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Electrochemical Functionalization of Carbon Surfaces by Aromatic Azide or Alkyne Molecules: A Versatile Platform for Click Chemistry

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Abstract: The electrochemical reduction of phenylazide or phenylacetylene diazonium salts leads to the grafting of azido or ethynyl groups onto the surface of carbon electrodes. In the presence of copper(I) catalyst, these azide- or alkyne-modified surfaces react efficiently and rapidly with compounds bearing an acetylene or azide function, thus forming a covalent 1,2,3-triazole linkage by means of click chemistry. This was illustrated with the surface coupling of ferrocenes functionalized with an ethynyl or azido group and the biomolecule biotin terminated by an acetylene group.

Keywords: click chemistry · cycloaddition · diazo compounds · electrochemistry · surface chemistry

Introduction

The development of strategies for controlling the functionalization of conductive surfaces, at the molecular level, with biological, redox-active, or photo/chemical sensitive molecules is of central interest in the development of molecular electronics,[1] energy conversion,[2] and chemical or biological sensors.[3] Among the large variety of surface chemistry that allows a high degree of control over how a conductive surface is modified, thiolates self-assembled monolayers (SAMs) on gold surfaces are the most popular.[4] Their popularity arises from their ease of preparation, the well-defined monolayer that results, and the possibility of introducing a vast number of terminal reactive groups that can serve subsequently to couple a wide range of species to the surface. However, most coupling methods are hampered by a difficulty in introducing the reactive groups, a lack of specificity, or a low yield.[5] A significant advance was recently proposed by Collman, Chidsey, and co-workers, who have used a click reaction to chemoselectively couple an acetylene functionality to an azide-terminated SAM on a gold electrode by means of a copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition.[6] This heterogeneous coupling strategy was found to be fast, quantitative, reproducible, resistant to side reactions, and highly tolerant to reaction conditions. It was hence advantageously used to attach in aqueous solution and in high yields complex functional molecules, such as oligonucleotides,[7] porphyrin redox catalysts,[8] receptors, and proteins,[9] on gold surfaces. Despite the great promise of such an immobilization strategy, SAMs on gold have a number of limitations, such as rather slow formation kinetics (that typically requires 24–36 h), a moderate chemical and thermal stability,[10] a tendency for photoxidation, and a narrow electrochemical potential window (1.2 V in aqueous media).[11] These limitations are aggravated by the use of short alkanethiols that are often required to ensure fast electron transfer through the SAM. To overcome these drawbacks, it would, therefore, be desirable to develop a surface chemistry that yields a stronger and faster covalent attachment of an azide- or acetylene-terminated layer on an electrode surface. One possibility that has been recently proposed consists of a chemical treatment of graphitic surfaces by iodine azide reagent.[12] Although this method was efficient for introducing azide groups on a graphitic surface, it needs safety precautions for handling IN_3, a highly explosive reagent. Another limitation is the relatively low azide surface coverage that results, with a preferential attachment of the azide groups at the edges of graphene sheets, which is not ideal for the formation of uniform monolayers. An alter-

