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CHARACTERIZATION OF AGAROSE AS IMMOBILIZATION MATRIX MODEL FOR A MICROBIAL BIOSENSOR

Microbial biosensors are promising tools for the detection of specific substances in different fields, such as environmental, biomedical, food or agricultural. They allow rapid measurements, no need for complex sample preparation or specialised personnel and easy handling. In order to enhance the managing, miniaturization and stability of the biosensor and to prevent cell leaching, bacteria immobilization is desirable. A systematic characterization procedure to choose a suitable immobilization method and matrix, was proposed in this study. Physical properties, storage stability, mass transport phenomena and biocompatibility were evaluated, employing agarose as the model matrix. Preliminary essays with bioluminescent bacteria detecting Tributyltin were also carried out.

The immobilization of bacteria is fundamental in order to achieve an efficient biosensor, miniaturized, stable, containing an optimal cell concentration, protecting bacteria from inhibitors and shear stress.

Different immobilization procedures are available, such as adhesion, adsorption, inclusion in carbon paste, containment within membranes, entrapment/ /encapsulation [1,2,3]; among these, entrapment offers the best potential in terms of stability, biocompatibility, mechanical resistance, chemical resistance, cell leaching, diffusion, sensibility, transparency, simplicity and cost [3]. Natural biopolymers are the most employed matrices for microbial biosensors, due to their biocompatibility and simple procedure, despite their poor resistance and stability [2,5,6,7]. Nevertheless, synthetic polymers show interesting potential although complex procedures and lower biocompatibility [8,9,10].

The "quality" and the "effectiveness" of the immobilization system are usually evaluated indirectly, from the biosensor output [2,6,8]. Nevertheless, the intrinsic characteristics of the immobilization matrix and its suitability to microbial biosensors has never been systematically analyzed. A characterization procedure of the immobilization matrix for a luminescent biosensor detecting pollutant agents in water was set up in this work. The rheological, mechanical and optical properties were studied, being fundamental for bacteria immobilization, polymer handling and signal transmission. Storage and biodegradation were also monitored, as the biosensor could be employed in marine/fluvial environment and wastewater. In order to verify whether the analytes would diffuse easily through the membrane, adsorption and diffusion were studied and modeled, with the purpose of prediction and optimization.

Agarose was chosen as the model matrix, showing high biocompatibility, transparency and simple thermal gelation [5,11,12]. Preliminary essays with bioluminescent bacteria detecting tributyltin [13,14] were also carried out. Some properties were compared with alginate, a natural biopolymer most commonly employed for cell encapsulation.

MATERIALS AND METHODS

Low-melting agarose (SoBiGel, Hendaye, France) solutions were prepared by dissolving 2% and 4% w/w of agarose in distilled water at 70°C. After homogenization, the solution was poured in plexiglass moulds. The polymer hardened in less than one hour, resulting in transparent and homogeneous membranes, with an average thickness of 5 mm.

Alginate membranes were prepared by pouring 2% w/w sodium alginate solution in the moulds, then immersed in 1% w/w $CaCl_2$ solution for gelation, in order to form opaque, non-homogeneous, multi-layered membranes.

The viscosity of 2% and 4% agarose solutions was measured using an AR100 Viscosimeter. The solutions were kept at 40°C during the measurement, in order to prevent thermal gelation. Agarose being a pseudoplastic solution, the value of the viscosity for the maximal shear rate (900 s^{-1}) was reported herein.

The mechanical resistance of 2% and 4% agarose membranes was measured using a Lloyd texture analyzer, by compressing the gel sample until rupture. The measurements were repeated in triplicates.

The transparency of the membranes was measured with a highly sensitive Raman Spectrometer, at the PCE laboratory of Le Mans; the average values of

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the transmission between 490 nm and 510 nm (the bacterial luminescence wavelength range) were reported.

The storage stability was tested through visual observation during storage in seawater drawn from Termoli harbour (Italy), in order to verify the membrane stability in marine environment.

