Direct laser immobilization of photosynthetic material on screen printed electrodes for amperometric biosensor

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(Received 2 December 2010; accepted 14 February 2011; published online 2 March 2011)

This letter demonstrates the direct laser printing of photosynthetic material onto low cost nonfunctionalized screen printed electrodes for the fabrication of photosynthesis-based amperometric biosensors. The high kinetic energy of the transferred material induces direct immobilization of the thylakoids onto the electrodes without the use of linkers. This type of immobilization is able to establish efficient electrochemical contact between proteins and electrode, stabilizing the photosynthetic biomolecule and transporting electrons to the solid state device with high efficiency. The functionality of the laser printed biosensors was evaluated by the detection of a common herbicide such as Linuron. © *2011 American Institute of Physics*.
[doi:10.1063/1.3562297]

The potential for producing a new generation of technological devices that integrate the knowledge coming from various fields—chemistry, biology, computer science, electronics, and engineering—is currently attracting increasing attention. This trend has led to the emergence of a new technological science based on the use of protein components that function as electronic parts. Several proteins have been explored for device applications, and, among others, photosynthetic protein complexes are presently the most largely utilized.[1,2] The fabrication of photosynthesis-based biosensors presents growing interest in recent years as these devices are advanced tools for environmental monitoring. Low cost detection of hazardous pesticides has become an important challenge for biosensor market due to their massive use in agriculture over the last decades. Herbicides such as triazines, triazinones, phenylureas, and phenols inhibit photosynthesis usually by targeting Photosystem II (PSII)—light dependent electron flow. Based on this principle of operation, several amperometric photosynthetic biosensors have been developed for environmental pollutant monitoring.[3–5] Various photosynthetic materials such as whole organisms, chloroplasts, thylakoids, and extracted PSII enriched membranes have been used as biological components in these biosensors.

The main problem with isolated photosynthetic materials is their intrinsic instability and their relatively short active lifetime that limits their effective use. Another problem is maintaining the high electron transfer conversion of the photosynthetic material at the conductor electrode. For this reason, various immobilization techniques and various metals and materials have been tested to improve the stability of the biomolecule both under storage and working conditions.[6] Photosynthetic material immobilization onto the electrodes surface includes both physical and chemical procedures.[6,7] The physical methods are based on the physical adsorption on specific matrixes or the inclusion of the photosynthetic material in natural synthetic gels.[8] In terms of simplicity and photosynthetic material vitality, the physical procedures are considered as the ideal immobilization method. However, this simple and mild technique leads to weak interaction forces, which cannot avoid the risk of high material leaking due to desorption phenomena. On the other hand, chemical methods provide covalent bonds between the photosynthetic material and the immobilizing agent resulting in low leaking of the material during the detection analysis.[7] The presence of the additional functionalization steps in the biosensors fabrication, which is usually time consuming and demands special care of the involved hazardous chemicals, is one of the main disadvantages of chemical immobilization methods. Moreover, the use of chemical reagents is usually harmful to sensitive photosynthetic material and therefore immobilization agents' selection requires in depth analysis in order to minimize delicate material's denaturation.

In our approach, we use the laser induced forward transfer (LIFT) process[9,10] as an advanced tool for direct immobilization of photosynthetic material onto low cost nonfunctionalized gold screen printed electrodes (SPEs). The high spatial resolution of the laser direct printing technique has led several research groups to employ the technique in order to transfer a wide range of functional materials for the fabrication of DNA,[11] protein[12] and cell[13] microarrays, amperometric and capacitive chemical sensors,[14,15] organic light-emitting diodes (LEDs),[16] and organic thin film transistors.[17] In this work, photosynthetic thylakoid membranes were extracted from *Spinacea oleracea* and printed on the sensors in liquid phase by using the LIFT process. The high kinetic energy of the transferred material induces high impact pressure and enhances physical adsorption onto the electrode surface. As a result, high photocurrent activity was achieved by using extremely low quantities of thylakoid membranes (0.06 μg of chlorophyll), indicating strong immobilization without the use of linkers. Furthermore, we have evaluated the laser printed sensors for the detection of a common herbicide such as Linuron.

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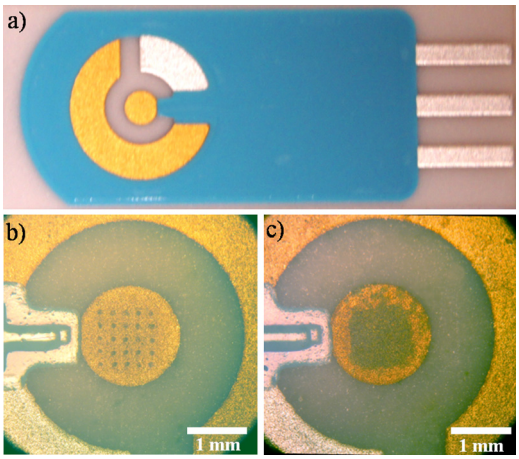

FIG. 1. (Color online) (a) A picture of a gold SPE (Dropsens C223AT) sensor. (b) Discrete thylakoid pixels printed by LIFT on the working electrode of the SPE sensor. (c) A continuous thylakoid layer printed by LIFT on the working electrode of the SPE sensor.

