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Formation of Glutathionyl Dinitrosyl Iron Complex Protects from Iron Genotoxicity

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The abbreviations used are: CIP- chelatable iron pool, ECD – electronic circular dichroism spectroscopy, DFO – desferrioxamine, DNIC -Dinitrosyl non-heme iron complex, EDRF- endothelium derived relaxing factor, GS- glutathionyl, GSH – glutathione, GSNO – S-nitrosoglutathione, GS-DNIC – dinitrosyl glutathionyl iron complex

ABSTRACT

Dinitrosyl iron (I) complexes (DNICs), intracellular NO donors, are important factors in nitric oxide-dependent regulation of cellular metabolism and signal transduction. It has been shown that NO diminishes toxicity of iron ions and *vice versa*. To gain insight into a possible role of DNIC in this phenomenon, we examined the effect of GS-DNIC formation on the ability of iron ions to mediate DNA damage, by treatment of pUC19 plasmid with physiologically relevant concentrations of GS-DNIC. It was shown, that GS-DNIC formation protects against the genotoxic effect of iron ions alone and iron ions in the presence of naturally abundant antioxidant, GSH. This sheds a new light on the iron-related protective effect of NO in the circumstances of the oxidative stress.

Keywords: Nitric oxide, iron ions, genotoxicity, glutathione

INTRODUCTION

Non-heme dinitrosyl iron complexes (DNICs) of the general formula: $[Fe(NO)_2(X)_2]^-$, are important players in nitric oxide-dependent regulation pathways in the cell [1–5]. NO itself is relatively nonreactive and in biological milieu reacts directly only with transition metals [6], with reactive radicals, such as superoxide, or with lipid peroxyl radicals [6, 7]. This on one hand determines its anti-oxidative properties and on the other, can have some deleterious implications. In the recent work of Folkes and O'Neill [8] NO was shown to protect plasmid DNA from radiation-induced lesions, but at the same time, NO-induced base modifications (such as xanthine and 8-azaguanine) were found responsible for subsequent replication-induced lesions. NO• was shown involved in DNA damage through a variety of different mechanisms, including DNA deamination, oxidation, strand breaks, inhibition of enzymes involved in DNA synthesis or repair, formation of cross-links. [9–13]. NO coexists in cells with nitrosothiols (SNOs) that serve

as endogenous NO carriers and donors; the most abundant nitrosothiol with proven role in NO signalling is S-nitrosoglutathione (GSNO). The liberation of NO from SNOs, as well as transnitrosylation reactions are catalysed by transition metals [14, 15].

In DNICs that are present in biological environment, X is usually a protein-based cysteinate residue or a low-molecular-weight thiol, e.g. glutathione (GSH). Alternatively, nitrosylated iron can coordinate imidazole rings present in residues such as histidine or purines [16]. Formation of high and low molecular-weight DNICs with thiol ligands has been observed in many kinds of organisms and in a wide spectrum of physiological conditions associated with inflammation, Parkinson's disease or cancer.

Accumulation of DNICs in tissue coincides with intensified production of nitric oxide in macrophages, spinal cord, endothelial cells, pancreatic islet cells and hepatocytes [17–19]. DNICs are also important in NO-dependent regulation of cellular metabolism and signal transduction [3–5, 20]. In biological systems NO is stored and transported in the two forms: as S-nitrosothiols (RSNO) or DNICs [21]. Depending on the micro-environment, LMW-DNICs can provide at least two types of nitrosylating modifications of proteins, forming either protein-*S*-nitrosothiols or protein-bound DNICs. Also, DNICs were shown to intermediate in iron-catalysed degradation and formation of *S*-nitrosothiols [15, 22]. A number of studies have described the complex relationships between iron and NO (as reviewed in [16, 23–26]): Toxicity of DNIC's components seems to be mutually dependent on each other [27, 28]. Presence of NO donors protects against iron-induced nephrotoxicity [29, 30] or neurotoxicity [31], whereas inhibition of NO synthesis attenuates this effect [32]. In line with attenuation of iron toxicity by NO, several studies revealed the ability of iron to rescue tumour cells from the anti-tumour activity of NO [33–35]. It was also shown that depletion of GSH rendered the cells vulnerable to NO donors [36]. According to some authors iron may play a protective role by inducing antioxidant species

