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Photonic lab-on-a-chip containing microbial-based alginate light waveguides for phenolic compounds determination

David Sanahuja Esteve

Supervised by Dr. Xavier Muñoz Berbel, (CNM)
Co-supervised by Prof. Nuria Vigués Frantzen, (UAB)
Co-supervised by Dra. Crina Maria Cojocaru, (UPC)

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Photonic lab-on-a-chip containing microbial-based alginate light waveguides for phenolic compounds determination

David Sanahuja Esteve
Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona (UAB), Edifici C, Bellaterra (Cerdanyola del Vallès) 08193, Spain.
Centre Nacional de Microelectrònica (IMB-CNM, CSIC), Bellaterra (Cerdanyola del Vallès) 08193, Spain.
E-mail: david.sanahuja@e-campus.uab.cat

Abstract. Phenolic compounds are one of the main causes of environmental pollution of soils and water for their toxicity and persistence. Currently, phenolic compounds detection is principally done with bulky and expensive equipment (e.g. HPLC) in the laboratory environment. This detection requires sample transport and sometimes pre-treatment which makes this determination long and expensive. An integrated, low-cost, portable and robust system for quick in situ determination of phenolic compounds would be, therefore, very desirable. According to this, the objective of this work is to develop a biophotonic lab-on-a-chip with an organic matrix of alginate incorporating genetically modified E.coli trapped inside to determine the presence of phenolic compounds, and particularly catechol, in water samples. The alginate hydrogel will be used as a light guide, confining the light inside of it. For this reason, physico-chemical properties of alginate hydrogels (e.g. optical, diffusional, structural, etc.) containing different alginate concentrations with and without bacteria were evaluated and optimized. Finally, the alginate light guide containing bacteria was used for catechol determination in water samples. This architecture should open the possibility for in situ determination of phenolic compounds.

Keywords: Photonic lab-on-a-chip, sodium alginate, genetically-modified bacteria, alginate/bacteria light guides, phenolic compounds.

1. Introduction.
Industrialization and massive use of fossil fuels such as oil are among the main causes of environmental pollution in soil and water. Some of the most dangerous pollutants, as much as for its toxicity as for its persistence in the environment, are phenolic compounds. These compounds, many of them hydrocarbon degradation products, have very harmful effects on the ecosystem that they pollute and are very difficult to remove. Currently, the detection of these compounds is mostly done with laboratory equipment, mainly chromatographic systems such as High-performance liquid chromatography (HPLC) [1]. Although these machines are very accurate, they are also expensive and bulky, making immediate data collection difficult and, consequently, delaying a possible action to prevent an environmental catastrophe. The design and manufacture of portable and low cost biosensors and bioassays appears as an interesting alternative to be considered.

From all them, lab-on-a-chip (LoC) architectures are currently positioning as one of the most attractive alternatives to traditional methods. A LoC is a kind of device, which includes one or more steps of the chemical analysis process. Such devices are generally cheap, robust and small sized (from only few square millimeters to centimeters), and have been applied to a large number of fields, ranging from environmental monitoring to clinical analysis [2, 3]. Initially,
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fabricated on silicon or glass, most of current LoCs are made on much cheaper and easy-to-use polymeric materials such as polydimethylsiloxane (PDMS), SU-8 or methacrylate, among others [4].

In this regard, I propose the development of a biophotonic LoC with an organic matrix of alginate containing genetically modified microorganisms for in situ determination of catechol. Alginate is a viscous polysaccharide (sugar), which, in the presence of calcium (or other divalent ions), is capable to produce a porous and stable gel matrix (hydrogel) [5, 6, 7]. Alginate is selected for presenting ideal properties for being the constituent material of the biophotonic LoC: (i) high transmittance in the VIS range, (ii) biocompatibility and low toxicity, (iii) gelation under soft reaction conditions, which allows for living cells trapping, (iv) high diffusion of hydrophilic compounds, (v) homogeneity and (vi) manipulation simplicity [8]. To selectively respond to the phenolic compounds, genetically modified microorganisms, Escherichia coli (E.coli) PtomAB Amp 100, are incorporated in the alginate matrix which confines the light enhancing analyte/light interaction. This microorganisms contains an enzymatic cascade that allows for the degradation of mono or bi aromatics hydrocarbons (such as catechol) to 2-hydroxymuconic semialdehyde [9, 10, 11], a compound with a clear absorption band at \( \lambda = 419\text{nm} \). Thus, phenolic compounds of the sample of interest may easily diffuse through the alginate matrix and reach the microorganisms that degrade them producing the absorbent molecule. This device may act as alarm system, reporting quickly and simply the presence of phenolic contamination in a sample. The use of this low cost, simple, stable and portable device opens the possibility for in situ determination of contaminant presence, reducing then, the environmental damage caused by accidental spills.