Biodegradation was evaluated immersing membranes in conical flasks containing 100 ml of mixed liquor drawn from an activated sludge wastewater treatment reactor. The suspension was kept aerated by sparging compressed air through ceramic diffusers for seven days. The integrity of the membranes was checked daily by visual observation.

Tests of metal ion adsorption were carried out with copper ions, which are often found in seawater and wastewater. Membranes were immersed in beakers containing copper chloride solutions at different concentrations between 60 mg/l and 800 mg/l, with a w:w ratio of membrane:solution of 1:3, then 500 μm samples were drawn every hour from the bulk solution and the copper ion content was measured by atomic absorption spectrometry. The performance of the agarose membranes was compared with that of alginate membranes.

For diffusion tests, a cell was designed in Plexiglas, with two stirred chambers, A and B, separated by a window (having a surface of 16 cm^2), containing the cast membrane. Three components were chosen as models for the diffusing compounds: copper chloride for heavy metals, potassium chloride for inorganic salts and paranitrophenol for aromatic compounds. Chamber A was filled with 120 ml of solution with the diffusing compound, chamber B was filled with 120 ml of distilled water. Samples (500 μl) were drawn from both chambers every hour. Atomic absorption spectrometry was employed to measure the copper ion content, a CD810 Tacussel conductometer to measure the KCl content and an Unicam UV spectrometer at 316 nm to measure the paranitrophenol concentration. The percentage of diffused compound was then calculated and the results fitted using a solution–diffusion model.

A preliminary test with immobilized bacteria was performed at the CBAC laboratory of La Roche sur Yon, employing a specific strain, *E. coli TBT3*, emitting light in the presence of tributyltin [3,13]. After overnight culture in glucose medium, two sets of microbial suspensions were centrifuged for 5 minutes at 7000 g, in different volumes in order to obtain cell optical densities between 0.1 and 0.5. The first set was suspended in 2% low-melting agarose at 35°C, the second set was suspended in $MgSO_4$ 0.1M, then both were cast in microtiter-wells (Nunc™) and in spectroscopy cuvettes for OD measurements by UV spectroscopy at 620 nm. Each well was injected with 150 μl of TBT (Sigma Aldrich) solution at concentrations between 0 and 5 μM. The microtiter plates were then incubated at 30°C: the suspended bacteria were induced for one hour and immobilized bacteria for two hours. n-Decyl aldehyde (25 μl) (Sigma Aldrich) 60 μM was introduced, following the procedure already set up for bioassays [13] and the luminescence was measured by microtiter plate luminometer (Microlumate L96V EGG Berthold). The measurements were repeated in triplicates.

RESULTS AND DISCUSSION

Rheological, mechanical and optical properties

The values of the viscosity, mechanical resistance and transparency of 2% and 4% agarose samples are shown in Table 1. As may be observed, the agarose concentration does not significantly influence the membrane transparency, which remained excellent. Nevertheless, a compromise had to be found between a higher mechanical resistance and a lower viscosity. 2% solutions were preferred, as they allowed easier handling and simpler membrane formation.

Table 1. Properties of the agarose solution and membranes

Agarose (%)	Viscosity at 900s^{-1} (Pa·s)	Mechanical resistance (kN/m^2)	Transparency at 490–510 nm (%)
2	0.09	26.7	99.95
4	0.85	55.4	98.95

Storage stability and biodegradation

After six weeks of storage in seawater, the agarose membranes maintained their integrity, without swelling, while the alginate membranes swelled and fell apart.

After one week in aerated activated sludge suspension, the agarose membranes maintained their integrity, but presented a thin film of microorganisms adhering on the surface, thus allowing light detection only from the side of the membrane not exposed to activated sludge. Alginate membranes showed a thicker biofilm and slow degradation.

