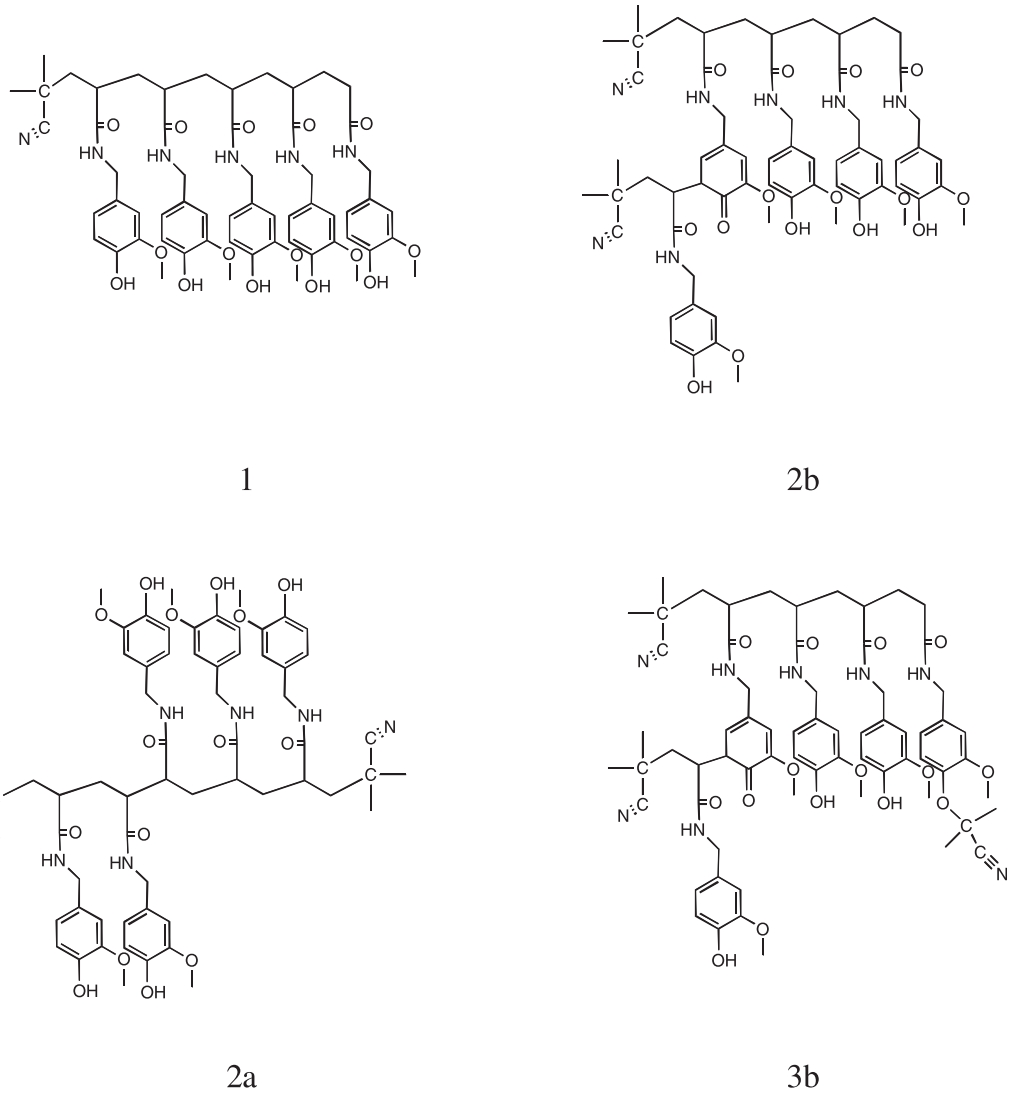


Fig. 4. Examples of chemical structures assigned to the main peaks observed on Fig. 3 (see Table 4 for details).

Table 4
MALDI-TOF experimental and theoretical molar masses of Na^+- ionized PNA with various chemical structures.

