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EPR Spectroelectrochemical Investigation of Guanine Radical Formation and Environment Effects

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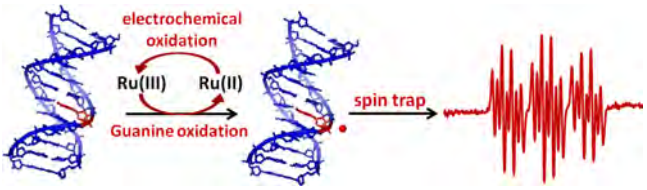
Supporting Information

ABSTRACT: Guanine radical detection was carried out by a new convenient and efficient method coupling electron paramagnetic resonance spectroscopy and indirect electro-oxidation of guanine in different biological environments, from the free nucleotide to several types of DNA substrates. Compared to the widely used photoirradiation method, this method appeared more selective in the choice of the electrochemical mediator. Carried out in presence of a ruthenium mediator and PBN as spin trap, this method revealed two types of EPR spectra depending of the environment of the guanine radical. Both EPR spectra show the trapping of the neutral guanine radical G(−H)$^{\bullet}$ obtained after fast deprotonation of the radical cation G$^{\bullet+}$. However, they differ by the atom where the trapped radical is centered. This difference highlights the structural dependency of the environment on the nature of the radical formed. This work gave the evidence of an innovative method to detect in situ the guanine radical.



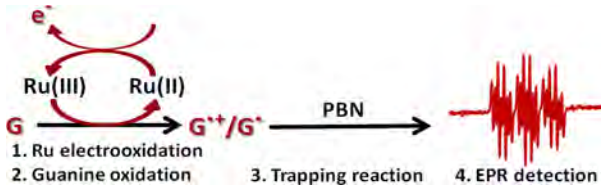
INTRODUCTION

Guanine radical is a key intermediate in the charge transfer processes responsible for DNA damage.[1] It is well-known that exposure to ionizing and ultraviolet radiation or reactive chemical species can lead to oxidative damage to DNA, a common event at the origin of mutagenesis, cell death and cancer.[2] Among the four bases, which composed the DNA, guanine is the main target as indicating by its lowest oxidation potential.[3] In neutral aqueous solution, the one-electron oxidation of guanine residues leads initially to the guanine radical-cation (G$^{\bullet+}$). This radical-cation is rapidly converted into a neutral radical by deprotonation or hydrolysis (G(−H)$^{\bullet}$ or G$^{\bullet}$), which gives various two- and four-electron oxidation products according to the media.[4,5] Herein, we propose a new convenient method to detect and analyze the guanine radical.

The guanine radical species detection involves specific techniques due to its short lifetime, including UV−visible spectra,[6] flash-quench technique,[7] pulse radiolysis,[8] laser flash photolysis[9] and electron paramagnetic resonance.[10] Studies on guanine radical are mainly focused on photoirradiation in the presence of photosensitizers including anthraquinones,[11] naphtalimides, riboflavin and ruthenium complexes,[12] in order to oxidize the guanine. To the best of our knowledge, only few reports took an interest in other guanine oxidation techniques including the hydroxyl ($^{\bullet}$OH) radical strong oxidant,[6] or electrooxidation, even though electrochemical oxidations are currently attractive.[13] Some studies focused on direct electro-oxidation of nucleobases using specific electrodes such as pretreated glassy carbon electrode,[14] tin-doped indium oxide coated glass plate[15] or carbon-fiber ultramicroelectrode,[16] while other studies centered in indirectly electrochemical oxidation of DNA. In fact, it has been demonstrated that some complexes generated in situ can oxidize guanine.[17] Furthermore, this indirect method does not require specific electrode, avoiding the surface functionalization step.[18]

In this report we present the detection of the guanine radical by EPR spectroelectrochemistry coupling the mediated electro-oxidation of guanine with the spin trapping method (Scheme 1). The in situ combination of electrochemistry and EPR spectroscopy make for an ideal partnership since a one-electron-transfer process from diamagnetic material must result in the formation of paramagnetic transient species. EPR spectroelectrochemical experiments provide information about the redox site activity by studying the contribution of various

Scheme 1. Electrochemical/EPR Coupling Method



nuclei to the molecular orbital occupied by the unpaired electron.[19]

At first, the electrochemical mediator, a ruthenium(II) complex, is oxidized at the electrode; (2) the oxidation of guanine by Ru(III) takes place; (3) the spin trap entraps the resulting radical, probably neutral; (4) the EPR spectrum is recorded during the whole process (Scheme 1).