Adsorption

Assays were carried out with agarose and alginate membranes for 20 days at different $CuCl_2$ concentrations. In order to better compare the results, the amount of Cu^{2+} adsorbed on the matrix (qads, expressed as $g_{Cu++}/g_{membrane}$) was calculated as follows:

$$q_{das} = (VC_0 - V_bC_{bulk})/m_{membr}$$

where V_0 and C_0 (expressed as $g_{Cu++}/l_{solution}$) are the volume and copper concentration of the bulk solution at the beginning of the experiment (t=0), V_b and C_{bulk} (expressed as $g_{Cu++}/l_{solution}$) are the volume and copper concentration of the bulk concentration at time t and m_{membr} is the mass of the membrane. The experimental data can be fitted using Freundlich's

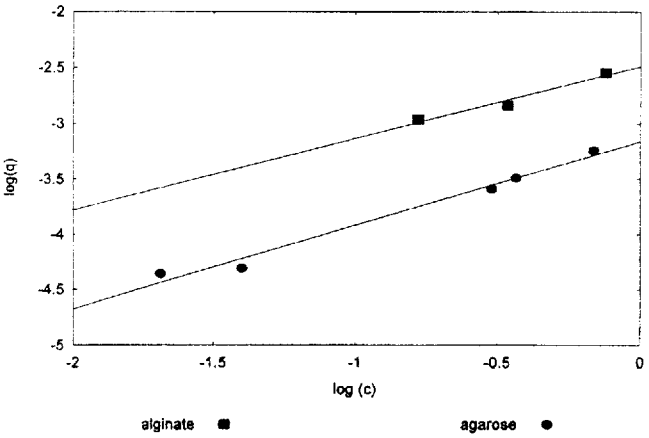

Figure 1. The adsorption of copper ion on alginate and agarose membranes. The experimental data were fitted using a Freundlich isotherm.

Table 2. Adsorption coefficients obtained by interpolating the exprimental data using a Freundlich isotherm

	Agarose	Alginate
Ka (l/g)	0.0007	0.0032
n	0.75	0.65

isotherm ($q = K_a c^n$) as shown in Figure 1. The values obtained for the coefficients are shown in Table 2. The higher adsorption capacity of alginate, containing COOH groups with an average pKa=3.5, may be due to the copper ions binding to the free COO^- groups, present in the matrix even after being reticulated with Ca^{2+}, or to Ca^{2+} replacement by copper ions. In any case, alginate seems inadequate as a matrix for environmental biosensors detecting metals or organometallic compounds.

Diffusion

In order to better compare the results, they are shown as the ratio of the concentration measured in chamber B, diffused through the membrane at different times, over the maximal concentration, which can be reached at the equilibrium ($C_{A0}/2$). A simple solution–diffusion model was proposed. The following differential equation was thus obtained:

$$V \cdot dC_B/dt = 2SKD_x/\delta\ (0.5\ C_{A0} - C_B)$$

where C_A is the bulk concentration in cell A (C_{A0} the initial concentration), D_X the diffusivity coefficient of the component through the membrane, V the volume of each chamber, S and δ the surface and the thickness of the membrane, and K the partition coefficient between the solution and the membrane. The following expression for the percentage diffused in to cell B was obtained by solving the differential equation:

$$R = 100 \cdot C_B/(C_{A0}/2) = [1 - \exp(-2t \cdot S \cdot K \cdot D_x/(V \cdot \delta)] \cdot 100$$

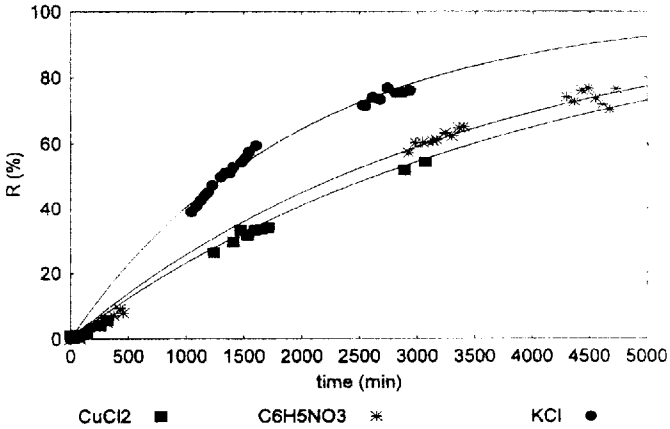

Figure 2. The diffusion of Cu^{2+}, KCl and $C_6H_5NO_3$ through 5 mm thick agarose membranes. The experimental data were fitted using a solution–diffusion model.