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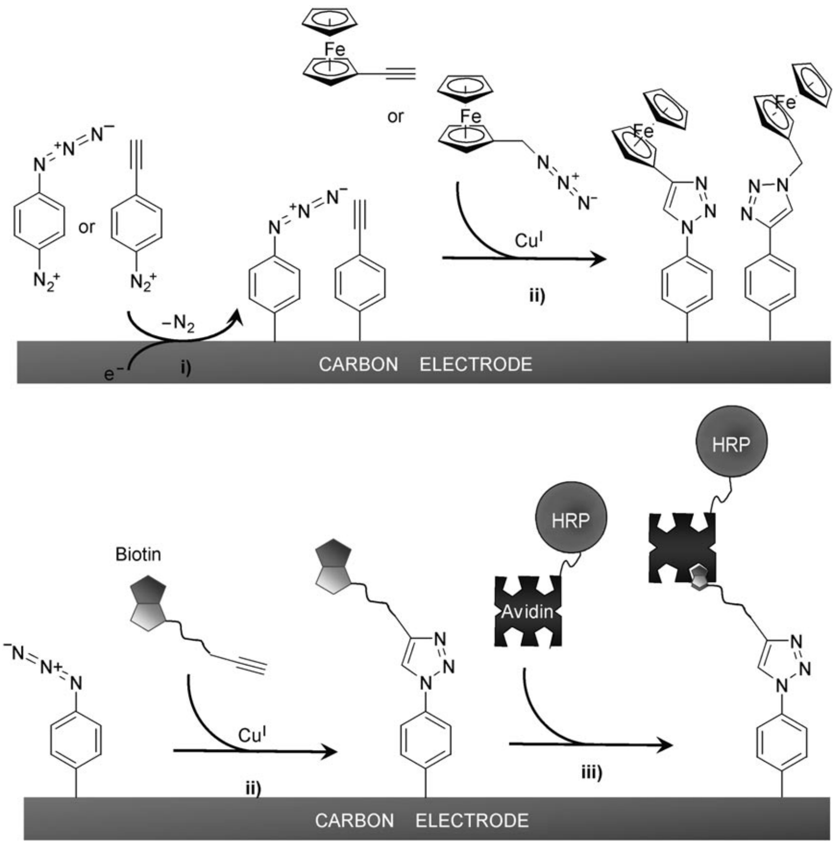
native consists of functionalizing carbon surfaces by electrochemical reduction of aryl diazonium ions.[13] Such a method was demonstrated for the electrografting of a wide variety of substituted aryl molecules,[14] but never for phenylazide or phenylacetylene diazonium salts. Generation of an aryl radical that couples with the underlying electrode to form a robust covalent C–C bond is the well-accepted mechanism.[15] Such an electrochemically assisted method offers several advantages over thiolates SAMs on gold: 1) a strong linkage of the modifier to the electrode surface, 2) a fast surface modification, generally requiring only few seconds for the formation of a saturated monolayer, 3) an easy control over functionalization coverage, and 4) the opportunity to electro-address the functionalization.

In the present work, we explored the possibility of grafting a phenylazide or phenylacetylene layer on carbon electrodes by means of electrochemical reduction of their corresponding phenylazide and phenylacetylene diazonium salts, and then coupling chemoselectively acetylene- or azide-bearing molecules by click chemistry, as depicted in Scheme 1. The surface coverage of reactive azide or ethynyl groups has been quantified electrochemically through the complementary coupling of simple redox-active probes, that is, ethynylferrocene (Fc–C≡CH) and azidomethylferrocene ($FcMeN_3$).[16] Finally, the utility of an azide-functionalized carbon electrode for fast and efficient attachment of a bioactive molecule in aqueous solution was demonstrated with the coupling of an acetylene-derivatized biotin that was subsequently indirectly revealed through the sequential specific binding and electrocatalytic detection of a neutravidin–horseradish peroxidase conjugate (N–HRP).



Scheme 1. Illustration of the multistep functionalization of carbon electrodes: i) Electroreduction of the diazonium salt for covalent immobilization of phenylazide or phenylacetylene molecules; ii) Cu^I-catalyzed Huisgen 1,3-dipolar cycloaddition between the immobilized azide or alkyne functions and the soluble molecules (ferrocene or biotin derivatives) bearing the corresponding ethynyl or azide group; iii) immobilization of an avidin conjugated to horseradish peroxidase (HRP).

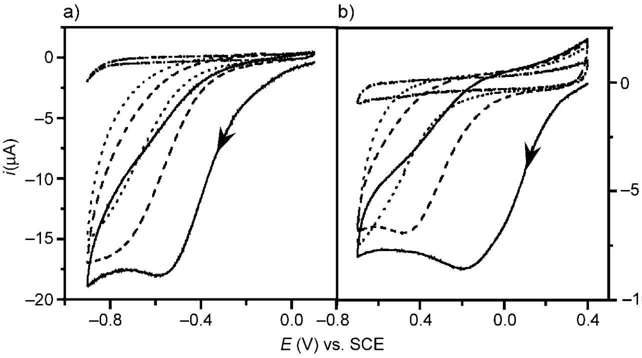


Figure 1. CVs of a) $N_3{-}C_6H_4{-}N_2^+$ and b) $HC{\equiv}C{-}C_6H_4{-}N_2^+$ (1.3 mM in 0.1 M HCl) at a PGE electrode (——: first scan, -----: second scan, •••••: third scan, –•–•: corresponds to the blank response in a diazonium-free HCl solution). Scan rate: 0.1 V s^{-1}; $T = 4$ °C.