2. Materials and methods.
The materials used in this work are detailed below:
- *Escherichia coli (E.coli) PtomAB Amp 100*;
- Alginate (different concentrations);
  - Acid alginic sodium salt (Aldrich Chemistry);
  - H₂O (Milli-Q);
- LB (Luria-Bertani);
- Anhydrous Calcium Chloride (CaCl₂) (different concentrations) (Panreac);
- Calcium sulphate (CaSO₄);
- Calcium carbonate (CaCO₃);
- Pyrocatechol (C₆H₄-1,2-(OH)₂) (different concentrations) (Sigma Aldrich);
- Ferricyanide (40mM);
  - Potassium Hexacyanoferrate (III) (K₃[Fe(CN)]₄) M=329.26g/mol (AppliChem Panreac);
  - H₂O (Milli-Q);
- Phosphate buffered saline (PBS).
  - Phosphate buffer (0.1M).
    - 2.625g potassium di-hydrogen phosphate (KH₂PO₄) M=136.09g/mol (Panreac)
    - 7.01g di-potassium hydrogen phosphate 3-hydrate M=228.22g/mol (Panreac)
    - 500ML H₂O (Milli-Q);
- Ringer (9.0%);
  - 9g Sodium Chloride (NaCl) (Panreac);
  - 1L H₂O (Milli-Q);
- Halogen light source (HL-2000-FHSA, Ocean Optics);
- Spectrometer (USB2000+XR extended range, Ocean Optics, wavelength range 200-1025nm, resolution 2nm FWHM, 20dB) with a control software (SpectraSuite 2008, Ocean Optics);
- Spectrophotometer (Smart Spec Plus Spectrophotometer, BIO-RAD) with a control software (Hyperterminal, Hilgraeve);
- ELISA plate reader (Multiskan Ex Primary EIA V. 2.3, Thermo Electron Corporation) with a control software (Ascent Software V.2.6. for Multiskan, Thermo Scientific);
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- Optical fibers (Thorlabs, core=200nm, cladding 230nm, NA=0.22);
- Optical engineering software program (TracePro® software, Lambda Research Corporation);
- Orbital water-bath shaker (Aquatron, HT Infors AG);
- Centrifuge (Centrifuge 5804R, Eppendorf);
- Vortex mixer (ZX Wizard, FB15013 Top Mix, Fisher Scientific);
- Magnetic stirrer (RCT basic safety control, IKA);
- Classic Balance (Classic PB 3002-S, METTLER TOLEDO);
- Antibiogram discs.
- polymethylmethacrylate (PMMA) alginate discs mould;
- PMMA straight waveguide system (figure 1 (b));
- PMMA lab-on-a-chip;

The phenolic compound employed in this work is the pyrocatechol (or catechol). This compound diffuses through the alginate hydrogel matrix with microorganisms trapped inside. In this case, these are genetically modified *Escherichia coli* with a plasmid containing a gene tomAB, able to degrade catechol, and the gene for resistance to ampicillin, 100 Amp.

With a spectrometer (USB2000+XR extended range, Ocean Optics), the detection of the absorption band, which indicates the presence of the catechol (actually, the detected compound is the degradation product of the catechol), is possible. It is at a wavelength of 419nm.

The hydrogel was used as a light guide. That light was confined and transmitted through the alginate matrix. For this reason, physico-chemical properties of the alginate hydrogel containing different concentrations of alginate (between 0.5 and 2.0% alginate) were evaluated and optimized.

