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# Biofunctionalization of Multiwalled Carbon Nanotubes by Irradiation of Electropolymerized Poly(pyrrole–diazirine) Films

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**Abstract:** A photoactivatable poly(pyrrole–diazirine) film was synthesized and electropolymerized as a versatile tool for covalent binding of laccase and glucose oxidase on multiwalled carbon nanotube coatings and Pt, respectively. Irradiation of the functionalized nanotubes allowed photochemical grafting of laccase and its subsequent direct electrical wiring, as illustrated by the electrocatalytic reduction of oxygen. Moreover, covalent binding of glucose oxidase as model enzyme, achieved by UV activation of electropolymerized pyrrole–diazirine, allowed a glucose biosensor to be realized. This original method to graft biomolecules combines electrochemical and photochemical techniques. The simplicity of this new method allows it to be extended easily to other biological systems.

**Keywords:** electrochemistry · enzymes · immobilization · nanotubes · polymers

## Introduction

Due to their large specific surface area, nanowire morphology, and intrinsic conductivity, carbon nanotubes are attracting wide attention for developing biointerfaces devoted to energy conversion in biosensing applications. In particular, carbon nanotube coatings were widely used as an electroactive skeleton for creating highly porous three-dimensional biological networks.[1] Among the different procedures for biofunctionalization of carbon nanotubes, the electrochemical polymerization of organic films is an attractive approach thanks to the electrochemical addressing of polymers. Due to this property, the film completely and uniformly covers the walls of the nanotubes.[2] Biomacromolecules were immobilized by an entrapment process during polymer growth or by a subsequent step based on affinity interactions.[3–6] However, these approaches have the disadvantage of low accessibility of the entrapped biomolecules or a possible loss of biomolecules due to the reversible character of affinity interactions. A new interesting research direction in biomolecule immobilization lies in creating direct covalent binding between biomolecules and polymers through irradiation of electropolymerized photoactivatable films.[7–10] The binding is stronger than that resulting from affinity interactions, and the procedure is spatially addressable by light and faster than those based on conventional covalent binding through activated ester groups.

In this context, we report here the synthesis and electropolymerization of a pyrrolic diazirine derivative and the use of the resulting polypyrrole film for the irreversible photobinding of glucose oxidase and laccase on MWCNT coatings. The two enzymes glucose oxidase and laccase were chosen as model proteins to illustrate the photografting properties of the polymer, since their immobilization is easily demonstrated through their catalytic activity. This immobilization method combines the advantages of the electrochemical addressing of the polymer with spatial localization of the protein by light. Compared to the first example of an electropolymerized film, namely, poly(pyrrole–benzophenone), which allowed reagentless covalent grafting of proteins on irradiation, the diazirine group exhibits better photostability and photoreactivity than benzophenone.[11–12] In particular, diazirine can react not only with C–H but also with N–H and O–H bonds and thus widens the field of applications. Moreover, compared to conventional photoactive groups used for photografting molecules to solid supports, such as diazo and diazopyruvate derivatives, azides and phenyl azides, tetrazoles, and benzophenone, diazirine has several advantages.[13–18] Diazirine is more easily and more efficiently activated by UV radiation of wavelength 330–370 nm, which preserves the native structure and activity of immobilized proteins (Scheme 1). In contrast to azides, diazirine is also essentially nontoxic and has better photostability. In addition, compared with benzophenone, diazirine does not require long irradiation times, which can cause serious damage to the protein configuration.