Results and Discussion

The diazonium salts of 4-azidobenzene and 4-ethynylbenzene were prepared by diazotation of their parent aniline derivatives and isolated as pure tetrafluoroborate salts. Their cyclic voltammograms (CVs; Figure 1) recorded at a pyrolytic graphite edge (PGE) electrode in 0.1 M HCl solution exhibit broad irreversible cathodic peaks at approximately −0.5 and −0.2 V (vs. SCE), respectively. During the second and third scans, the peak intensity dramatically decreases and the peak potential shifts towards more negative values, consistent with the formation of a grafted layer on the electrode surface. At glassy carbon (GC) electrodes, a faster peak inhibition was observed, exhibiting very low or undetectable currents after the first scan (not shown).

Because many parameters could affect the density and quality of the resulting grafted films, it was necessary to adopt standard conditions for the grafting reaction. The diazonium salt concentration was fixed at 1 mM in 0.1 M HCl maintained at 4 °C. The functionalization of the carbon electrode was carried out by stepping the electrode from open circuit potential to a constant applied potential, $E_{appl.}$, and holding at that value for a given electrodeposition time, $t_{appl.}$ The influence of $E_{appl.}$ was examined for both diazoniums and the best results were obtained for an applied potential 0.2–0.3 V more negative than the reduction peak potential of the diazonium salts. Values of $E_{appl.} = -0.8$ and -0.4 V were thus selected for the 4-azidobenzene and 4-ethynylbenzene diazoniums, respectively. The influence of $t_{appl.}$ was also examined and an electrodeposition time ≥ 1 min was enough for saturating the surface with a phenylazide or phenylacetylene monolayer (vide infra). Once formed, the azide- or acetylene-modified electrodes were thoroughly rinsed with water and ethanol to remove any physisorbed species and then immersed at room temperature (16–20 °C) in an aqueous click solution containing appropriate $Fc{-}C{\equiv}CH$ or $FcMeN_3$ (10 µM) and a large excess of 1:1 $CuSO_4$/ascorbic acid (10 mM) catalyst precursor. After undergoing the click reaction for a given incubation time, the electrodes were copiously washed with water and ethanol, and scanned in 0.1 M KPF_6 by cyclic voltammetry. Figure 2 shows typical CVs of modified PGE or GC electrodes after click reaction. A well-defined pair of symmetric peaks ($E^0 = (0.33 \pm 0.02)$ V vs. SCE), characteristic of the reversible oxidation/reduction of immobilized ferrocene groups, were obtained. The peak current was found to vary linearly with the scan rate from 0.02 to 4 V s^{-1}, which is consistent with a surface-immobilized ferrocene. The peak potential difference, ΔE_p, at 0.1 V s^{-1} was fluctuated from 45 to 55 mV, depending on the nature of carbon electrode, whereas the full-width at half-maximum of the anodic and cathodic peaks was roughly 0.17 V. These values are greater than those expected for an ideal Nerstian behavior, which suggests some heterogeneity or interaction among attached ferrocenes.[17] No peaks corresponding to the ferrocene redox couple were observed when an azide-modified GC electrode was incubated with $Fc{-}C{\equiv}CH$ in the absence of $CuSO_4$/ascorbic acid (Figure 2b, -----) or when an unmodified GC electrode was immersed in the click solution (not shown). Identical results were obtained for the reversed approach involving the coupling of $FcMeN_3$ to an acetylene-modified electrode. These control experiments clearly show that the click reaction is highly specific and that there is no significant adsorption of $Fc{-}C{\equiv}CH$ or $FcMeN_3$ onto the bare or modified electrode.