formation and scavenging NO during oxidative stress [37–39]. It was demonstrated, that free iron incorporation into DNICs significantly attenuates Fenton's activity of iron ions [40, 41]. Simultaneously, our studies showed [42], that up to 50% of NO scavenged by Fe to form dinitrosyl complexes is formed with transiently chelated iron, so called chelatable iron pool (CIP). This is in line with the results obtained in other laboratories reporting that amounts of DNICs formed upon NO treatment are equal to or greater than the measured amounts of chelatable iron [43–45]. Beside the immediate CIP-scavenging effect, the long-term exposure to NO causes an increase in DNIC-bound iron levels, most likely via disruption of iron proteins [45–49]. It has been reported that the release of nitrosonium ion (NO^+) from DNIC-thiosulfate induced apoptosis in human leukemia cells on the Bcl-2 and caspase independent pathway [50].

It must not be missed that dinitrosyl-dithiol-iron possesses endothelium derived relaxing factor (EDRF) activity [1, 51]. An important aspect connecting the roles of GSH and DNIC is related to the ability of the latter to modulate cellular antioxidant activity through the inhibition of glutathione-dependent enzymes; regulatory action of DNICs regards enzymes such as reductase, transferase, and peroxidase of glutathione [52–54].

Iron, due to its propensity to enter the Fenton reaction is highly toxic, and several mechanisms were revealed, which allow the strict regulation of the iron levels and its availability inside the cell [55]. For the same reason iron was thought to be found relatively rare in the vicinity of the DNA, However, recently several reports have been published on the inorganic iron–sulfur cofactors (Fe-S clusters) present in some nucleic acid binding proteins ([56] and references therein). The cofactor's presence maintains the protein structure required for the nonspecific DNA binding and thus is essential for the activity of DNA binding enzymes [57, 58]. On the other hand iron-sulfur clusters were shown to readily scavenge NO via formation of DNIC- and Roussin-Red-Ester-type complexes (RNS) [59, 60]. This implies the possibility of

DNICs occurrence in the proximity of the DNA strand in the conditions of the elevated RNS production. The well-established anti-apoptotic role of iron in tumour cells [33, 61] inspired us to formulate a question: Does NO scavenging by iron protect the DNA from the NO-induced damage during oxidative stress?

Herein presented results on thiolate-type dinitrosylato-iron complex complement the previously published work on the small-molecular dinitrosyl complex of iron with histidines as auxiliary ligands [62].

MATERIALS AND METHODS

Reduced L-Glutathione (98%) was supplied by Avocado Organics. All other chemicals were supplied by Sigma–Aldrich. EPR spectra were recorded on X-band Bruker ESP 300 in 77 K, at microwave power 1 mW, microwave frequency 9.5 GHz and modulation amplitude 0.3 mT. Evaluation of *g* factors was performed using SimFonia 1.25 software (Bruker, DE).

Preparation of S-nitrosoglutathione: S-nitrosoglutathione (GSNO), an NO donor, was prepared according to the literature [63]. Briefly, to the solution containing 20 mM GSH pH 2, an equimolar amount of $NaNO_2$ was added. Mixture was allowed to react for 10 min and pH was brought to 6 with NaOH. The concentration of formed GSNO was calculated according to the molar coefficient 922 M^{-1} cm^{-1} at 335 nm [64].

Preparation of GS-DNIC: Dinitrosyl iron complex with glutathione was obtained according to the method described in the literature [63], anaerobically. The complex was prepared in an average of 200-fold excess of GSH and 20-fold (unless otherwise indicated) excess of GSNO. Briefly, deoxygenized solution of GSH (20 mM) was brought to pH 6 with NaOH. GSNO was added to the desired concentration (400 μM – 2 mM), as indicated. Then, acidic solution of $FeSO_4$ (pH=4, 11.9 mM) was added to various final concentrations in the range of 1 μM –240

μM. Formation of DNIC was confirmed by UV/VIS at 403 nm (Varian Cary 1E spectrophotometer).

Evaluation of DNIC(GS) stability: In order to estimate the stability of the obtained DNICs, and relate it to the previously studied histidinyl complex [62], the preparation was incubated with ten times molar excess of deferoxamine (170 μM DNICs*vs* 2 mM DFO) for 20 min. at room temperature.