Table 3. Permeability coefficients obtained by interpolating the experimental data using the solution–diffusion model

Diffusing component	KD (cm^2/s)
Cu^{2+}	$4.91 \cdot 10^{-4}$
KCl	$9.66 \cdot 10^{-4}$
$C_6H_5NO_3$	$7.79 \cdot 10^{-4}$

The model was applied to the diffusion of Cu^{2+}, K^+ and $C_6H_5NO_3$ through membranes having a thickness of 0.5 cm. As reported in Figure 2, good agreement between the experimental data and theoretical model was obtained. The values found for the permeability KD_x are shown in Table 3. The diffusion tests were repeated with 2 mm thickness membranes (δ = 0.2 cm) and the experimental data were fitted using the model proposed herein employing the permeability $KD_{Cu^{2+}}$ of the model formerly found, as shown in Figure 3. The results confirm the suitability of the diffusion model

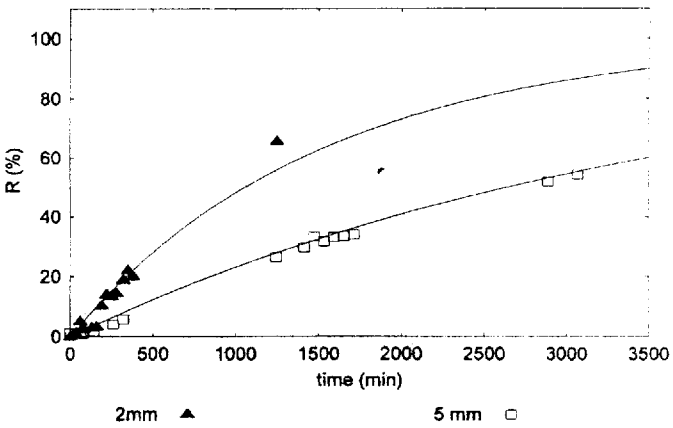

Figure 3. The diffusion of Cu^{2+} through agarose membranes having a thickness of 2 mm and 5 mm. The experimental data were fitted using a solution diffusion model.

to predict the diffusion rates for membranes of different thickness and as a basis of a more complex diffusion–reaction simulation.

Preliminary test with immobilized bacteria

The system was tested with luminescent bacteria *E. coli TBT3*, which will eventually be employed to build the biosensors. As the study is in progress, very preliminary results are shown herein, in order to show the behaviour of immobilized bacteria, and previous optimization of the procedure. Figure 4 shows the light signal (expressed in relative light units per second) emitted in time by *E. coli TBT3* immobilized with an optical density of 0.3, induced with TBT at different concentrations (1–5 μM). The luminescence peak appeared after n–decyl aldehyde was added (t = 90 s), promoting light production. These first results show that immobilized bacteria, induced by TBT and n–decyl aldehyde, produce the light signal, but at low intensity. The immobilization procedure still requires optimization,

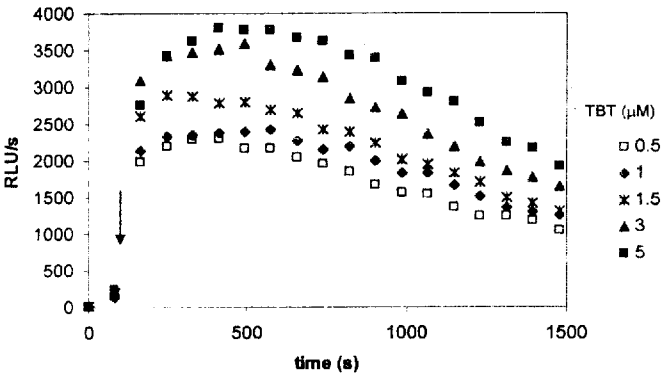

Figure 4. Light emission due to E. coli TBT3 immobilised in 2% agarose, with a final OD=0.3. The peak is observed after the addition of n–decyl aldehyde (arrow).