### 2.1. Microorganism characterization

These genetically-modified bacteria were supplied by Dra. María Sánchez Contreras from the Universidad Autónoma de Madrid. Bacterial metabolic activity was characterized using the ELISA (enzyme-linked immunosorbent assay) plate reader. The production of 2-hydroxymuconic semialdehyde for different *E.coli* (0, 10⁷, 10⁸ and 10⁹ CFU/mL, where CFU is the abbreviation for colony-forming units, an indirect plate count which only takes into account the living cells in the sample) and catechol concentrations (0.0%, 0.1%, 0.2%, 0.5%, 1.0%, 2.0%, 4.0% and 8.0%) was monitored with time. Optical measurements were performed at 405nm instead of 419nm due to the lack of a more suitable filter in the instrument. Unfortunately, biomass scattering interfere in the determination of cell metabolism at 405nm and an additional measurement at 620nm was necessary to correct the biomass influence, as detailed later (section 3.1). After correction, absorption at 405nm was only caused by the degradation product of the catechol.

Next, to observe how the alginate affects absorbance, the previous procedure was repeated but, this time, with cells trapped in a 0.5% alginate matrix. In this case, absorbance at 620nm, where catechol degradation products did not absorb, allowed us to eliminate the scattering produced by alginate matrix gelation and biomass.

Although bacterial assays are usually performed with phosphate buffered saline (PBS) or Ringer medium (sodium chloride), both culture mediums present important drawbacks in this case (phosphate prevented alginate gelation and sodium chloride in the alginate matrix affected optical measurements). For this reason, in this work, alginate was always prepared with Milli-Q water.

### 2.2. Optical properties of hydrogel alginate

To understand how the alginate hydrogel confines the light and how the light passes through an alginate light guide, absorption spectrum and refractive index of the alginate matrix at different concentrations was determined.

Refractive index determination was very challenging. Several processes were performed without exciting results. First, ellipsometry was not suitable since required very thin layers that,
for the own nature of alginate, dehydrate too fast to be able to provide any reliable data. Second, using index matching (changing the refractive index of the medium until matching with the refractive index of the material), results were not consistent. Finally, the refractive index was indirectly determined by comparing the aperture of the light crossing hydrogels with different alginate concentrations with theoretical data from ray tracing simulations. Experimental data were obtained using the setup showed in the figure 1 (a) and alginate discs of approximately 9mm diameter and 5mm thickness. These discs were made using a mould. The fabrication process consisted of: (i) introduction of an antibiogram paper in the bottom of the mould, (ii) inoculation of 60µL of 200mM CaCl$_2$ and, subsequently, (iii) inoculation of 180µL of the corresponding alginate, depending of the concentration you want. Antibiogram paper in the bottom allowed for slow diffusion of the CaCl$_2$ into the alginate ensuring homogeneous gelation. With suitable imaging systems, it was possible to determine the aperture of the light beam after crossing the alginate disc. Alginate discs with concentrations between 0.5% and 2.0% were analysed. Experimental data was compared with theoretical aperture values considering discs of the same size and known refractive indexes ranging from 1.33 (water) to 1.62. Theoretical values were obtained with the ray tracing simulation TracePro®.

On the other hand, absorption spectra of alginate matrices at different alginate concentrations were determined also using two protocols. First, the setup showed in figure 1 (a), using air spectrum as reference, and second the one showed in figure 1 (b). In this second case, absorption spectra of alginate at different concentrations (taking water as reference) were also compared with non-gelified alginate samples.

![Figure 1.](image)

Figure 1. (a) Alginate disc between two fibers (the output fiber is connected to a spectrometer, and the input fiber to a halogen light source), to measure the spectre of the light when crosses the alginate disc. (b) PMMA straight waveguide system between two fibers (the output fiber is connected to a spectrometer, and the input fiber to a halogen light source), to measure the spectre of the light when crosses the alginate guide.

2.3. Physical properties of hydrogel alginate

Relevant physical properties of the alginate matrix were analysed. That is, gelation homogeneity, gelation kinetics and diffusion.

In terms of homogeneity, three different gelation inducers were tested: CaSO$_4$, CaCO$_3$, and CaCl$_2$. From all of them, the one providing with the most homogenous matrix was CaCl$_2$, which was selected as the ideal inducer.