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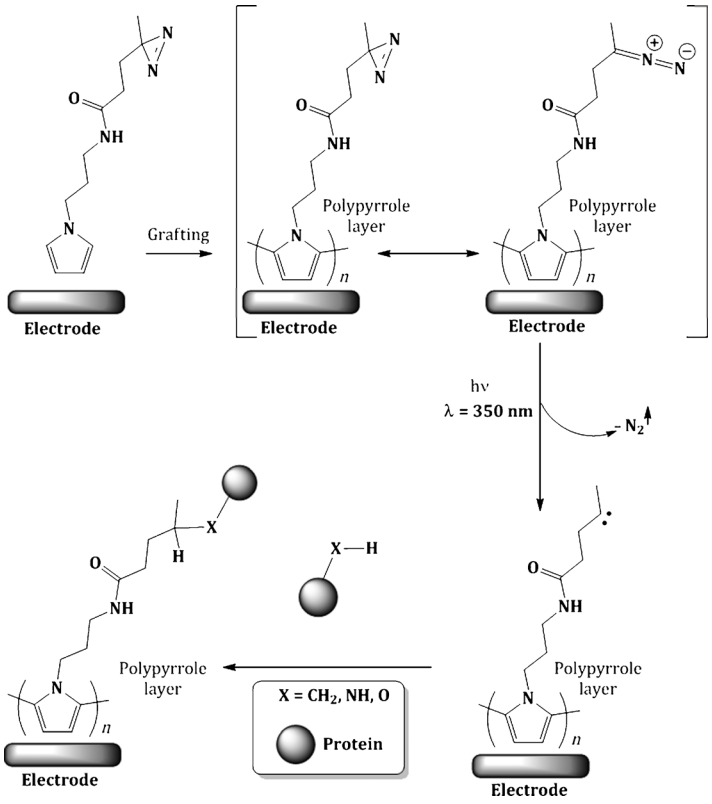
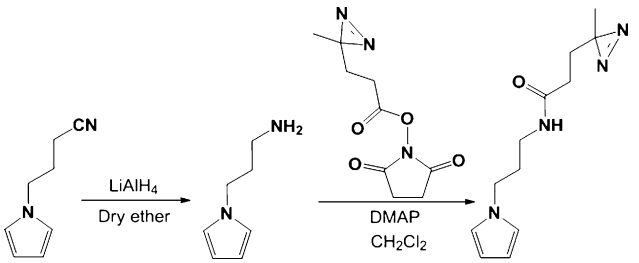



Scheme 1. Schematic of biomolecule photografting onto films of poly(pyrrole–diazirine) electrogenerated on a Pt or MWCNT electrode.

## Results and Discussion

**Synthesis and characterization of pyrrole–diazirine 1**: Pyrrole-functionalized diazirine monomer **1** was prepared by amide coupling of *N*-(3-aminopropyl)pyrrole with NHS-diazirine in the presence of 4-dimethylaminopyridine (DMAP) in dichloromethane (Scheme 2).The pyrrolic diazirine derivative was purified by silica-gel chromatography and characterized by $^1$H NMR spectroscopy, MALDI-TOF MS, and cyclic voltammetry.




Scheme 2. Synthesis of pyrrole–diazirine **1**.

The electrochemical behavior of pyrrolic diazirine **1** ($2\times10^{-3}$ mol L$^{-1}$) was investigated in CH$_3$CN + 0.1 mol L$^{-1}$ tetrabutylammonium perchlorate (TBAP). On oxidative scanning, monomer **1** exhibits an irreversible peak at 1.05 V (vs. Ag/Ag$^+$), which corresponds to the well-known oxidation of the pyrrole group (Figure 1, inset). Polymerization of **1** ($2\times10^{-3}$ mol L$^{-1}$) was carried out by a galvanostatic method or by controlled-potential electrolysis of the monomer in CH$_3$CN + 0.1 mol L$^{-1}$ TBAP. The resulting electrode was transferred with thorough rinsing to a CH$_3$CN solution free of monomer. The cyclic voltammogram of this electrode exhibits a poorly reversible peak system at 0.40 V reflecting the electroactivity of the formed polypyrrole (Figure 1). The apparent surface coverage of poly(pyrrole–diazirine) of $1.25\times10^{-8}$ mol cm$^{-2}$ was determined from the charge recorded under the oxidation wave of the polypyrrole backbone (electropolymerization yield: 56%).

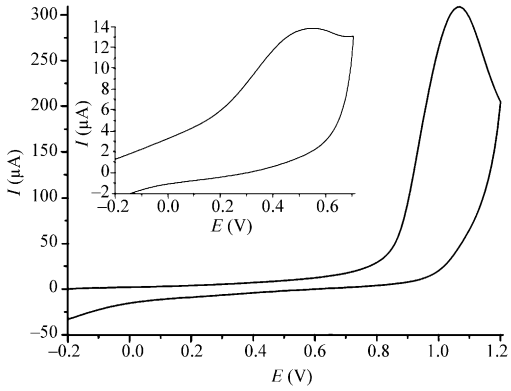



Figure 1. Cyclic voltammogram of **1** ($2\times10^{-3}$ mol L$^{-1}$) in CH$_3$CN + TBAP (0.1 mol L$^{-1}$) recorded at a Pt electrode (5 mm diameter). Inset: Cyclic voltammogram of poly(pyrrole–diazirine) (obtained by potentiostatic method at 0.90 V, $Q$ = 1 mC) and transferred into CH$_3$CN + 0.1 mol L$^{-1}$ TBAP free of monomer.