Series	Experimentally mass (m/z)	Assumed possible structure	Calculated mass	Assignment
1	1127.5	1 $\left(R\{NA\}_5H, Na\right)^+$	1127.5	Disproportionation
2	1198.5	2a $\left(R\{NA\}_5R, Na\right)^+$ 2b $\left(R\{NA\}_3\underset{\underset{R}{\vert}}{NA}\text{-}H, Na\right)^+$	1194.1	2a: recombination 2b: disproportionation and radical coupling (R-NA°)
3	1263.5	3a $\left(R\{NA\}_3\underset{\underset{R}{\vert}}{NA}\{NA\}_2H, Na\right)^+$ 3b $\left(R\{NA\}_2\underset{\underset{R}{\vert}}{NA}\text{-}\underset{\underset{R}{\vert}}{NA}\text{-}H, Na\right)^+$ 3c $\left(R\{NA\}_3\underset{\underset{R}{\vert}}{NA}\text{-}R, Na\right)^+$	1261.6	3a: disproportionation and radicals coupling (2 R°) 3b: disproportionation and radicals coupling (R° and R-NA°) 3c: recombination and radicals coupling (R-NA°)

R corresponds to the AIBN fragment obtained after thermal decomposition $CN(CH_3)_2C$- NA corresponds to one monomer unit.

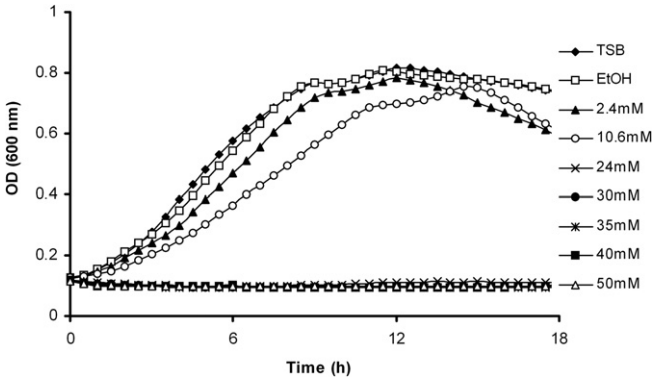


Fig. 5. Determination of the MIC of guaiacol.

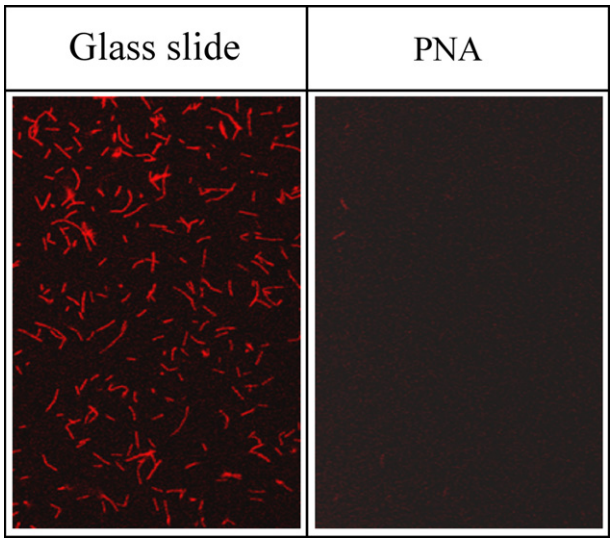


Fig. 6. CLSM images after an adhesion time of 3 h.

them, so that its effect does not reach distant areas from the material. Furthermore, the antibacterial effect of the immobilized component is mainly bacteriostatic as the agent cannot penetrate through the cell wall or membrane unlike free antimicrobials. Thus Rojo et al. [14] showed that eugenyl containing materials inhibit the growth of different bacterial species.