Gelation kinetics was determined using the system showed in figure 1 (b). For this purpose, alginate and CaCl$_2$ were mixed in the liquid reservoir and optical measurements were performed every 30s for the duration of the experiment (10-15min). In order to determine the effect of CaCl$_2$ concentration in the gelation process, samples containing 0mM, 0.2mM, 0.4mM, 0.8mM, 4mM, 10mM, 20mM, 40mM, 100mM, and 200mM CaCl$_2$ were evaluated. Next, gelation process was evaluated by changing the concentration of alginate (0.5%, 0.7%, 1.0%, 1.5% and 2.0%). Finally, gelation kinetics of the alginate samples without bacteria were compared with alginate samples containing 10$^9$ CFU/mL _E.coli_ to determine the influence of cells in the gelation process.
Lastly, using the same setup as before (figure 1 (b)), diffusion kinetics of the alginate matrices were analysed using ferricyanide (40mM). Ferricyanide is a small molecule with an intense absorption band at 420nm. The measurement protocol consisted of inoculation of ferricyanide in already gelified and stable alginate matrices containing different alginate concentrations (0.0%, 0.5%, 0.7%, 1.0% and 2.0%). Ferricyanide diffusion was determined by monitoring the changes at 420nm.

2.4 Catechol determination using the biophotonic LoC
Alginate/bacteria matrices were used to determine the catechol concentration on water samples. For this reason, alginate/calcium/bacteria mixtures were introduced into the biophotonic LoC until homogenous and stable gelation. After that, catechol samples (ranging from 0.0 to 2.0% catechol) were inoculated into the LoC and the degradation product formation was monitored at 419nm with time. Bacterial metabolism kinetics was used for fast determination of catechol concentration in water samples.

3. Results and discussion.
The obtained results are detailed below:

3.1. Microorganism characterization
As previously detailed, the possibility to use the genetically-modified *E.coli* bacteria was first evaluated by monitoring the formation of the metabolic product 2-hydroxymuconic semialdehyde with time. In figure 2 (a), the variation of absorbance at 405nm is monitored with time by different catechol concentrations. As shown, absorbance magnitude increased with time due to the bacterial degradation of catechol and the formation of the 2-hydroxymuconic semialdehyde with a wide absorption band around 419nm. Additionally, this absorbance increase is proportional to the catechol concentration until 1.0% catechol. This fact is even clearer when analysing figure 2 (b) where the slope (variation of the absorbance magnitude with time) is represented against the catechol concentration using three different bacterial concentrations. It is clearly observed that there is a linear relationship between slope and catechol concentration until 1.0% catechol for all bacterial concentrations. From that point, the toxic effect of catechol is too high and progressively kills bacteria. This result confirms the possibility to use these bacteria as catechol reporters at least for low catechol concentrations.

Once demonstrated the viability to use these bacteria as catechol reporters, the same viability should be demonstrated under the alginate matrix. For this, identical experiments were performed but, in this case, including catechol and CaCl$_2$ (the gelation inducer) to generate the hydrogel inside each well. In this case, apart from the previously observed absorbance changes associated to the bacterial activity, the alginate gelation process also produce a variation in the absorbance magnitude at 405nm that hardly interfere the monitoring of bacterial metabolism. For this reason, biomass and alginate interference was removed by using the measurement at a second wavelength (620nm) where the 2-hydroxymuconic semialdehyde did not absorb.

At 405nm the absorption is as:

$$\text{Abs}_{405} (\text{A.U.}) = \text{Abs}_{405}^{\text{cat}} (\text{A.U.}) + \text{Abs}_{405}^{\text{alginate}} (\text{A.U.}) + \text{Abs}_{405}^{\text{E.coli}} (\text{A.U.})$$  (1)

At 620nm the absorption is as:

$$\text{Abs}_{620} (\text{A.U.}) = \text{Abs}_{620}^{\text{alginate}} (\text{A.U.}) + \text{Abs}_{620}^{\text{E.coli}} (\text{A.U.})$$  (2)

The obtained slope of the calibration line (figure 2 (d)) is as:

$$\text{Abs}_{620}^{\text{interf}} (\text{A.U.}) = 0.8569 \cdot \text{Abs}_{405}^{\text{interf}} (\text{A.U.}) - 0.1044$$  (3)

Then, the absorption at 405nm, only caused by the degradation of the catechol is, in this case, as:

$$\text{Abs}_{405}^{\text{cat}} (\text{A.U.}) = \text{Abs}_{405} (\text{A.U.}) - \frac{\text{Abs}_{405}^{\text{interf}} (\text{A.U.}) - 0.1044}{0.8569}$$  (4)

Where the interferential absorption is as:

$$\text{Abs}_{\text{interf}} (\text{A.U.}) = \text{Abs}_{\text{alginate}} (\text{A.U.}) + \text{Abs}_{\text{E.coli}} (\text{A.U.})$$  (5)
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Unfortunately, even with this correction, it was not possible to obtain a clear correlation between absorbance at 405nm and catechol concentration when alginate was not stably gelified due to the heterogeneity and wavelength-dependence of the gelation process. Thus, an alternative system should be prepared to be able to determine catechol concentration with this approach.