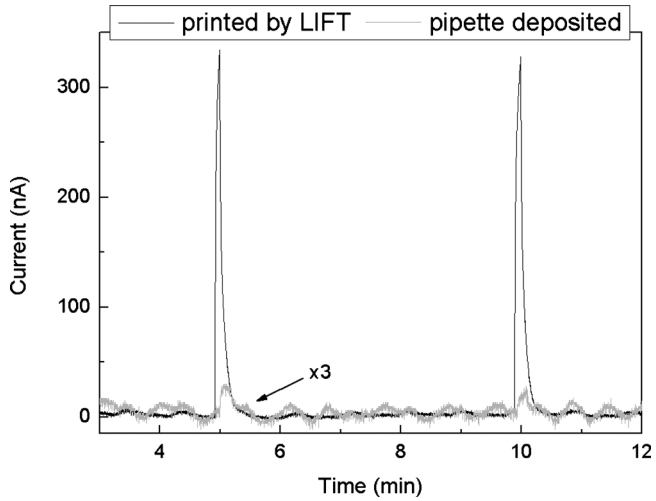

FIG. 2. For comparison, typical electrical signals for printed by LIFT (black line) and pipette deposited (gray line) sensors.

Thylakoid membranes were extracted from fresh spinach leaves (*Spinacea oleracea L.*) using a protocol, which has been previously described in detail.[3] In the final step, thylakoid membranes were resuspended in a buffer containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) *p*H=6.0, sucrose 70 mM, and NaCl 15 mM. The photosynthetic activity of the thylakoid membranes was tested using the chlorophyll fluorescence induction technique. Tests were performed using the plant efficiency analyzer (Hansatech instruments LTD, U.K.).

The deposition of the thylakoid membranes onto the working gold electrode was carried out by a pulsed Nd:YAG (yttrium aluminum garnet) laser (266 nm wavelength, 4 ns pulse duration) and a high power imaging micromachining system, which has been previously described in detail.[12,14] The target substrates were prepared by drop casting 10 μl of the thylakoid solution onto 1 in. quartz plates, coated with a 40 nm titanium laser absorbing layer. The final concentration of the thylakoid solution on the target substrate was 7.6 mg/ml. Two-dimensional patterns of discrete thylakoid droplets and continuous thylakoid layers were printed on the electrodes by adjusting droplets separation distance as it can be seen in Figs. 1(b) and 1(c), respectively. The transfer was carried out in such a way that each droplet was deposited by a single pulse and the optimum distance between the target and the substrate was found at 300 μm. Following the optimization study, the laser transfer was performed at 470 mJ/cm^2 and the laser beam size on the donor substrate was 60 μm in diameter. By using these laser parameters the deposited thylakoid droplets on the electrode surface were 150 μm in diameter.

Time dependent amperometric measurements with the immobilized photosynthetic material on SPEs were performed using the amperometric system AMPBIOSPE (Biosensor srl-www.biosensor.it). Thylakoid membranes were deposited over a gold working electrode surface (1.6 mm ∅) of a Dropsens SPE (*ref. C223AT*) (DropSens SL Spain) [Fig. 1(a)]. Amperometric detection of electric current generated by thylakoid membranes was measured after 5 s of illumination by two red LEDs (peak wavelength 652 nm, with light intensity 130 μmol photons m^{-2} s^{-1}) in the presence of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) at applied potential 0.2 V. The electrode was continuously washed with the measuring buffer, containing 20 mM tricine *p*H=7.8, 70 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 30 μM DCPIP with a flow rate 130 μl/min. All measures were carried out at 25 °C.

The measurement of the PSII activity was based on amperometric registration of the reduced form of an artificial electron acceptor under the specific potential. Under illumination the immobilized thylakoid membranes reduced the artificial electron acceptor which is finally reoxidized on the surface of the gold electrode. Figure 2 shows typical electrical activity signals of gold SPEs printed by using LIFT and the reference pipette spotting method. In both cases the total mass of the chlorophyll on the electrode was 0.06 μg. Gold SPEs were illuminated every five minutes and the resulted current peaks were proportional to the immobilized photosynthetic material. The printed by LIFT gold SPEs present increased photocurrent activity indicating strong immobilization of the laser printed photosynthetic material onto the gold electrode surface without the use of linkers. Moreover, the high electrical signal is combined with an extremely high signal to noise ratio. For comparison, the same thylakoid material, deposited using pipette, has measured very low activity indicating weak immobilization of the photosynthetic material on the sensor surface.