DNA interaction measurements: A salmon testes DNA stock solution (1 mg/mL in phosphate buffer) was mixed with solutions containing dinitrosyl iron complex, in the presence of glutathione (20 mM) in the phosphate buffer. Concentration of DNA was checked spectrophotometrically using the DNA extinction coefficient ε = 6600 at 260 nm. The stability of mononucleated and bi-nucleated forms of GS-DNIC in the presence of DNA was assessed by UV/VIS. Additionally, the stability of the paramagnetic mononucleated form of GS-DNIC was examined by EPR as follows: increasing amount of salmon testes DNA (see Figure 1) was added to the solution of 10 μM GS-DNIC pH6 for 2 min prior to freezing of the sample in the EPR quartz tubes (Spectrosil). The spectra of these preparations were measured as described above.

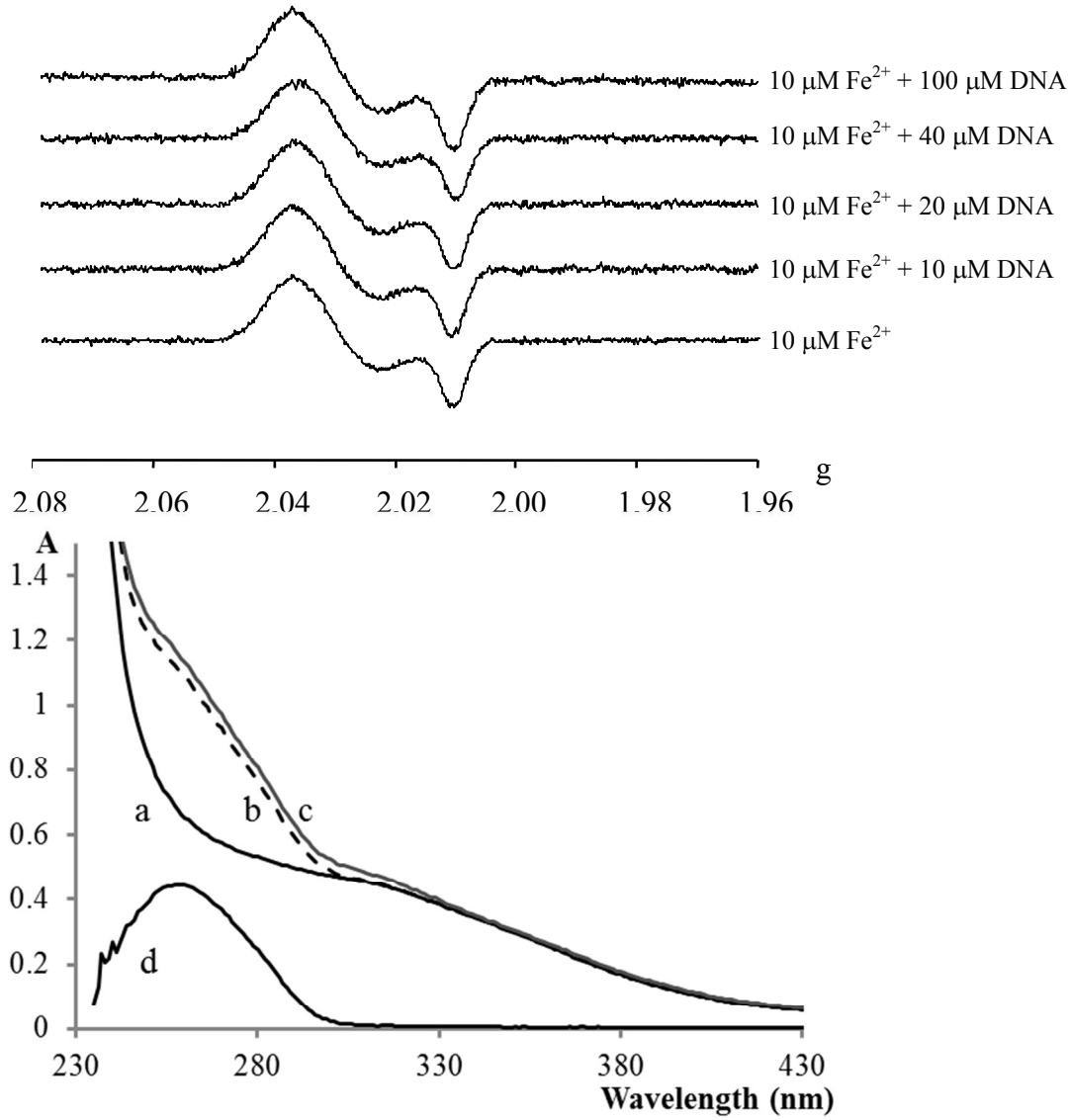


FIGURE 1. Stability of the 10 μM GS-DNIC in the presence of DNA. Fe^{2+} (10 μM) was added to GSH (15 mM)/GSNO (266 μM) in pH 6, 10 mM PBS). To thus obtained DNIC increasing amounts of DNA in 10 mM phosphate buffer, pH 6 were added. EPR spectra of the paramagnetic form of DNIC (mononucleated) were recorded (**upper graph**). The fate of both the mono- and the bi-nucleated forms can be examined by UV/VIS (**lower graph**). DNIC solution, prepared by addition of Fe^{2+} (133 μM) to GSH (15 mM)/GSNO (266 μM) in pH 6 (10 mM PBS) buffer (**a**), upon addition of 66 μM bp of salmon testes DNA after 1 min. (**b**), 30 min. (**c**), DNA alone (**d**); The slight increase in the spectrum after incubation of mixture is probably due to the partial precipitation/denaturing of the DNA, consistent with the plasmid nicking data.

DNA complexing measurements: A calf thymus DNA stock solution (1 mg/mL in phosphate buffer) was mixed with solutions containing increasing concentrations of GS-DNIC (12 μM; 60 μM; 120 μM; 240 μM) in phosphate buffer. The GS-DNIC–DNA interaction was evaluated by ECD spectroscopy. All ECD spectra were recorded at 293 K, 2 min. after sample preparation. Influence of pH on GS-DNIC–DNA interaction was evaluated in pH 6 and 7. Concentration of DNA was checked spectrophotometrically using extinction coefficient ε = 6600 at 260 nm.

