Synthesis of (poly)gallic acid in a bacterial growth medium

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ABSTRACT

Bioelectronics requires versatile, efficient, and low-cost interfaces between the biological entities and the conductive unit. Conductive polymers represent a valid choice to assemble such interfaces able to extract or impinge charges between the biological units and the conductive electronic systems. A drawback in the use of such systems is that the polymerization reaction often takes place in environments whose chemical and physical characteristics clash with the mild conditions required for living biological systems. In the present work, we successfully prove that the conductive polymer poly(gallic acid) can be synthesized in medium designed for bacterial growth, characterised by the presence of several adverse conditions including numerous chemicals, high ionic strength, and almost neutral pH. The gallic acid successfully polymerizes within few hours and with a 40% yield, by exploiting the catalytic activity of the enzyme laccase from the polypore mushroom Trametes versicolor. The resulting polymer is characterised by absorption and Nuclear Magnetic Resonance spectroscopies. The viability of Rhodobacter sphaeroides culture, assessed via the coffee-ring technique, shows an important, but not complete detrimental effect of the gallic acid on the bacterial growth.

INTRODUCTION:

Clean energy production is a tight requirement for the growing needs of the population on Earth and for the sustainable development of the planet. A large scientific
community is actively looking at the exploitation of metabolically active bacteria to the purpose of producing environmental safe energy. This requires an intimate contact between ad-hoc designed devices able to collect from or donate to the bacterial cells charges produced by the metabolic activity. Such bioelectronic devices must be fully compatible with the living bacterial cells.[1-3] Soft conductive materials are often used as active films for interfacing the electrodes with the living cells or proteins.[4] Organic polyconjugated semiconductors [5-8] represent a valid option as conductive soft materials in biodevices. Among many conductive polymers, poly(gallic acid) (PGA) represents an interesting candidates as biocompatible interface for whole, living bacterial cells as its structure makes it quite adapt for interacting with the quinol/quinone moieties of several biomolecules.[9] The production of gallic acid dimers or polymers can be obtained with high yield and rapidly under several conditions that include electropolymerization and organometallic synthesis [10], which are too harsh for biological systems. In particular, organometallic processes exploit high temperature, prolonged timings of exposure to toxic organic solvents and the use of heavy metal salts, conditions which are not compatible for biological interfaces. Successful attempts show that PGA forms in presence of laccase, an oxydoreductase (EC number 1.10.3.2) obtained from the polypore mushroom Trametes versicolor, at pH 5.0 in acetate buffer at high ionic strength. Under the published condition, PGA forms with high yield using air bubbling. [11] The following data present the polymerization of the Gallic acid (GA) as a result of the enzymatic reaction of laccase in much milder conditions. Here, GA is solubilised and polymerised in a water solution containing all the nutrients required for the growth of bacteria, including organic and inorganic salts, in phosphate buffer that maintains the pH at 6.9. The results show that under the proposed conditions, the PGA forms in absence of external sources of feeding oxygen. with the good yield. The results indicate that the polymerization is amenable to be performed in growth media in the presence of metabolically active bacteria, including the photosynthetic anoxygenic bacteria under investigation for their relevance in energy conversion [12] and bioremediation [13-14] processes.

RESULTS AND DISCUSSION

Characterization of PGA

PGA was obtained after 1 day of reaction with laccase in the culture medium, starting from the water soluble gallic acid monomer, in a bacterial culture medium and under milder condition than those ones previously reported in literature. The prolonged time of reaction was used to ensure the maximal monomer consumption. An insoluble brownish precipitate was observed after completion of the reaction. Conversely to data reported in literature, about laccase-dependent synthesis of (poly)gallic acid at pH 5 in acetate buffer, which sets a 82% m/m yield after 24 h reaction, our polymer was produced with a 40% m/m yield. Once purified, PGA was characterized via $^1$H-NMR. As shown in Figure 1(a), PGA $^1$H NMR spectrum exhibits a shift in the position of the aryl peak with respect to the lone GA monomer from 7.14 to 7.03 ppm. This shift is related to the formation of a $\pi$-conjugated poly(arylene)s with terminal aryl protons. Furthermore, the presence of peaks at 6.65-6.55 and 6.42-6.33 indicates the co-presence of oxygen bridged teafavin like moieties (Figure 1(a)iii)[15] and catechin like structures (Figure 1(a)ii). These signals can be ascribed to intermediate complex structures reported due to collateral oxidative polymerization and found in the final polymer structure.[16] Moreover, the polyconjugated backbone emerges from the optical spectrum. The PGA absorption extends from 350 nm to 700 nm and above, while the flavin or catechin-like
moieties absorption is limited to 400 nm. In the specific case, the formation of C-C coupling was underlined by the increase of peak at 450 nm, which corresponds to π-π* transition.[17]