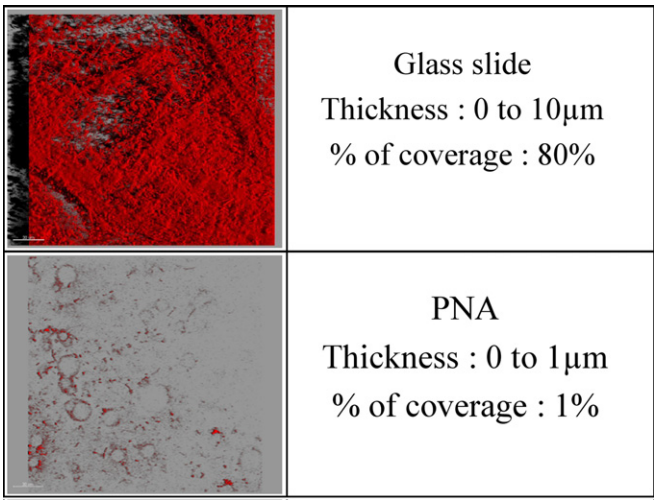


Fig. 7. CLSM images after 3 days culture of biofilm.

Park and co-workers [30] have synthesized vinyl monomers and polymers with phenol pendent groups. The antimicrobial activities were explored using the halo zone test after a contact time of 72 h. Surprisingly the polymerization of the monomers significantly decreased their antimicrobial activities. The authors stated that, even though the antimicrobial activity of the polymers is much lower than that corresponding of the monomers, they could be coated on glassy polymers. The concentration of antimicrobial agents on the coated polymeric material surface could then be higher than that on the surface of the polymer compounded with low molecular weight antimicrobial agent showing identical or even superior antimicrobial activity. That seems to be confirmed by the results we obtained with the only difference that we have used a glass surface instead of a glassy polymer surface.

Interestingly polymer surfaces containing quaternary ammonium salts have been clearly identified as biocidal surfaces leading to cell death by disrupting cell membranes allowing release of the intracellular contents. The killing effect was explained in that case by the highly charged surface that can induce what is essentially an ion exchange between the positive charges on the surface and structurally critical mobile cations within the membranes. Upon approaching a cationic surface, the structurally essential divalent cations of the membrane are relieved of their role in charge cations of the membrane components and are thus free to diffuse out of the membrane. The loss of these structural cations results in a loss of membrane integrity. Murata et al. [31] showed that the biocidal efficiency depends both on chain length and grafting density i.e. short chains with high grafting density and long chains with low grafting density were equally effective against E. Coli. We could then imagine a rather similar behavior of amphiphilic components of the membrane influenced by the guaiacol moiety of our PNA polymer grafted on a surface. The electrostatic interaction would then be replaced by hydrophobic and hydrogen bounding interactions.

4. Conclusion

A novel monomer (NA) derived from guaiacol was synthesized by Friedel–Craft alkylation between guaiacol and *N*-hydroxymethylacrylamide or with a three steps synthesis from vanillin. Conventional radical polymerizations of NA were carried out in solution at different temperatures using AIBN as the initiator. Average molar masses and polydispersity index of PNA were in the range of 3000–11,000 g mol^{-1} and 1.3–1.8, respectively.

Preliminary microbiological studies were carried out against *B. subtilis*. The planktonic tests showed that guaiacol has a MIC of 35 mM against 10^6 CFU ml^{-1} of *B. subtilis*. It was impossible to test the MIC of NA and PNA, because of their poor solubilities in aqueous alcohol solution. However, anti-adhesion test showed that PNA is highly effective against bio-adhesion of *B. subtilis*. Moreover, results of anti-biofilm tests suggested that PNA has strong anti-biofilm effects against *B. subtilis*. Thus NA and PNA could be potentially used in preparation of antibacterial materials.

The successful synthesis of a new antibacterial polymer from natural resources opens a new avenue toward development of a variety of renewable polymeric materials as potential replacement for petroleum-derived plastics. This work will be continued by grafting NA on polymer surfaces using grafting from methodology in the aim of inhibiting bacterial adherence and prevent biofilm formation [32].

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