Figure 2. (a) Absorbance magnitude variation (at 405nm) with time at different catechol concentrations for genetically modified bacterial suspensions (E.coli $10^8$ CFU/mL). (b) Obtained slopes of the absorbances for each used concentrations of E.coli. (c) Absorbances of the degradation product of the catechol (E.coli $10^8$ CFU/mL), in alginate (0.5%) at 405nm (noise cleaned). (d) Calibration line for the absorbances for each used concentrations of E.coli in alginate 0.5% (cat = 0.0%, time = 0).

### 3.2. Optical properties of hydrogel alginate

Considering the fact that alginate should be the constituent material of the biophotonic LoC, its optical properties should be carefully analysed. In this case, absorbance spectrum and refractive index were determined as detailed in previous sections. In terms of refractive index and as already exposed, it was very difficult to determine with conventional methods and a heterodox protocol was used. It consists of preparing discs with different alginate concentrations and injecting light on them. Then, determining the aperture width of the transmitted light cone (which depends on the refractive index of the material) and comparing this with theoretical values from ray tracing simulations. Theoretical aperture widths (figure 3 (a)) from simulated ray tracing (figure 3 (b)) showed a clear linear relationship with the refractive index of the constituent material of the disc, as shown in figure 3 (d). With the experimental set-up detailed in the experimental section, it was possible to obtain pictures of alginate discs with different alginate concentrations (figure 3 (c)) and to determine their experimental aperture widths. Figure 3 (e) shows a linear correlation between experimental aperture width and the alginate concentration of the disc. When compared with theoretical data, it is possible to obtain the refractive index of each alginate concentration. According to figure 3 (f), the refractive index of these samples increase from 1.4 to 1.6 with the alginate concentration. These values ensured light confinement into the alginate matrix since it is much bigger than the medium (around 1.33).
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Figure 3. (a) Simulations of the flux of the light crossing the alginate discs with different RI (1.33 and 1.51). (b) Simulation of the light beams crossing an alginate disc (RI=1.33). (c) Light crossing the discs of alginate (0.5% and 1.0%) and CaCl₂ (0.2M). (d) Approximated simulated aperture widths vs. RI. (e) Experimental aperture widths of the alginate discs. (f) Approximated refractive index, for each alginate disc.

Figure 4. Absorbances of the alginate (concentrations between 0.5% and 2.0%), between 300 and 900nm: (a) Alginate discs (air), (b) Gelified alginate straight guide (H₂O).

Absorbance spectra of each alginate concentration were obtained using two set-ups as explained in the experimental section: (i) the alginate discs (reference = air) and (ii) a methacrylate system where alginate gelifies generating a straight light guide (reference = water). In both cases, all alginate samples showed similar spectra with a wide absorption band around 400nm. Surprisingly, the intensity of this band did not depend on the alginate concentration and it is more accentuated after the gelation process (it is almost inexistent in non-gelified alginate). Thus, this band may be associated to the gelation process, probably to the formation of small scattering centres (porous) of around 400nm in the alginate matrix. Although this absorption band coincides with the absorption peak of 2-hydroxymuconic semialdehyde, the absorption magnitude of this band (below 0.3 A.U. in all cases) should not be a problem in the catechol determination (if it does not change during catechol determination). For this, a complete characterization of the gelation process would be necessary.
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3.3. Physical properties of hydrogel alginate

As previously stated, the stability of the alginate matrix is essential for the use of this compound as constituent material of the biophotonic LoC. For this reason, the gelation process was monitoring with time by introducing alginate and CaCl$_2$ in the methacrylate system already detailed and monitoring optical changes. The variation of light intensity at 450nm (at 405 the number of counts was too small) was monitored with time for different CaCl$_2$ and alginate concentrations (figure 5 (a) and (c)). As shown, in all cases intensity decrease with the gelation process until stabilization at around 10 min. The gelation process seems to be quite complex and heterogeneous and random increases and decreases are recorded during the process in all cases. However, the homogeneity of the final matrix differed depending on the calcium and alginate concentrations. Thus, homogenous matrices after 10 min of gelation were only obtained by 100 mM CaCl$_2$ and alginate concentrations below 1.0% and thus, 100mM CaCl$_2$ with 0.5% alginate concentrations, were considered optimal and used for the preparation of all biophotonic LoCs.