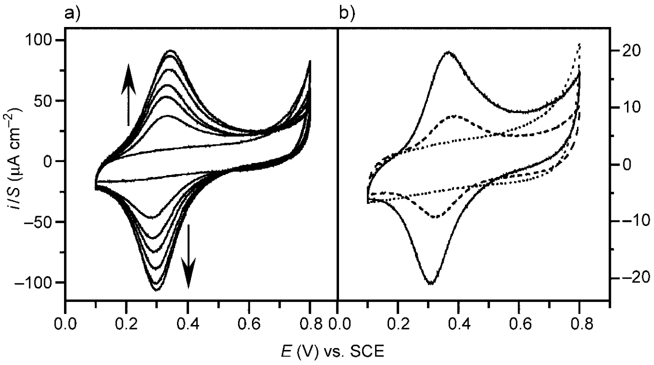


Figure 2. a) CVs of an azide-grafted PGE electrode after coupling with $Fc{-}C{\equiv}CH$ (from bottom to top: after 0, 10, 20, 30, 50, 90, 120 min incubation in an aqueous solution of 10 µM $Fc{-}C{\equiv}CH$ and 10 mM $CuSO_4$/ascorbic acid). b) CVs of (—— and •••••) azide- or (-----) acetylene-modified GC electrodes after incubation (30 min) in a click solution containing 10 µM of the appropriate $Fc{-}C{\equiv}CH$ or $FcMeN_3$ molecule and 10 mM of $CuSO_4$/ascorbic acid (•••••: without $CuSO_4$/ascorbic acid). Electrolyte: aqueous solution of 0.1 M KPF_6; scan rate: 0.1 V s^{-1}; $T = 20$ °C).

Progress in the heterogeneous click reaction was obtained by recording the voltammetric response of ferrocene as a function of incubation time into the click solution. A typical set of CVs obtained at a same PGE electrode is depicted in Figure 2a. The area under the oxidation or reduction peaks can be used to calculate the surface concentration of attached ferrocenes per unit of geometric electrode area, Γ_{Fc}. The variation of Γ_{Fc} as a function of the reaction time is plotted in Figure 3a at both PGE and GC electrodes. Completion of the reaction was achieved relatively rapidly since the maximal coverage at the PGE electrode was reached in less than 1 h and at the GC electrode in less than 20 min. It is worth noting that higher concentrations of $CuSO_4$/ascorbic acid did not change the shape and magnitude of the kinetic curves of Figure 3a. The maximal surface concentration (Γ_{Fc}^{max}) obtained at the GC electrode (i.e., $(3.3 \pm 0.9) \times 10^{-10}$ mol cm^{-2}) is consistent with the theoretical value that can be calculated for a hexagonally close-packed monolayer of ferrocene moieties on a perfectly flat surface (i.e. 4.5×10^{-10} mol cm^{-2}, assuming that the ferrocene headgroup is a

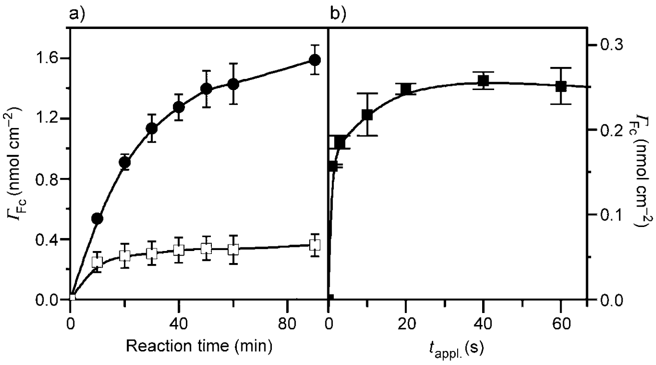


Figure 3. a) Electrochemically determined coverage of clicked ferrocene at azide-modified electrodes ($t_{appl.} = 1$ min) as a function of reaction time into a click solution containing 10 µM $Fc{-}C{\equiv}CH$ and 10 mM $CuSO_4$/ascorbic acid (●: PGE electrode; □: GC electrode). b) Ferrocene coverage at azide-modified GC electrodes as a function of $t_{appl.}$ applied during the electrochemical grafting of $N_3{-}C_6H_4{-}N_2^+$ (1 mM). The click reaction time was 60 min when using the same click solution as that in (a). Error bars represent the standard deviation from three electrodes.