Plasmid nicking assay In order to estimate the extent of DNA damage caused by the glutathionyl dinitrosyl iron complex *versus* the aquated or GSH-complexed Fe^{2+}, a plasmid cleavage test was carried out. In this test, the abundance of DNA bands, corresponding to the supercoiled (CCC), open circular (OC) and linear (L) forms of the plasmid, visualized after electrophoresis is directly related to DNA damage. Plasmid pUC19 (40 μg/mL) was incubated at 22°C for 10 min. under N_2 atmosphere in dark with a physiologically relevant mixture of mono- and bi-nucleated GS-DNIC (pH 6 or 7) formed by addition of 3 μM Fe(II) iron sulfate heptahydrate, 20 mM GSH and 2 mM GSNO (pH 6 or 7), to the final Fe(II) conc. 1 μM, that corresponds to cellular CIP concentrations [65] in the presence of either 1 mM or 100 μM H_2O_2. For comparison with the effect of the unchelated iron ions, the plasmid was analogically incubated with 1 μM Fe, in the presence of 1 mM or 100 μM H_2O_2 (in pH 6 and 7). In order to relate the studied effect to that of the GSH-chelated iron, the plasmid was analogically incubated with 1 μM Fe, in 7 mM GSH in the presence of 1 mM or 100 μM H_2O_2. A set of three controls was also prepared, containing: (a) 7 mM GSH, 0.7 mM GSNO and pUC19 (line 7) (b) native pUC19 plasmid (line 8), (c) pUC19 cleaved L form (line 9).

The incubation mixtures in a total volume of 6 μL contained 450 ng pUC19 plasmid DNA. All experiments were done in an MBraun glovebox under N_2 atmosphere (N6.0). Each experiment was repeated thrice. The reaction products were resolved electrophoretically on 1.5% agarose gel containing 0.25 μg/mL ethidium bromide. To obtain a linear form, the plasmid was cleaved with SmaI endonuclease (Fermentas). The DNA bands were visualized under UV light, photographed and the intensity of bands was estimated by ImageJ, a public domain Java image processing program (http://rsbweb.nih.gov/ij/index.html).