At this point, laser printing was proven to be an advanced tool for both printing and immobilization of the photosynthetic material on the gold SPEs surface. The explanation for this phenomenon is given by investigating the physical aspects of the LIFT process and the high roughness surface of the gold SPEs, which is 0.5 μm. Surfaces with protrusions present two wetting states, namely, Cassie state (partial wetting) and the Wenzel state (complete wetting). It has been reported that an applied pressure on a drop (e.g., manually applied force, high speed projectiles, etc.) can force the liquid into the valleys between the projectiles, and thus force the Cassie state to the Wenzel state resulting in complete wetting of a rough surface.[18] It has been observed, by time resolved imaging of the liquid phase LIFT, that the liquid film detaches the target through jet formation mechanism.[19] Laser printing of reference glycerol solutions with rheological properties similar to the thylakoid suspension produces a liquid jet that reaches the receiving substrate

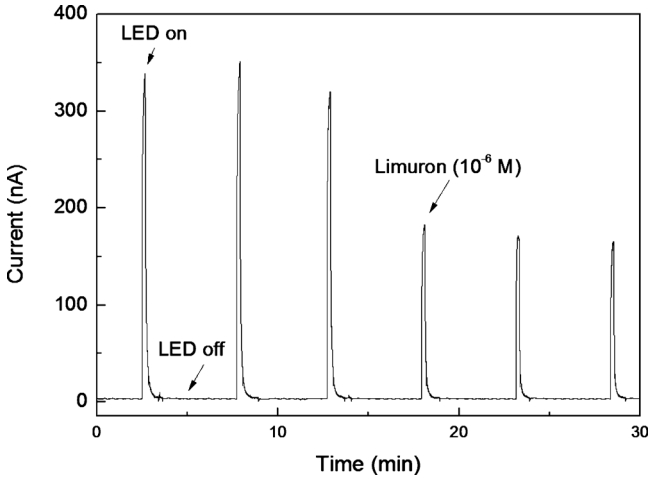

FIG. 3. Herbicide inhibition, as chronoamperometric measurements, of the spinach thylakoids immobilized onto gold SPE. The herbicide (Linuron 10^{-6} M) has been added after three light pulses. The duration of light pulses was set to be 5 s with a frequency of 5 min.

in few microseconds with a front velocity on the order of 100 m/s.[19,20] The ejected droplets induce high impact pressure on the sensors' surface, which is calculated a few megapascal. This pressure is higher to the barrier which is needed in order to transit from the Cassie to the Wenzel state,[18] and thus dramatically improves the wetting of the gold SPEs by removing the trapped air in the high roughness surface (Wenzel state). As a result of this wetting state transition, physical adsorption of thylakoid membranes on the electrode surface is enhanced resulting in high activity signals. Similar results were also obtained for LIFT printed carbon nanotube and graphite SPEs.

Following the laser immobilization investigation and prior to herbicide detection, a detailed study was performed in order to optimize electrical activity signal of gold SPEs. This homogeneous cover of the electrode surface, achieved by the laser printing, eliminates the "coffee ring" phenomenon[21] which is unavoidable with the conventional printing methods.[22] A total chlorophyll mass of 0.06 μg was defined to be the optimum selection to produce a high activity signal (335 ± 13 nA) combined with a high signal to noise ratio.

In order to test the functionality of the laser printed photosynthetic biosensors, we have detected a common herbicide [Linuron, 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea]. Figure 3 shows the inhibition of the photosynthetic activity of the immobilized photosynthetic material in the presence of the herbicide Linuron (10^{-6} M). The activity of the sensor was initially recorded in the absence of herbicide. Then, the herbicide-containing sample was loaded to the flow cell and the residual activity was registered. In the presence of the mediator (DCPIP), the herbicide detection is based on the herbicide induced decrease in the electron transfer between the photosynthetic membranes and the mediator. This, result in the reduction in the generated electric current and it is indicated as a decrease in the biosensor signal. Thylakoid membranes have already been immobilized onto SPE for biosensor purposes either by chemical or physical immobilization.[3,4,6,23,24] Compared with those types of immobilization the LIFT method provides the following: higher activity photocurrent signal (335 nA), an excellent signal to noise ratio, lower response time (5 min), no diffusion problems, and higher life time (48 h).

In conclusion, we present the use of a laser transfer technique for direct printing of thylakoid membranes for the fabrication of photosynthetic-based amperometric biosensors. Laser printing is an excellent tool for direct immobilization of the transferred photosynthetic material onto nonfunctionalized electrodes due to the high impact pressure of the transferred droplets. The use of this laser transfer technique enabled complete wetting of the rough electrodes' surface, which could not be achieved with conventional printing/spotting methods. Both immobilization and activity of the photosynthetic material were confirmed by high photocurrent signals combined with a high signal to noise ratio. Our results indicate laser printing as a very promising technique for direct immobilization of photosynthetic protein complexes onto high rough surfaces, such as low cost SPEs, which are widely used in biosensors' market.

Financial support from European Commission (ICT: e-LIFT, Grant Agreement No. 247868 and BEEP-C-EN FP 7-SME-2008-01, Grant Agreement No. 231082) is gratefully acknowledged.

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