EXPERIMENTAL METHODS

General. Guanosine 5′-monophosphate, adenosine, cytidine, thymidine and *N-tert*-butyl-α-phenylnitrone (PBN) were purchased from Sigma-Aldrich and used without further purifications. Poly(dG-dC)$_2$ was obtained from Sigma and 18-mer oligonucleotides were obtained from Eurogentec SA. Tris(2,2′-bipyridyl-4,4′-bis(hydroxymethyl))Ruthenium(II) hexafluorophosphate complex was synthetized following a reported procedure.[20]

Sample Preparation. Solutions were prepared in phosphate buffer (pH 7.5, PBS 10 mM and NaCl 0.1 M) at room temperature with 1 mM of ruthenium complex, 25 mM of PBN and between 0.2 to 5 mM of duplex DNA or GMP, in a 2 mL flat quartz cell adapted to electrochemical measurements, using a three-electrode setup: the working and counter-electrode were platinum and the reference electrode was a silver wire. Solutions were thoroughly degassed with Argon prior to use.

Cyclic Voltammetry. Voltammetric experiments were carried out at room temperature by using an Autolab 20 potentiostat (EcoChemie). A classical three electrodes cell was used. The working electrode was a Pt disc (diameter 0.5 mm) or a GC disc (glassy carbon, diameter 1 mm, BioLogic). The counter electrode was a platinum wire. The reference electrode was a saturated calomel electrode (SCE). All experiments were carried out in phosphate buffer (pH 7.5, PBS 10 mM and NaCl 0.1 M) under argon atmosphere. Voltammetric results were analyzed with the help of DigiElch Software.

Electrochemical Coupled Electron Paramagnetic Resonance. The EPR spectrometer was coupled to a potentiostat-galvanostat (EG&G Princeton Applied Research-Model 362). A PHN 81 (Tacussel) voltameter was used to control the applied potential. A flat quartz cell adapted to electrochemical measurements (Bruker, Wissembourg, France) was used for analysis. The electrochemical reduction was performed using a three-electrode setup: the working and counter-electrode were platinum and the reference electrode was a silver wire (its potential was 0.056 V/SCE).

EPR spectra were obtained at X-band at room temperature on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). WINEPR and SIMFONIA software (Bruker, Wissembourg, France) were used for EPR data processing and spectrum computer simulation. Typical scanning parameters were: scan number, 120; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 20 mW; sweep width, 100 G; sweep time, 41.94 s; time constant, 20.48 ms; center field, 3 480 G; receiver gain 2×10^6.

RESULTS AND DISCUSSION

Mediated Electrochemical Oxidation of Guanine. Previous works reported that ruthenium(III) complexes, with an adequate potential, oxidize guanine by electron transfer.[21] In phosphate buffer at pH 7.5, an hydrophilic ruthenium complex tris(2,2′-bipyridyl-4,4′-bis(hydroxymethyl))ruthenium(II) hexafluorophosphate[20] was studied. The cyclic voltammograms of this ruthenium complex showed the Ru(III)/Ru(II) redox couple which appears reversible (Figure 1, curve A black). The

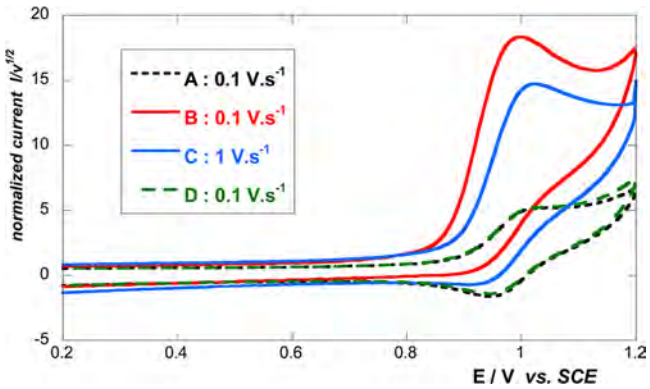
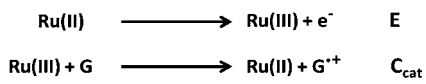