**Characterization of the biomaterial functionalized by glucose oxidase (GOx)**: With the aim of demonstrating its photochemical grafting properties, poly(pyrrole–diazirine) and a control polymer based on the same skeleton but without diazirine groups were irradiated in the presence of glucose oxidase (GOx), an enzyme chosen as protein model. A solution of GOx (20 μL, 4 mg mL$^{-1}$) was incubated with poly(pyrrole–diazirine) and regular polypyrrole films under irradiation for 10 min. This enzyme catalyzes the oxidation of glucose in the presence of oxygen with production of $H_2O_2$. The activity of grafted GOx can thus be monitored in phosphate buffer by the electrochemical oxidation of $H_2O_2$ at the underlying electrode surface potentiostated at 0.7 V (vs. SCE).

Figure 2 shows the anodic current response of the enzyme electrodes as a function of glucose concentration. The sensitivity of the poly(pyrrole–diazirine)/GOx electrode determined by the slope of the linear part of the calibration curve is 0.49 mA mol$^{-1}$ L cm$^{-2}$ with a maximum current density ($J_{max}$) of around 24 μA cm$^{-2}$. This sensitivity appears to be similar to or higher than those previously reported for the formation of a compact enzyme monolayer based on affinity interactions between biotinylated polymers and biotinylated GOx (0.15–0.3 mA mol$^{-1}$ L cm$^{-2}$).[19–20]

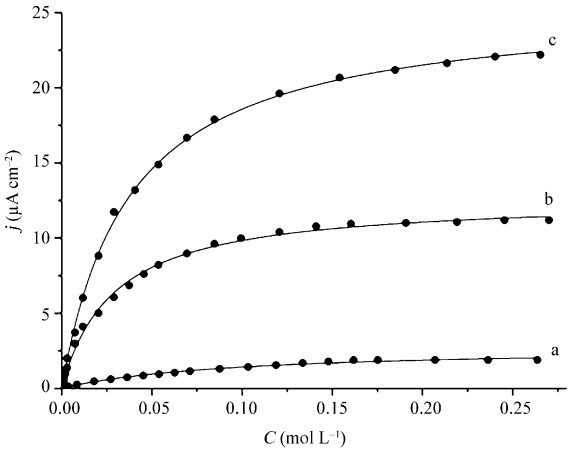



Figure 2. Calibration curves for glucose a) at an irradiated polypyrrole/GOx electrode, b) at a poly(pyrrole–diazirine)/GOx electrode, and c) at an irradiated poly(pyrrole–diazirine)/GOx electrode in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0). Applied potential: 0.7 V vs. SCE.

Moreover, the maximum current density was higher than or similar to those reported for these modified bioelectrodes or for poly(pyrrole–nitrilotriacetic acid)/$Cu^{2+}$/histidine-tagged GOx or between biotinylated polymers and β-cyclodextrin-tagged GOx (3.4–23 μA $cm^{-2}$).[21,22] Since the $J_{max}$ value is directly proportional to the enzyme loading, these results illustrate efficient covalent binding of GOx and good permeability of the poly(pyrrole–diazirine) for $H_2O_2$. Moreover, the apparent $K_M$ value (29 mmol $L^{-1}$) obtained for this enzyme electrode is similar to that reported for GOx in solution ($K_M = 33$ mmol $L^{-1}$), which reflects the absence of denaturation due to photochemical grafting.[23]

In contrast, performing the same incubation and irradiation process on regular polypyrrole film led to a modified electrode with almost no electroenzymatic activity, and thus the role of diazirine is corroborated (Figure 2).

A control experiment was carried out in which poly(pyrrole–diazirine) and GOx were exposed to visible light without specific irradiation. Surprisingly, the resulting modified electrode provided sensitivity and $J_{max}$ values of 0.48 mA $mol^{-1}$ L $cm^{-2}$ and 11 μA $cm^{-2}$, respectively. Although the $J_{max}$ value, which is directly proportional to the enzyme loading, is markedly lower than that observed with irradiation, these values reflect the ease of diazirine photoactivation.