RESULTS

Spectra of dinitrosyl diglutathionyl iron complex: According to the literature, the mononucleated $[(RS)_2Fe(NO)_2]^-$ complexes (M-DNICs) in the biologically relevant pH, thiol and NO concentrations are in equilibrium with their diamagnetic bi-nucleated counterparts (B-DNICs) according to the following reaction:

$$2[(RS)_2Fe(NO)_2]^- \rightarrow [Fe_2(\mu\text{-}SR)_2(NO)_4]+2RS^- \quad (1)$$

The diamagnetic properties of the B-DNICs are provided by antiferromagnetic interaction of two $Fe(NO)_2$ groups through sulphur bridges [66, 67]. M-DNICs are stable in solution only in the presence of a large excess of thiol and at the range of higher pH (depending on the pK_a of the given thiol) that assures sufficiently high concentration of deprotonated RS^- ligands [66, 68, 69]. In case of the GSDNIC, as reported in the recent paper of Borodulin [69], at neutral pH the B-form of DNIC dominates despite the presence of a 10-fold excess of free glutathione, while the prevalence of the M-form is found at pH as highly alkaline as 10.4.

The herein applied procedure for DNIC preparation can be treated relevant to the conditions available in natural milieu: The 200-fold excess of GSH and 20-fold excess of GSNO versus iron

relate to the biological ratio of cellular GSH (0.5- 10 mM, [70]) to the cellular labile iron (lower μM range, [71]) and NO levels during NO burst (several up to tens of micromoles [72–74]). The well documented occurrence of paramagnetic M-DNICs in the living tissue [75], together with the conclusions on paramagnetic and EPR-silent DNICs equilibrium in the solution account for the use of thus prepared mixture of mono- and bi-nucleated DNICs. Formation of GS-DNIC was confirmed by UV/VIS at 403 nm (Figures 2a for pH 7 and 2b for pH 6; kinetics of DNIC formation are depicted). Immediately after addition of iron ions to the 400 μM solution of GSNO in an excess (20 mM) of GSH an intense peak at *ca* 350 nm and another one of moderate intensity in the region around 475 nm emerged. These spectra are analogous to the one observed for the reaction of the formation of mononitrosyl complex with aquated iron ion, as described in [76]. During the next 20 min the spectrum evolved, the two bands at 350 and 475 nm decreased along with the increase of absorption at about 400 nm (Figure 2). In pH 7, after 20 min. time the reaction was completed and the spectrum characterised with molar coefficient 3000 M^{-1} cm^{-1} at 403 nm, in accordance with the literature data [63]. The kinetics of DNIC formation reveal a two-step process, and are consistent with the results of Lo Bello et al [63]. In pH 6 complex formation could still be seen in UV/VIS, however the spectrum had a slightly different shape, with characteristic peak shifted hypsochromically.