Figure 1. Normalized cyclic voltammograms versus the potential scan rate (current $I/v^{1/2}$) in phosphate buffer (pH 7.5) at a GC electrode of Ru(II) complex 10^{-3} M, potential scan rate v: (A) complex alone; (B and C) complex after addition of guanosine 5′-monophosphate 6.2×10^{-3} M; (D) complex after addition of adenosine 5×10^{-3} M.

oxidation peak current (forward) is linearly related to the Ru(II) complex concentration and to the square root of the potential scan speed v as for a simple diffusion controlled electron transfer (Scheme 2, step E). The redox potential $E°$ is

Scheme 2. EC Catalytic Pathway of Guanine Oxidation by Ru(III)



estimated by a mean value of $E° = [\mathrm{Ep(forward)} + \mathrm{Ep(backward)}]/2 = 0.986$ V vs SCE. When guanosine 5′-monophosphate (GMP) was added (Figure 1, curves B and C), the oxidation peak of Ru(II) was enhanced and the reverse peak disappeared (curve B); when the potential scan rate v was increased (curve C), the backward peak appeared (reduction of Ru(III)). At a potential scan rate $v = 0.1$ V.s^{-1}, the oxidation peak is 4 times higher than that of Ru(II) complex; at $v = 1$ V·s^{-1}, the ratio drops to 3. The forward peak current grew also when the GMP concentration increased.

This behavior can be explained by an EC$_{cat}$ scheme (Electron transfer followed by a catalytic reaction) according to Scheme 2. Although the G$^{\bullet+}$/G potential (1.04 V vs SCE[22]) is slightly higher than the Ru(III)/Ru(II) potential, the irreversibility of GMP oxidation draws the overall process.

Simulations carried out with the DigiElch software gave an order of magnitude of 2.5×10^3 l.mol^{-1}.s^{-1} for the chemical oxidation of GMP; however, it appears that the mechanism is more complex. Such cyclic voltammetry experiments were

carried out with the other nucleobases separately. When adenosine was added, the cyclic voltammograms where unchanged (Figure 1, curve D), which means that no reaction took place between Ru(III) and adenosine. For cytidine and thymidine (Figure 2), the same conclusions can be drawn.

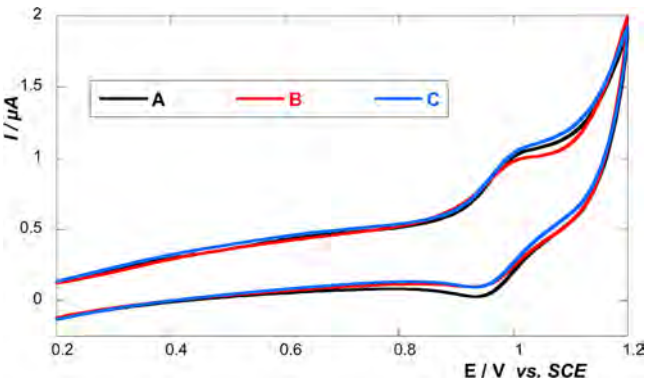


Figure 2. Cyclic voltammograms in phosphate buffer (pH 7.5) at a Pt electrode of Ru(II) complex 10^{-3} M, potential scan rate 0.1 V.s^{-1}: (A) complex alone; (B) complex after addition of cytidine 5×10^{-3} M; (C) complex after addition of thymidine 5×10^{-3} M.

Taking into account these results, the Ru(III) complex provides the oxidation of guanine to the radical-cation, which is further converted in the medium:[4,5] the process is quantitative and is named "indirect or mediated electrooxidation" of guanine. Moreover, the oxidation is selective because the other nucleobases are not oxidized.