hard sphere of 6.6 Å diameter)[18] and is also in agreement with the values obtained for ferrocene-terminated SAMs on gold (Γ_{Fc}^{max} ranging from 1.5×10^{-10} to 6×10^{-10} mol cm^{-2}).[19] A higher Γ_{Fc}^{max} was obtained at the PGE electrode (1.4×10^{-9} mol cm^{-2}), which corresponded approximately to three equivalent close-packed layers. This higher value is not surprising since the polished-edge plane of pyrolytic graphic was shown to be highly porous, with a pore-size distribution spanning from the micro to nanometric range, thus leading to a real electrode area much larger than the geometric one.[20,21] The mesoporosity of PGE suggests that some of the grafted azide or acetylene groups should be less accessible to the coupling reagents. This steric and/or diffusional constraint may explain the slower rate of ferrocene surface coupling at PGE than at the GC electrode (Figure 3a). The influence of electrodeposition time on the density of azide groups grafted on the electrode surface was evaluated indirectly through the determination of Γ_{Fc}^{max} obtained after the click reaction under optimal conditions. Considering that every grafted phenylazide group is able to quantitatively react with one ethynylferrocene, the resulting curve in Figure 3b suggests that a complete phenylazide monolayer can be obtained after only 20 s of electrochemical grafting in a 1 mM 4-azidobenzene diazonium solution. This result illustrates the remarkable efficiency of the electrochemical grafting, a process that is considerably much faster than the formation of SAMs of alkylthiols on a gold surface.

Table 1 summarizes the Γ_{Fc}^{max} values obtained at different functionalized carbon electrodes. A rapid inspection of data shows that there is a significant difference between azide- and acetylene-modified electrodes. The ferrocene surface concentration is doubled upon passing from acetylene- to azide-modified GC electrodes, and nearly tripled in the case of PGE electrodes. A first hypothesis that should explain such a difference is the production of a lower density of grafted reactive groups during the electrodeposition of 4-ethynylbenzene diazonium than of 4-azidobenzene diazonium. This lower density may be attributable to a surface coupling of the delocalized phenylacetylene radical at the C(1) position of the acetylene function rather than through the aromatic ring. A second hypothesis is a lower click-coupling reactivity of the grafted phenylacetylene layer versus the phenylazide layer. This is supported by the mechanism of copper-catalyzed azide–alkyne cycloaddition, which was proposed to pass through the formation of an intermediate multinuclear copper complex involving more than one acetylene group in the coordination sphere.[22] Although it is unclear what the nature of this intermediate might be, the multiple binding of acetylene suggests that the surface reaction should be prone to steric effects, thus leading to a lower maximal coverage of the clicked molecule with acetylene- than the azide-modified surface. The voltammetric response of ferrocene-functionalized electrodes was observed to be quite stable upon prolonged immersion into aqueous solution. The current response was, for example, decreased by only 15–20 % after seven days. The grafted ferrocene layer was also extremely difficult to remove since after ultrasonication for 1 min in ethanol, the cyclic voltammetric signal remains nearly unchanged. Once prepared, the azide- or acetylene-grafted electrodes can be stored in a dry state in air and in the dark without loss of their click-coupling capacity. For example, after one week storage, we found no difference in the clicked-ferrocene coverage compared with freshly functionalized electrodes.

Table 1. Maximal ferrocene surface concentrations obtained at different functionalized carbon electrodes.[a]

Functional group grafted onto the electrode	Type of carbon electrode	Γ_{Fc}^{max} [$\times10^{-10}$ mol cm^{-2}]
N_3–	GC	3.3 ± 0.9
N_3–	PGE	14.3 ± 1.0
HC≡C–	GC	0.9 ± 0.1
HC≡C–	PGE	2.7 ± 0.2

[a] For all electrodes, $t_{appl.}$ was 1 min and the click reaction time was 60 min.