**Photografting of laccase**: To validate this procedure for the functionalization of carbon nanotube deposits by proteins, a poly(pyrrole–diazirine) film was electropolymerized by controlled-potential electrolysis ($E_{applied} = 0.9$ V, $Q = 12.5$ mC $cm^{-2}$) onto electrodes modified by a MWCNT coating and irradiated in presence of laccase. This enzyme catalyzes the reduction of oxygen and has been widely exploited for the elaboration of biocathodes and hence the fabrication of biofuel cells. The enzyme activity of the resulting nanostructured bioelectrode was investigated in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). In the presence of oxygen, ABTS is enzymatically oxidized to its stable cation radical form $ABTS^{\bullet+}$ at pH 5. This process was monitored by UV/Vis spectroscopy; absorption bands at 420 nm characteristic of the one-electron oxidation of ABTS (associated with a shoulder at 650 nm) appear and increase with time (Figure 3). The presence of an isosbestic point at 375 nm demonstrates the selectivity of the oxidation.

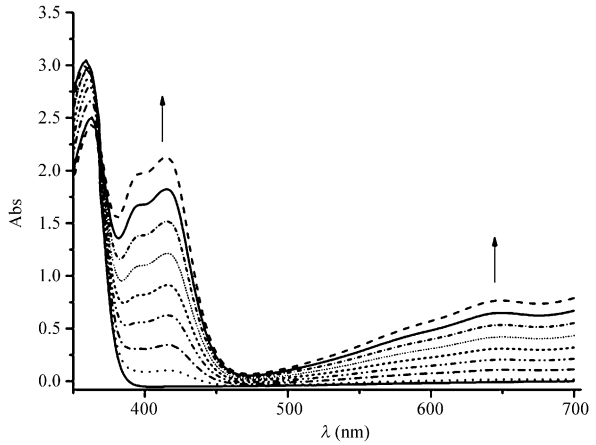



Figure 3. Activity of laccase immobilized by irradiation on MWCNT electrodes previously modified with poly(pyrrole–diazirine). Enzymatic activity was evaluated by UV/Vis spectroscopy at $\lambda = 420$ nm in acetate buffer (0.1 mol $L^{-1}$, pH 5) containing ABTS ($1.48 \times 10^{-4}$ mol $L^{-1}$) for 15 min.

By comparison with spectrophotometric measurements performed with the free enzyme, the bioelectrode exhibits an immobilized activity of 3.2 U $mg^{-1}$. This corresponds to the immobilization of 60.7 μg $cm^{-2}$ of laccase, assuming that the immobilized enzyme retains its full activity.

Since laccase and avidin have the same molecular weight (66 kDa), a close-packed single-molecular layer of laccase should correspond, by analogy, to $3.3 \times 10^{12}$ molecules $cm^{-2}$ or 362 ng $cm^{-2}$. Hence, the photochemical grafting process results in the immobilization of $5.53 \times 10^{14}$ molecules $cm^{-2}$ of laccase corresponding to 167 equivalent compact monolayers of laccase and thus indicates a three-dimensional structure of the conductive composite biocoating.

The performance of the resulting composite bioelectrode in oxygen reduction was investigated by cyclic voltammetry in acetate buffer (pH 5). In presence of oxygen, a catalytic cathodic current appeared at 0.61 V (vs. SCE), close to the redox potential of laccase (Figure 4 A). Chronoamperometric measurements at 0.3 V demonstrated the presence of a steady-state catalytic current (303 μA $cm^{-2}$) reflecting the activity of the immobilized laccase (Figure 4 B). This corroborates the efficient photografting of laccase and reflects also the direct electrical connection of this enzyme by laccase/poly(pyrrole–diazirine)/MWCNT/GC. The latter shows an electroenzymatic activity of 47 mU $cm^{-2}$ towards oxygen reduction and corresponds to 25 % of the preceding spectrophotometric enzyme activity. This difference may be ascribed to the thickness of the polypyrrolic film, which could