![Figure 5](image)

When comparing the gelation process with ($10^9$ CFU/mL E.coli) and without bacteria, no relevant differences were observed: in both cases light intensity decreases with time until stabilization around 10 min (figure 6). Thus, the presence of bacteria did not significantly affect the gelation process.

Another important property to be evaluated is the diffusion of hydrophilic molecules inside the alginate matrix. Figure 7 (a) shows the light intensity decrease associated to ferricyanide diffusion into the alginate matrix. Unexpectedly, diffusion is much faster with high alginate concentrations (1.5 and 2.0%) than with low concentrations (from 1.0 to 0.5%). This may be associated to the heterogeneity of the high concentration alginate matrices that may favour ferricyanide diffusion. In figure 7 (b) the ferricyanide peak is shown to increase with time during diffusion, which links the light intensity decrease with ferricyanide diffusion into the matrix (0.5% alginate).
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Figure 6. Gelation kinetics, at 450nm, of the 0.5% alginate with CaCl$_2$ 100mM and the E.coli $10^9$ CFU/mL.

Figure 7. (a) Diffusional kinetics, at 420nm, of the ferricyanide 40mM through the alginate (concentrations between 0.5% and 2.0%) with CaCl$_2$ 100mM. (b) Absorbance variation (0.5% alginate) during the time (253s) at each wavelength between 350 and 800nm.

Figure 8. (a) Degradation kinetics, at 419nm, of the catechol (concentrations between 0.0% and 2.0%) through the alginate (concentrations between 0.5% and 2.0%) with CaCl$_2$ (100mM) and the E.coli ($10^9$ CFU/mL). (b) Slope variation with the concentration of catechol.

3.4 Catechol determination using the biophotonic LoC

Finally, homogenous matrices containing genetically-modified bacteria and introduced in a PMMA biophotonic LoC were used for the determination of catechol in water samples. For this purpose, homogeneous matrices of 0.5% alginate containing $10^9$ CFU/mL genetically-modified E.coli were prepared under optimal conditions (100mM CaCl$_2$ for 10 min). After suitable gelation, water samples containing catechol were inoculated into the biophotonic LoC and the degradation kinetics was optically monitored at 419nm. The degradation kinetics of several catechol samples (between 0.0% and 2.0% catechol) was analysed using this protocol. Experimental results are plotted in figure 8 (a). In this figure, light intensity at 419nm is shown...
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to decrease with time for all catechol concentrations different to 0. Additionally, the intensity decrease was directly proportional to the catechol concentration until 1.0% catechol. As shown in figure 8 (b), there is a clear relationship between the slope (intensity at 419nm versus time) and the catechol concentration until 1.0% catechol. Since this plot has been obtained considering just the first 10 minutes of degradation, this approach represents a low cost, simple and robust alternative for quick determination of catechol (10 min assay) in water samples. The sensitivity and quick response of the system open the possibility to in situ determination of phenolic compounds contamination.

Alginate hydrogels have some very interesting characteristics for the development of photonic biosensors as, for example: (i) alginate hydrogel can be structured in a mould, (ii) it can gelificate at room temperature (iii) it is biocompatible and non toxic, (iv) the microorganisms (E.coli) inside of the alginate structure can be maintained in good conditions along large periods of time, (v) alginate has a RI higher than the RI of the medium (allowing light confinement), (vi) it has low absorption in the visible spectral range, (vii) and it is hydrophilic and porous, allowing fast diffusion of analytes (in this case, catechol). Alginate hydrogel and the genetically-modified E.coli has been characterized, considering optimal these hydrogels with high homogeneity, low absorption in the VIS range, fast gelation process and where substrates can diffuse fast enough to allow a quick response of the system.
All these characteristics allow the development of a small size alginate-based PMMA device, with a microbial-based alginate hydrogel matrix acting as a light guide, which could be used for in situ determination of the presence of phenolic compounds in the medium.

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References