The practical interest of azide-functionalized carbon electrodes was demonstrated with the covalent coupling of an acetylene-terminated biotin followed by the binding of the enzyme conjugates N–HRP. We have selected this redox enzyme because its catalytic activity towards H_2O_2 reduction can be easily monitored by cyclic voltammetric in the presence of a soluble redox mediator.[23] The presence of biotin on the electrode surface can thus be indirectly revealed from the electrocatalytic current generated by N–HRP specifically attached through avidin–biotin recognition. Figure 4 shows the cyclic voltammetric current responses (normalized here to the geometric electrode area, *S*) recorded in the presence of $[Os^{III}(bpy)_2Clpy]^{2+}$ (bpy = bipyridine, py = pyridine) as mediator and excess H_2O_2. A typical sigmoidal catalytic wave with a well-developed steady-state cathodic current was observed at the azide-grafted GC electrode. This result is in marked contrast to the voltammetric current response recorded at an underivatized GC electrode, which showed only the reversible wave of the mediator with no significant catalytic current. From the magnitude of plateau

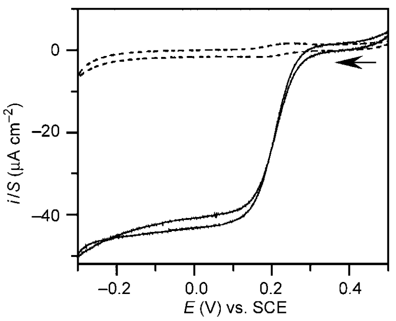


Figure 4. Electrocatalytic responses obtained at (-----) an unmodified and (——) a phenylazide-modified GC electrode ($t_{appl.} = 2$ s in a solution containing 0.1 mM N_3–C_6H_4–N_2^+). All of the electrodes were immersed first for 60 min in a click solution containing the acetylene-terminated biotin, followed by incubation for 2 h in 0.2 μM N–HRP. The CV curves were recorded in a 0.1 M phosphate buffer (pH 7.4) containing 20 μM $[Os^{III}(bpy)_2Clpy]^{2+}$ and 1 mM H_2O_2 (scan rate: 20 mV s^{-1}; $T = 20$ °C).

current, the N–HRP kinetic rate constants, and the rate equation relating the steady-state catalytic current to the enzyme coverage, one can estimate the N–HRP surface concentration.[23b] Taking into account the previously determined rate constants of N–HRP,[23b] a surface concentration of 3.15 $\mathrm{pmol\,cm^{-2}}$ was calculated. This value is consistent with the formation of a saturated monolayer on the surface.[23b] The catalytic response of the enzyme electrode was finally observed to be quite stable over time, since no decrease was noticed after several days storage in an aqueous buffer. Such good stability demonstrates the strong adhesion of the immobilized enzyme on the clicked layer of biotin.

Conclusion

We have developed a versatile, simple, and fast method to covalently attach functional molecules on carbon surfaces through an efficient combination of electrochemical grafting and click chemistry. The electrochemical grafting offers a high degree of control over the functionalization, with an addressing capability, whereas the click coupling promises a wide range of applications owing to the wide variety of functional groups that are orthogonal to the click reaction. A last interesting aspect that was previously demonstrated on the gold electrode[24] and that might be applied in the present case, is the electro-addressing of the click reaction by electrochemical reduction of the copper(II) catalyst precursor. We are currently investigating this possibility.

Experimental Section

Reagents: The ethynylferrocene was purchased from Aldrich and used without further purification. Hydrogen peroxide (50 wt %) was supplied by Prolabo (reagent-grade product). Its concentration was determined by permanganate titration. The complex $[\mathrm{Os^{III}(bpy)_2Clpy}](\mathrm{PF_6})_2$ was prepared as previously described[23] and the azidomethylferrocene was synthesized under argon by following a published procedure.[25]