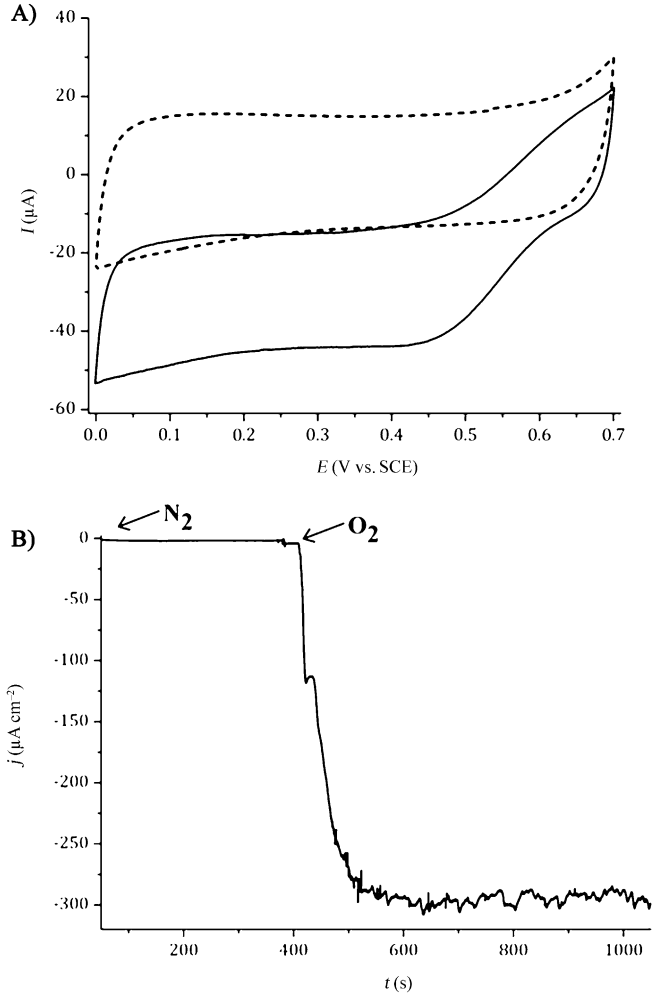



Figure 4. A) Laccase/poly(pyrrole–diazirine)/MWCNT/GC electrode (3 mm diameter disk) in 0.1 mol $L^{-1}$ acetate solution (pH 5.0) without (dashed line) and with oxygen (straight line). Scan rate: 10 mV $s^{-1}$. B) Chronoamperometric response of the laccase/poly(pyrrole–diazirine)/MWCNT/GC electrode potentiostated at 0.3 V (vs. SCE) in 0.1 mol $L^{-1}$ acetate buffer (pH 5.0) before and after oxygen saturation.

partly prevent direct electron transfer. Moreover, photochemical grafting of laccase can lead to random orientation of the electroactive T1 copper center. The location of the active center opposite to the surface of the electrode can thereby prevent electron exchange with the electrode surface. In contrast, the freely diffusing ABTS can assure electrical wiring of all immobilized enzymes whatever their position on the polymer and thus lead to higher enzyme activity. Moreover, under oxygen-saturated conditions, a stable open-circuit potential of 0.61 V was reached for more than 1 h (Figure 5).

## Conclusion

We have demonstrated easy and electrochemically controlled elaboration of a poly(pyrrole–diazirine) film and the suitability of the polymerized diazirine for photochemical

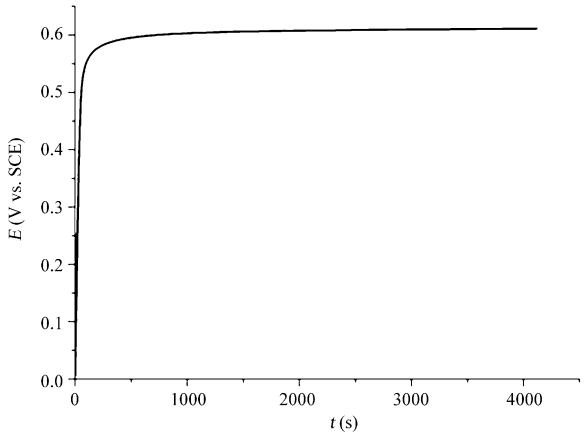



Figure 5. Evolution of the open-circuit potential of a laccase/poly(pyrrole–diazirine)/MWCNT/GC electrode under oxygen-saturated conditions in acetate buffer (0.1 mol $L^{-1}$, pH 5).

immobilization of proteins by formation of strong and stable chemical bonds. This procedure combines the advantages of light-dependent immobilization with those related to the electrochemical addressing of polymers, in particular, the possibility of precisely electrogenerating a photoreactive polymer film on nanoobjects such as carbon nanotubes. The potential of this fast and generally applicable photoelectrochemical approach to biomolecule immobilization for biosensor fabrication was illustrated with GOx as enzyme model. The resulting biosensor characteristics showed the efficiency of this concept, and nondenaturation of the catalytic properties of GOx under UV irradiation was demonstrated. As illustrated with laccase, this method is an efficient and fast way for the functionalization of carbon nanotube deposits by proteins. Moreover, the photoactivatable poly(pyrrole–diazirine) allows direct electron transfer between MWCNT and redox enzymes. It is thus expected that this new reagentless procedure for immobilization of biological macromolecules on three-dimensional carbon nanostructures will be useful for the development of bioarchitectures exhibiting extremely high biological activities