Figure 1: (a) $^1$H NMR of gallic acid (grey line; GA, D$_2$O, 500 MHz): s, 7.142 δ (ppm); $^1$H NMR of poly(gallic) acid (black line; PGA, D$_2$O, 500 MHz): s, 7.032; m, 6.58; m, 6.373 δ (ppm); (b) UV-vis spectra of GA and PGA.

**R. sphaeroides** biomass experiments using GA as monomer for feeding

*R. sphaeroides* bacteria were fed with gallic acid to check for cell viability at three different concentrations of the monomer, namely 0.5, 1 and 2 mg/mL in the presence of laccase (0.5 mg/mL). The formation of PGA composites at very late stage of cell growth was confirmed by variation of colour of the bacterial culture. Cell accumulation was monitored after an adaptation period of 12 h in the dark at room temperature. Figure 2 shows biomass accumulation of the *R. sphaeroides* control, and in presence of 0.5, 1 and 2 mg/mL of gallic acid monomer at the coffee ring interface with medium spotted over glass slides. A partial inhibition of the bacterial growth occurs in the presence of GA monomers, which increases with the increase of the initial GA concentration as qualitatively evidenced by the total biomass reduction [18] with respect to control growth.

Figure 2: Optical reflection microscopy images of biomass accumulation of *Rhodobacter sphaeroides* in (a) control experiment, in presence of (b) 0.5 mg/mL, (c) 1 mg/mL and (d) 2 mg/mL of gallic acid monomer at the coffee ring interface with medium spots on glass.

**EXPERIMENTAL SECTION**
Materials

Laccase from *Trametes versicolor* and gallic acid were purchased from Sigma Aldrich. Optical absorption spectra were recorded with a Cary 5000 UV-Vis-NIR spectrophotometer (Agilent), $^1$H NMR spectra were collected at room temperature on a Varian Inova or on a Bruker Avance AM at 500, using D$_2$O as solvent. Chemicals for the Bacterial growth medium are given below

**Bacterial growth medium**

The polymerization of the gallic acid was tested in the growth medium 27 of the German Collection of Microorganisms and Cell Cultures. This culture medium consists [19] of KH$_2$PO$_4$ (0.5 g), MgSO$_4$·7 H$_2$O (0.8 g), NaCl (0.4 g), NH$_4$Cl (0.4 g), CaCl$_2$·2 H$_2$O (0.05 g), D,L-malic acid (1.5 g), yeast extract (2.0 g), p-amino benzoic acid (1.0 mg), SL6 solution (1 ml), iron(II) citrate solution (5 ml), ethanol (1 ml), and deionized water up to 1000 ml. The pH of the medium was adjusted to 6.9 by adding drops of 5 M NaOH. The iron(II) citrate solution was prepared dissolving FeSO$_4$ (0.608 g), Na$_2$SO$_4$ (0.528 g), citric acid (1.680 g), and deionized water up to 1000 ml. To avoid rapid iron oxidation the solution was flushed with N$_2$ throughout the preparation. The SL6 solution contained ZnSO$_4$·7 H$_2$O (0.1 g/L), MnCl$_2$·4 H$_2$O (0.03 g/L), H$_3$BO$_3$ (0.3 g/L), CoCl$_2$·6 H$_2$O (0.2 g/L), CuCl$_2$·2 H$_2$O (0.01 g/L), NiCl$_2$·6 H$_2$O (0.01 g/L), and Na$_2$MoO$_4$·2 H$_2$O (0.03 g/L). All chemicals were purchased from Merck, while yeast extract was purchased from Sigma. The growth media and all experimental materials used were sterilized at 120 °C for 20 min.