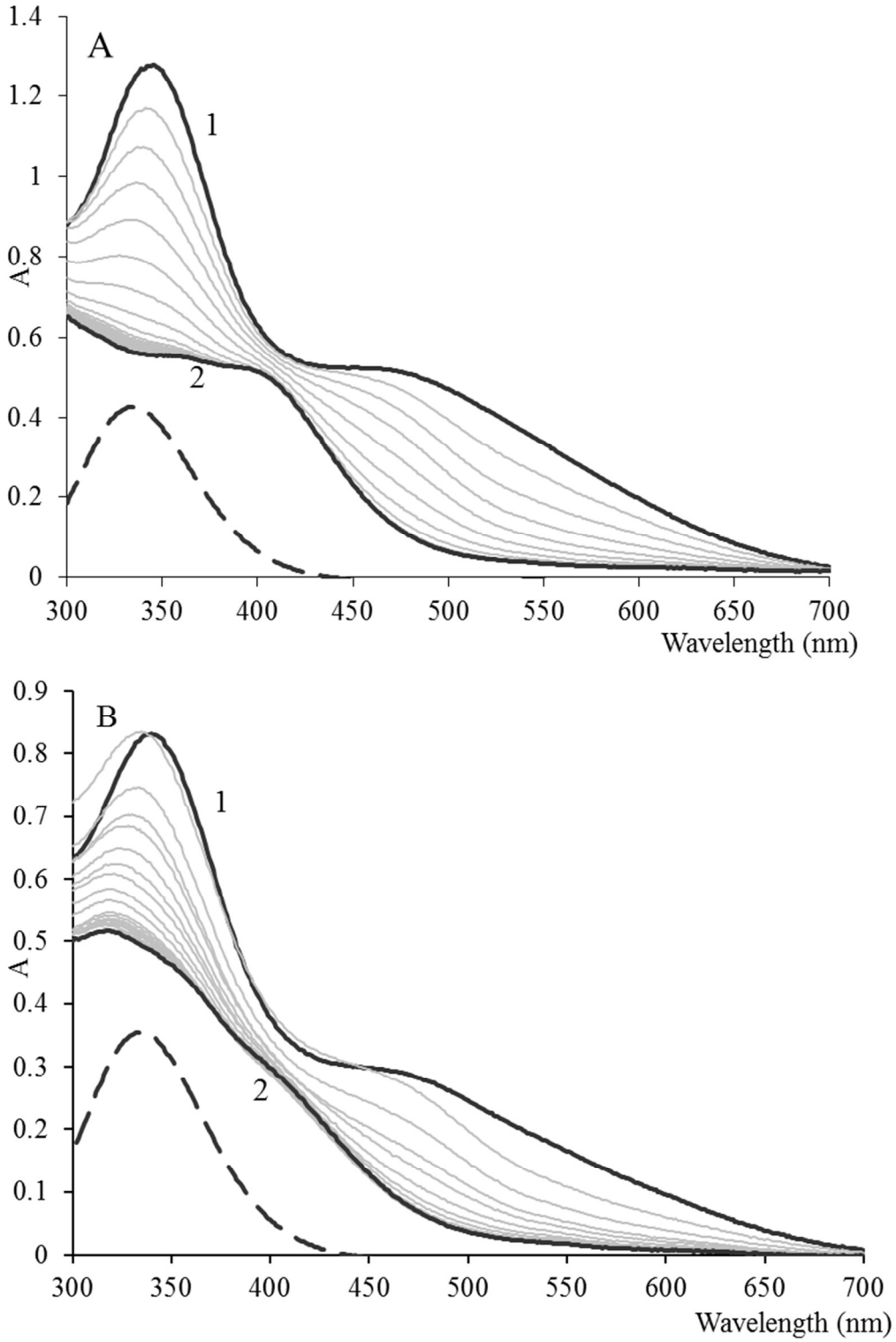


FIGURE 2. Formation of GS-DNIC after addition of Fe^{2+} (200 μM) to GSH (20 mM)/GSNO (400 μM) in pH 7 (a) and pH6 (b); 1 - immediately (1 min.) after Fe addition, 2 - after 20 min. time; light grey lines depict kinetics of DNIC formation, dashed line shows the spectrum of starting GSH/GSNO solution.

Formation of the complex was also observed in EPR. Glutathionyl DNIC, both in pH 6 and pH 7 had an EPR signal characteristic for DNIC with low-molecular thiol ligands, with $g_{\perp} \cong 2.03$, and $g_{\parallel} \cong 2.01$, a spectrum widely published in the literature, e.g. [77–79] (see Figure 1, 4, 5). The g values, calculated by simulation, were: x = 2.04, y = 2.03, z = 2.01.

As it follows from the mentioned work of Borodulin et al. [80], and as can be seen in the presented UVVIS spectra, the herein used GS-DNIC preparation is a mixture of the mono- and binuclear-DNIC (M- or B-DNIC, respectively). Thus there arises a question on the ratio of these species in the solution in the conditions given. It is hardly possible to estimate M-DNIC to B-DNIC ratio from the UV/VIS spectra, since such spectra represent a sum of absorption of M- and B-DNIC and as well contain an unknown absorption of the excess ligands. Thus, based upon the cited reference [80], the pH of the test solutions was brought to 11.0, where in the presence of a free glutathione excess DNIC are predominantly represented by the M-form. The decrease of the EPR signal of M-DNIC upon shifting pH from 11.0 to 7.0 and finally to 6.0 corresponds to the increases in concentration of B-DNIC at lower pH.