EPR Spectroscopy. To demonstrate the formation of radical intermediates accompanying the one-electron mediated oxidation of guanine, electrochemistry was coupled with EPR spectroscopy using PBN (*N-tert*-butyl-α-phenylnitrone) as a spin trap. Experiments were indeed carried out at room temperature and guanine radical itself is not stable long enough to be observed. Two milliliter and 0.5 mL flat quartz cells adapted to electrochemical measurements were used for analysis. The electrochemical reduction was performed using a three-electrode setup: the working and counter-electrode were platinum, and the reference electrode was a silver wire. The applied potential was chosen to be the oxidation potential of Ru(II): $E_{applied}$ = 1 V. EPR spectra were obtained at X-band for samples containing Ru(II) complex 1 mM, PBN 25 mM and GMP 5 mM. All experiments were performed in phosphate buffer (10 mM PBS, 100 mM NaCl, pH = 7.5).

Experiments were first carried out on GMP. The EPR spectrum recorded in the presence of Ru(II), GMP and PBN after electrolysis (120 scans) consists in a multilined spectrum characterized, as demonstrated by simulation, by the hyperfine splitting constants $a_{N\ PBN}$ = 12.106 G, $a_{N'}$ = 1.48 G, $a_{H\ PBN}$ = 3.5 G, $a_{H'}$ = 2.6 G, and g = 2.0098 (Figure 3a). No peak was recorded in the absence of PBN, GMP, or Ru(II) (Figure S1, Supporting Information). Furthermore, control experiments with the other nucleosides showed no radical formation (Figure 3b).

Numerous experimental and theoretical studies showed the formation and evolution of the guanine radical $G^{\bullet+}$. It is now well established that this radical is not stable under physiological conditions and it rapidly deprotonates.[5,23] The deprotonation of $G^{\bullet+}$ has been described in terms of a release of the N1 proton (pK_a = 3.9) to form the G(N1−H)$^\bullet$ neutral

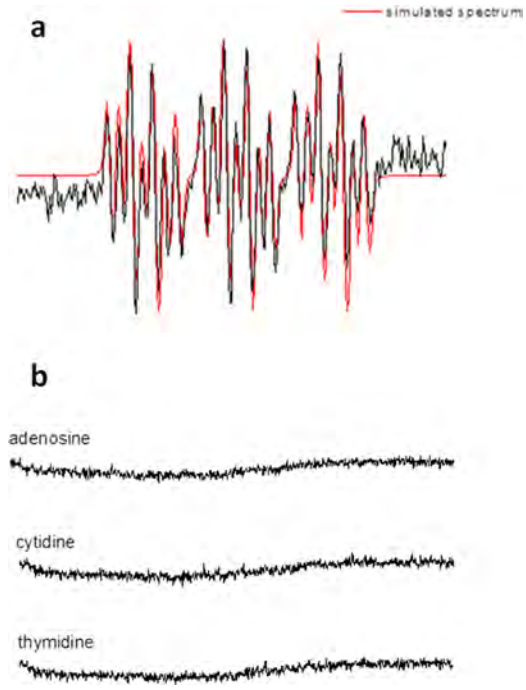


Figure 3. (a) EPR spectrum of the spin-trapped guanine radicals produced by the electrolysis of Ru(II) complex in the presence of PBN in PBS buffer at pH 7.5 at room temperature from GMP 5.10^{-3} M (black) and simulated spectrum (red). (b) EPR spectra recorded in the same conditions with other nucleosides.

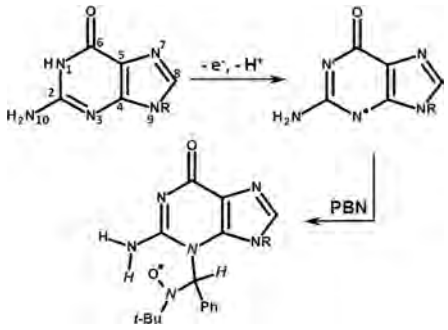
radical.[23] Some studies also suggested the formation of G(N10−H)$^\bullet$, in which the exocyclic nitrogen is deprotonated.[5] However, both calculations and experiments in water media are either more consistent with the N1 deprotonation mode or suggest a tautomerization process leading to G(N1−H)$^\bullet$ from G(N10−H)$^\bullet$.[24] The G(N1−H)$^\bullet$ neutral radical has several possible resonance structures (Scheme S1). Indeed, various forms of G(N1−H)$^\bullet$ have been described, including oxygen,[23,25] C8, C5,[26] and N3[27,28] centered radicals depending on the oxidation mode.