## Experimental Section

**Materials**: Acetonitrile (HPLC grade) used for electrochemistry measurements was obtained from Rathburn and used without further modification. Tetrabutylammonium perchlorate (TBAP, Fluka) was used as the supporting electrolyte in organic media. Glucose oxidase (GOx, 168 U $mg^{-1}$), laccase (13.6 U $mg^{-1}$), and glucose were purchased from Sigma. All reagents and chemicals purchased from Aldrich were of reagent-grade quality and used as received unless otherwise mentioned. Commercial-grade thin multiwalled carbon nanotubes (MWNTs, 9.5 nm diameter, purity >95%), obtained from Nanocyl, were used as received without any purification step. NHS-diazirine (succinimidyl 4,4-azipentanoate) was purchased from Thermo Scientific.

For laccase experiments, the glassy carbon (GC) disk used as working electrode was polished, rinsed, and then sonicated with water before each measurement.

The MWCNT electrode was fabricated by using the following method: MWCNTs (5 mg) were added to 1 mL of *N*-methyl-2-pyrrolidone (NMP). The suspension was homogenized by an ultrasonic generator for 30 min. For electrochemical investigations, a portion (10 μL) of this suspension was dropped onto a freshly polished GC disk electrode (3 mm diameter) and the solvent was removed under reduced pressure. The electroactive surface was estimated as previously reported.[24]

For cross-linking reactions between enzymes and diazirine, 20 μL of enzyme solution (5 mg mL$^{-1}$ laccase or 4 mg mL$^{-1}$ GOx) was deposited on modified electrodes and then irradiated with an Hg/Xe lamp (Oriel Instruments) through an optical fiber for 10 min. Filters from Oriel were used to select the wavelength and to block IR rays to prevent overheating of the optical fiber.

**Instruments**: Electropolymerization and cyclic voltammetric experiments were performed with an Autolab Potentiostat 100 (Ecochemie, Utrecht, the Netherlands). All electrochemical experiments were carried out in a conventional three-electrode cell. The amperometric measurements were performed with a Tacussel PRG-DL potentiostat in phosphate buffer solution (0.1 mol L$^{-1}$, pH 7) or with an Autolab Potentiostat 100 in acetate buffer (0.1 mol L$^{-1}$, pH 5). All enzyme electrodes were prepared on bare platinum electrodes or on electrodes previously modified with an MWCNT deposit. An aqueous saturated calomel electrode (SCE) was used as reference electrode, and a Pt wire placed in a separate compartment containing the aqueous electrolyte as counter electrode. An $Ag^+$/Ag ($10^{-2}$ mol L$^{-1}$) electrode was used as reference in acetonitrile.

**Synthesis**: The pyrrole amine was prepared by reduction of 1-(2-cyanoethyl)pyrrole with lithium aluminum hydride in diethyl ether. A suspension of $LiAlH_4$ (35 mL, 1.0 mol L$^{-1}$ in diethyl ether) was stirred under a nitrogen stream. A solution of 1-(2-cyanoethyl)pyrrole (7.2 g) in anhydrous diethyl ether (35 mL) was then added dropwise. The resulting solution was heated to reflux for 18 h under nitrogen. The resultant reaction mixture was allowed to cool, and excess $LiAlH_4$ was destroyed by successive addition of water (3.5 mL), 15% NaOH solution (3.5 mL), and water (10 mL). The organic layer was separated, heated to 40 °C for 2 h, and filtered through powdered Celite/anhydrous sodium sulfate (1/1). The filtrate was washed twice with diethyl ether (20 mL) and the resulting solution evaporated in vacuo providing a yellow oil, which was used in the next reaction step without further purification.

A mixture of *N*-(3-aminopropyl)pyrrole (0.25 g), NHS-diazirine (0.45 g), and 4-dimethylaminopyridine (0.08 g) in dichloromethane (10 mL) was stirred in a flask covered with aluminum foil to prevent irradiation of the product under nitrogen at room temperature for 5 days. The resulting solution was filtered, and dichloromethane was removed in vacuo. The resulting crude product was purified by column chromatography on silica gel eluted with dichloromethane to give a colorless oil in 57% overall yield. $^1$H NMR (Bruker, 400 MHz, $CDCl_3$): $\delta = 1.02$ (s, 3H), 1.72–1.97 (m, 6H), 3.25 (d, 2H), 3.96 (d, 2H), 5.29 (s, 1H), 6.18 (d, 2H), 6.68 ppm (d, 2H); MS (MALDI-TOF): $m/z$: 234.30.

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