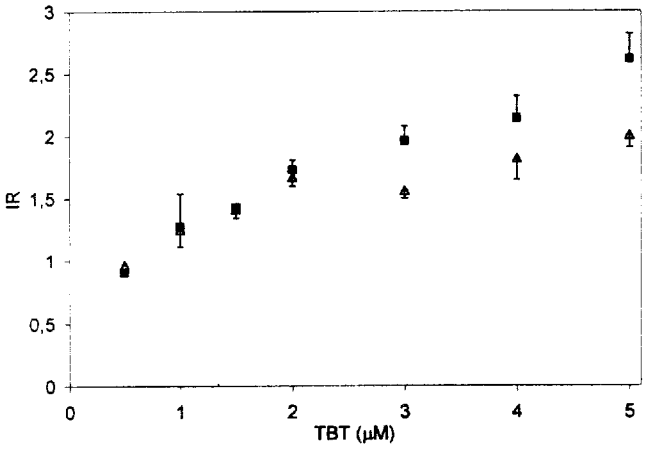

Figure 5. Induction ratio emitted by E. coli TBT3 immobilized in 2% agarose (full squares) and in suspension (empty triangles), with an average optical density of 0.3.

with the purpose of maximizing the intensity of the light signal.

In order to normalize the light signal, the results are evaluated in the form of the induction ratio (IR), calculated as follows:

$$IR = RLU_{TBT}/RLU_0$$

where RLU_{TBT} is the light signal due to different TBT concentrations and RLU_0 is the signal due to the reference solution (containing no TBT, but induced with n–decyl aldehyde). The data reported in Figure 5 show a linear response of the sensor with immobilized bacteria up to about 5 μM. These results are better than those obtained with free bacteria, that show a lower response at concentrations higher than 2 μM, and are probably due to physical protection of the matrix. By optimising and enhancing the cell culture and immobilization, higher values of the induction factors and, therefore, higher sensor sensitivity may be attained, as already shown in previous works with suspended bacteria [13,14].

CONCLUSIONS

The work presented herein aimed to set up a standard and systematic procedure to characterize an immobilization matrix for microbial biosensors, employing agarose as a model. The rheological, optical and mechanical properties are important to compare different materials and concentrations. The study of adsorption and diffusion are fundamental to predict the fate of analytes and substrate through the membrane, necessary for the choice of the matrix, and to eventually optimise the membrane thickness. Preliminary tests with luminescent bacteria showed that immobilized bacteria induced with TBT were able to emit a light signal proportional to the pollutant concentration, thus showing promising results for pollutant detection, though showing the need for further investigation and optimization.

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IZVOD

KARAKTERIZACIJA AGAROZE KAO IMOBILIZACIONE MODEL MATRICE ZA MIKROBNE BIOSENZORE

(Naučni rad)

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Mikrobni biosenzori su potencijalno atraktivni uređaji za određivanje specifičnih jedinjenja u različitim oblastima, kao što su oblasti zaštite životne sredine, biomedicine, proizvodnje hrane, poljoprivrede. Oni omogućavaju brza merenja, ne zahtevaju kompleksnu pripremu uzoraka ili obučeno osoblje, i jednostavni su za korišćenje i održavanje. U cilju jednostavnijeg rukovanja, minijaturizacije i povećanja stabilnosti rada biosenzora, kao i sprečavanja spiranja ćelija, poželjno je izvršiti imobilizaciju ćelija bakterija. U ovom radu je predložena sistematska procedura izbora odgovarajuće metode i matrice za imobilizaciju. Za agarozu kao model matricu utvrđene su fizičke karakteristike, stabilnost pri čuvanju, fenomeni prenosa mase i biokompatiblinost. Takođe su izvedena preliminarna ispitivanja primene bioluminiscentnih bakterija za detektovanje tributiltina.

Ključne reči: Agaroza • Biosenzor • Imobilizacija • Luminiscencija •
Key–words: Agarose • Biosensor • Immobilization • Luminescence •