In our hands, the radical detected corresponds to the radical adduct formed between the spin trap PBN (nitrogen and hydrogen coupling) and a radical derived from guanosine. The hyperfine splitting constants $a_{N'}$ and $a_{H'}$ exclude in this case an addition to O, C5, or C8[29] and suggests an addition to nitrogen. The second hydrogen coupling would in this case be attributable to an H−N10 atom. In our experiment, we cannot determine on which exact nitrogen of the guanidine moiety (N1, N3, or N10) the radical was trapped, but according to previous studies,[27,28] a N3-centered radical is more probable (Schemes 3 and S2).

The following step consisted of applying this method in a biological environment, in order to detect the guanine radical in DNA substrate. DNA environment can strongly cause modifications in comparison with a single nucleotide. For example, it is well-known that nucleobase oxidation potentials vary according to their environment.

The same experiments were thus performed with 18-mer synthetic double-stranded oligonucleotides containing 0, 1, 2, 3, and 4 adjacent guanines, in order to determine potential sequence effect on the structure of the trapped radical, with following sequences respectively: 5′-TATTAATTATAATTAA-TA-3′; 5′-TATTATAAGTAATTATTA-3′; 5′-TATTATAA-

Scheme 3. PBN-Trapped N3-Centered Guanine Radical in GMP[a]



[a]Atoms coupling with PBN oxygen radical are in italic.

GGTAATTATA-3′; 5′-TATTATAGGGTAATTATA-3′, and 5′-TATTATAGGGGTAATTAT-3′ and their complementary strands.

Indeed, in duplex DNA, guanine oxidation potential varies with the sequence particularly at multiple guanine sequences such as GG, GGG, or GGGG for which oxidation potential gradually dropped with increasing number of stacked guanine.[30] A last experiment was carried out with long polynucleotide poly(dG-dC)$_2$. The corresponding spectra are presented in Figure 4a,c, respectively. First, spectrum recorded with the oligonucleotide without guanine showed as expected no signal at all (Figure 4d). The other spectra are the same for poly(dG-dC)$_2$ and DNA duplex whatever the number of guanine bases. As demonstrated by the simulation carried out for 18-mer DNA with 4G (Figure 4b), the EPR spectra are characterized by the hyperfine splitting constants $a_{N\ PBN}$ = 11.2 G, $a_{H\ PBN}$ = 3.2 G, and $a_{H'}$ = 2.1 G with g = 2.0098. In double-strand DNA, the $G^{\bullet+}$ radical is also not stable and rapidly deprotonates to form the $G(-H)^{\bullet}$ radical but in a slower way than in the case of free guanosine. This slower deprotonation is due to the prototropic equilibrium with cytosine, which stabilizes the cation form by sharing the proton between the guanine and the cytosine along the hydrogen bond.[25,27] The deprotonation process is thus in competition with an hydration process, leading to the 8-hydroxy-7,8-dehydroguanyl $G(OH)^{\bullet}$ radical, which is the intermediate in the formation of 8-oxo-7,8-dehydroguanine, the most common oxidative damage in DNA.[22] The nature of the hyperfine splitting constants is in favor of the trapping of the deprotonated radical form. Indeed, the disappearance of the second nitrogen coupling points out addition of PBN to a carbon and confirms that the radical trapping may takes place before hydration. In this case, as shown in scheme 4, the addition would take place through C8. The second hydrogen coupling would be attributable to the H−C8 atom. The differences between the radical trapped in the case of GMP and in duplex DNA can be explained with the structure of the double helix. O, N1 and N10 are involved in the hydrogen bonds with cytosine and thus located in the middle of the helix. On the other hand, C8 is the most accessible atom for PBN, and along all the resonance forms of the guanine radical, the C8-centered one is thus the most easily trapped.