Synthesis of 4-azidobenzene diazonium tetrafluoroborate $[\mathrm{N_3{-}C_6H_4{-}N_2}](\mathrm{BF_4})$: A cold solution of $\mathrm{NaNO_2}$ (45 mg, 0.65 mmol) in Milli-Q water (0.25 mL) was added slowly to a cold solution (over an ice bath) of 4-azidoaniline hydrochloride (100 mg, 0.59 mmol) in 1 M HCl (1 mL). The mixture was left to react at 4 °C for 1 h, and then 0.6 mL of a saturated solution of $\mathrm{NaBF_4}$ in Milli-Q water was added. The off-white precipitate was filtered and rinsed twice with cold ether (10 mL). The crude product was purified by reprecipitation from acetonitrile in the presence of an excess of diethyl ether to give the diazonium salt as an off-white powder (78 mg, 57 %). The solid was dried in a desiccator and stored in the dark. ^{1}H NMR ($\mathrm{CD_3CN}$): $\delta = 8.42$ (m, 2H, $J_{AB} = 9.2$, $J_{AA'} = 3.0$, $J_{AB'} = 0.3$ Hz; $\mathrm{C}\mathit{H}{-}\mathrm{C}{-}\mathrm{N_2}^+$), 7.49 ppm (m, 2H, $J_{AB} = 9.2$, $J_{BB'} = 3.5$, $J_{AB'} = 0.3$ Hz; $\mathrm{N_3{-}C{-}C}\mathit{H}$); ^{13}C{^{1}H} NMR ($\mathrm{CD_3CN}$): $\delta = 156.1$ ($\mathrm{N_3{-}}\mathit{C}$), 135.6 ($\mathit{C}\mathrm{{-}C{-}N_2}^+$), 123.1 ($\mathrm{N_3{-}C{-}}\mathit{C}$), 107.4 ppm ($\mathit{C}\mathrm{{-}N_2}^+$); MS (ES): calcd: 129.0447; found: 129.0453 $[M]^+$; elemental analysis calcd (%) for $\mathrm{C_6H_4N_5BF_4}$: C 30.93, H 1.73, N 30.07; found: C 29.25, H 1.62, N 28.16.

Synthesis of 4-ethynylbenzene diazonium tetrafluoroborate $[\mathrm{HC{\equiv}C{-}C_6H_4{-}N_2}](\mathrm{BF_4})$: The same procedure as for $[\mathrm{N_3{-}C_6H_4{-}N_2}]\mathrm{BF_4}$ was used, starting from 4-ethynylaniline. The diazonium product was recovered as a crystalline claret-colored powder (51 %). ^{1}H NMR ($\mathrm{CD_3CN}$): $\delta = 8.45$ (d, 2H; $\mathrm{C}\mathit{H}{-}\mathrm{C}{-}\mathrm{N_2}^+$), 7.98 (d, 2H; $\mathrm{HC{\equiv}C{-}C{-}C}\mathit{H}$), 4.26 ppm (s, 1H; $\mathrm{{-}C{\equiv}C}\mathit{H}$); ^{13}C{^{1}H} NMR ($(\mathrm{CD_3})_2\mathrm{SO}$): $\delta = 135.0$ ($\mathrm{HC{\equiv}C{-}C{-}}\mathit{C}\mathrm{H}$), 134.6 ($\mathrm{HC{\equiv}C{-}}\mathit{C}$), 134.0 ($\mathit{C}\mathrm{H{-}C{-}N_2}^+$), 116.5 ($\mathit{C}\mathrm{{-}N_2}^+$), 92.3 ($\mathrm{HC{\equiv}}\mathit{C}$), 82.3 ppm ($\mathrm{H}\mathit{C}\mathrm{{\equiv}C}$); MS (ES): calcd: 146.0461; found: 146.0467 $[M]^+$.

Apparatus: The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 250 spectrometer operating at 250.13 MHz. Electrospray ionisation mass spectrums were recorded on a Bruker microTOF-Q instrument in positive ion mode.