**Growth of the bacterium Rhodobacter sphaeroides**

*Rhodobacter (R.) sphaeroides* is a Gram-negative bacterium belonging to the genus of Rhodobacteraceae class of the alfa-proteobacteria. It has a very versatile metabolism, including both anoxygenic photosynthesis and respiration. The wild type of this bacterium, *R. sphaeroides* 2.4.1, can grow photosynthetically in presence of a very low partial pressure of oxygen, while the carotenoidless mutant R26 – used in the present experiments – requires strict anaerobiosis for its photosynthetic growth. Bacterial cells were grown in the medium 27 of the German Collection of Microorganisms and Cell Cultures (DSMZ), described in details in a previous work.[20-21] *R. sphaeroides* was grown into 8 mL glass vials with a diameter of 1 cm. Vials were inoculated by ~ 0.4 ml of $10^9$ CFU/ml starting culture in exponential growing phase and completely filled. For the photosynthetic growth, the vials were kept in the dark for 4-6 h for allowing the residual dissolved oxygen to be consumed and then exposed to light. The cultures illumination was achieved by 100 W tungsten filament light bulbs placed at 25 cm from the vessels.

**Spectrophotometric investigation of laccase activity on GA-dimer production**

The optimal concentration of enzyme for the PGA biosynthesis was chosen by testing different amounts of laccase from *Trametes versicolor* (0.01, 0.05, 0.1, 0.5 and 1 mg) in 1 mL of the bacterial culture medium in the presence of 2 mg of gallic acid. The mixture was kept at room temperature in an open vial. The reaction was monitored spectrophotometrically after 30 minutes, by measuring the absorbance increase at 380 nm.[22] The band at 380 nm (see inset in Figure 3) is associated to the dimeric GA, which is easily detectable and it can be considered a precursor of the PGA. In Figure 3
the calibration curve obtained by fitting the amount of dimeric GA formed during the enzymatic reaction, as a function of the initial concentration of the enzyme has been reported. This curve was obtained at constant concentration of the GA substrate. Data fits to a Michaelis Menten function; laccase shows a $K_m$ value of 5.5 mM. The concentration of laccase chosen in the polymerization experiments is 0.5 mg of enzyme per 1 mL of culture medium. Higher amount would not increase the yield of PGA significantly.

**Figure 3:** (a) Absorbance changes in the first 30 minutes due to dimeric gallic acid formation recorded at 380 nm vs the amount of laccase added to the bacterial growth medium. The Michaelis Menten equation was fitted to the data and the fitting results is presented as continuous line. The typical absorption spectrum of the dimeric GA is presented in the inset; (b) Evolution of the polymerisation reaction at three different time intervals after GA addition to the solution; (c) chemical structures of GA monomer, hypothetical GA dimer and poly-GA.

**Purification and characterization of PGA**

PGA was allowed to polymerize for a full day in presence of laccase enzyme in the culture medium. After the reaction, samples were acidified with HCl 17% and extracted with ethyl acetate. After organic phase collection, the crude fraction was dried over anhydrous Na$_2$SO$_4$, filtered, and the solvent was vacuum pumped from the crudes and weighted. The reaction mixture lead to a PGA as brown solid in 40% yield.[23] PGA was characterized via $^1$H-NMR and UV-vis spectroscopy.
**R. sphaeroides viability in presence of ga as monomer feeding**

*R. sphaeroides* was grown in anoxygenic conditions. GA monomer was used in the starting inoculum in anoxygenic conditions, in DMSZ culture medium. Final concentrations of the GA monomer were set at 0.5, 1 and 2 mg/mL. GA polymerization was conducted after a cell optical density (OD) between 0.1 and 0.3 (final volume: 8.3 mL) in temporary oxygenic conditions, by introducing a laccase stock solution. After PGA formation in the culture medium, the qualitative evaluation of biomass accumulation was performed via coffee ring technique. A number of three spots of culture medium (3x15 µL) containing treated or bare cells were deposed on a glass slide and samples were analysed via optical reflection microscopy imaging. Biomass depth was qualitatively evaluated at the coffee ring of the medium formed over a glass slide.

**CONCLUSION**

The aim of the present work is the building of soft conductive polymers scaffold for interfacing microbial systems with electrodes. We tested the possibility of polymerizing gallic acid to form the conductive polymer (poly)gallic acid in a culture medium of general use in bacterial growth. The polymerization was successfully achieved by the use of the biocatalytic enzyme laccase with a yield of conversion of 40%.

The polymerization experiments were also conducted in a growing culture of the photosynthetic bacterium *Rhodobacter sphaeroides*. Although only qualitatively, it was possible to show that notwithstanding the gallic acid inhibits the bacterial growth, not negligible amount of bacterial biomass does accumulate in treated samples. The assembly of (poly)gallic acid in very mild chemical and physical conditions allows to tackle the polymerization using an enzymatic synthetic approach in aqueous solution, which are basically set for bacterial growth. The synthesis of PGA under these conditions can be considered sustainable and with very little ecological impact.

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