All these results confirmed the one-electron mediated oxidation of guanine, isolated or in DNA environment, by the detection of the radical adduct formed between the spin trap PBN and a guanine radical intermediate. Moreover, the efficiency and the interest of this EPR spectroelectrochemical coupled method are demonstrated. Indeed, the observed EPR spectra are different according to the substrate: either free nucleotide or duplex DNA. Deprotonation pathway is confirmed for the first one-electron oxidation product, $G^{\bullet+}$ in agreement with previous reports. Furthermore, no difference in the signal was observed between the different sequences with one or more guanine, which supports the idea of a single oxidation occurring mainly at the 5′-G of multiple guanine sites such as GG or GGG. The difference observed in the EPR signals, between free guanosine and DNA, is due to the different accessibility of the neutral guanine cation for the PBN

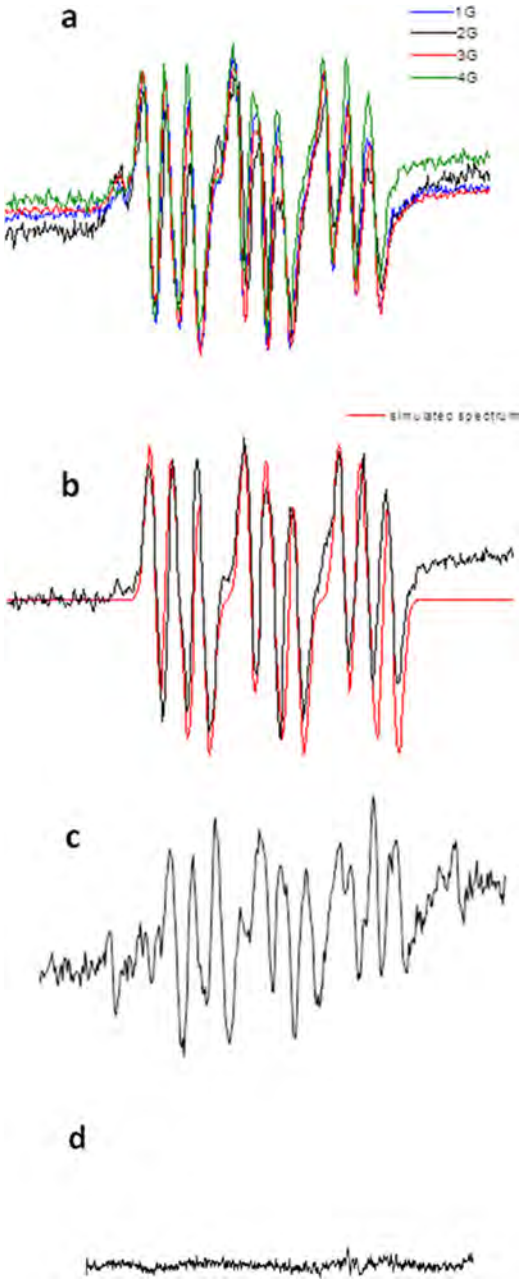
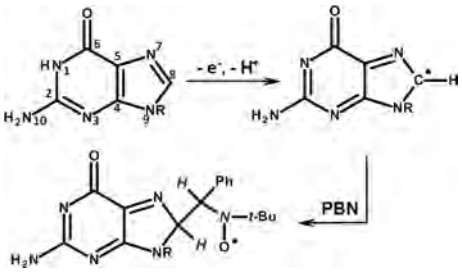


Figure 4. EPR spectra of the spin-trapped guanine radicals produced by the electrolysis of Ru(II) complex 1×10^{-3} M in presence of PBN 25×10^{-3} M in PBS buffer at pH 7.5 at room temperature from (a) double strand 18-mer DNA 5×10^{-4} M with 1G, 2G, 3G, and 4G; (b) double strand 18-mer DNA 5×10^{-4} M with 4G and simulated spectrum (red); (c) poly(dG-dC)$_2$ 3.75×10^{-2} M in base pairs; and (d) double strand 18-mer DNA 0.2×10^{-3} M without G.

Scheme 4. PBN-Trapped Guanine Radical in Duplex DNA[a]



[a]Atoms coupling with PBN oxygen radical are in italic.

trapping and highlights the dependency of the environment on the nature and structure of the radical formed.

■ CONCLUSION

In conclusion, the indirect electrochemical oxidation/EPR spectroscopy coupling method has been shown to be a new attractive technique for the *in situ* guanine radical detection with its easier setup (i.e., working at room temperature) compared to other methods and a relevant tool to understand oxidative damage in DNA. Moreover, the guanine radical analysis has revealed two types of spectra depending of the environment of the guanine radical (free or in duplex DNA). Elucidation of other radical processes in DNA with this method is currently under investigation and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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