Electrochemical experiments: Cyclic voltammetry was performed in a standard three-electrode water-jacketed cell by using an Autolab potentiostat (PGSTAT 12, Autolab) interfaced to a PC computer and piloted with GPES software (version 4.7). A saturated calomel electrode, isolated from the solution by a glass frit, and a platinum wire were used as reference and counter electrodes, respectively. Working electrodes were either glassy carbon (3 mm diameter) or pyrolytic graphite edge (0.1 $\mathrm{cm^2}$). Glassy carbon electrodes were polished successively by silicon carbide grinding paper (grit 1200) and by a 3 and 1 μm alumina slurry on a cloth polishing pad and then washed in ethanol under sonication, whereas PGE electrodes were just polished successively with a 3 and 1 μm alumina slurry and thoroughly rinsed with water and ethanol. CVs of ferrocene-modified electrodes were systematically recorded in 0.1 M $\mathrm{KPF_6}$ at 20 °C.

Electrode modification: Surface derivatization of carbon-based electrodes was carried out at 4 °C in a deaerated solution (with argon) of 0.1 M HCl (3 mL) containing 1.3 mM of 4-azidobenzene diazonium or 4-ethynylbenzene diazonium ($\mathrm{BF_4}$ salts). The reductive electrodeposition of the aryl diazonium salts was achieved amperometrically by stepping the potential at a sufficiently cathodic value. Unless otherwise stated, for the reduction of 4-azidobenzene diazonium or 4-ethynylbenzene diazonium, the working electrode potential was set for 1 min at a value $E_{\mathrm{appl.}} = -0.8$ or -0.4 V, respectively. After functionalization, the electrodes were thoroughly rinsed with Milli-Q water and next with ethanol to remove any adsorbed species.

Surface coupling of ferrocene probes by copper(I)-catalyzed azide–alkyne cycloaddition: In a typical experiment, arylazide- or arylalkyne-modified electrodes were immersed in a 15 mL Milli-Q water solution containing $\mathrm{CuSO_4}$ (10 mM), L-ascorbic acid (10 mM), and the corresponding ethynylferrocene or azidomethylferrocene (10 μM). The click reaction was complete after ≈60 min. The ferrocene-modified electrodes were then rinsed with copious amounts of Milli-Q water and ethanol to ensure that any physisorbed species were washed off, and finally stored in aqueous buffer before being scanned by means of cyclic voltammetry.

Click surface coupling of acetylene-terminated biotin: Acetylene-terminated biotin was purchased from an Invitrogen kit (Click-iT™ C33372 kit). For the coupling reaction, the other reagents contained in the kit (i.e., copper catalyst and buffers) were also used according to an adapted protocol. In a reaction tube, the following reagents were successively added: 38 μL "component B", 2 μL acetylene-terminated biotin in DMSO, 20 μL $(\mathrm{NH_4})_2\mathrm{SO_4}$ buffer (50 mM, pH 8.7), and 4 μL Milli-Q water. After stirring, 4 μL $\mathrm{CuSO_4}$ (40 mM) and 4 μL "component D" were added, followed after 5 min by 8 μL "component E" ("component D" and "component E" were first reconstituted in 100 and 500 μL Milli-Q water). The resulting mixture was spotted onto the azide-modified GC surface for 1 h under a controlled humidity atmosphere. The electrodes were thoroughly rinsed with Milli-Q water and sonicated in ethanol for 30 s. We also verified that the click reaction was working by using our own conditions. For this purpose, a 80 μL solution containing $\mathrm{CuSO_4}$ (1 mM), L-ascorbic acid (1 mM), and 2 μL of biotin alkyne was prepared. 8 μL of this solution was deposited on the active surface of each modified electrode for 1 h, and then the electrodes were rinsed with Milli-Q water and sonicated in ethanol for 30 s.

N–HRP immobilization and electrocatalytic detection: The biotin-clicked electrodes were immersed for 2 h in 0.1 M phosphate buffer (PB, pH 7.4) containing 0.2 μM N–HRP, and next copiously rinsed with PB and stored in the same buffer until used. The catalytic response of HRP-modified electrodes were obtained by scanning the electrodes in an electrochemical cell maintained at 20 °C and filled with a PB solution containing 20 μM $[\mathrm{Os^{III}(bpy)_2Clpy}](\mathrm{PF_6})_2$ and 1 mM $\mathrm{H_2O_2}$.

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