The EPR data point out that in pH6 M-DNIC represents *ca* 21%, whereas in pH7 *ca* 64% of the M-DNIC observed in pH 11, for the solution containing 200 μM Fe^{2+}. For the lower, 1 μM Fe^{2+} concentrations these values were found 26% and 49%, respectively.

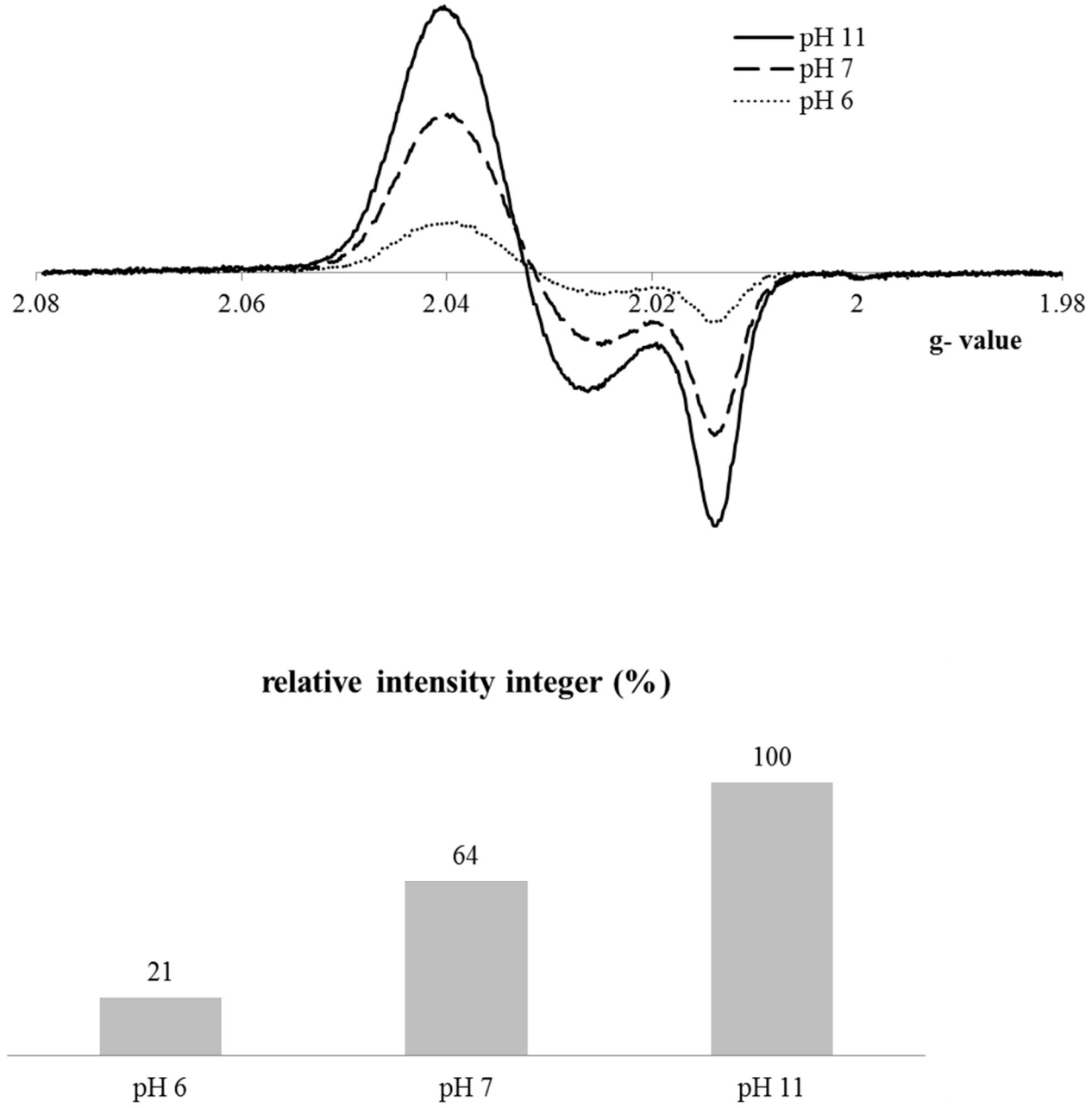
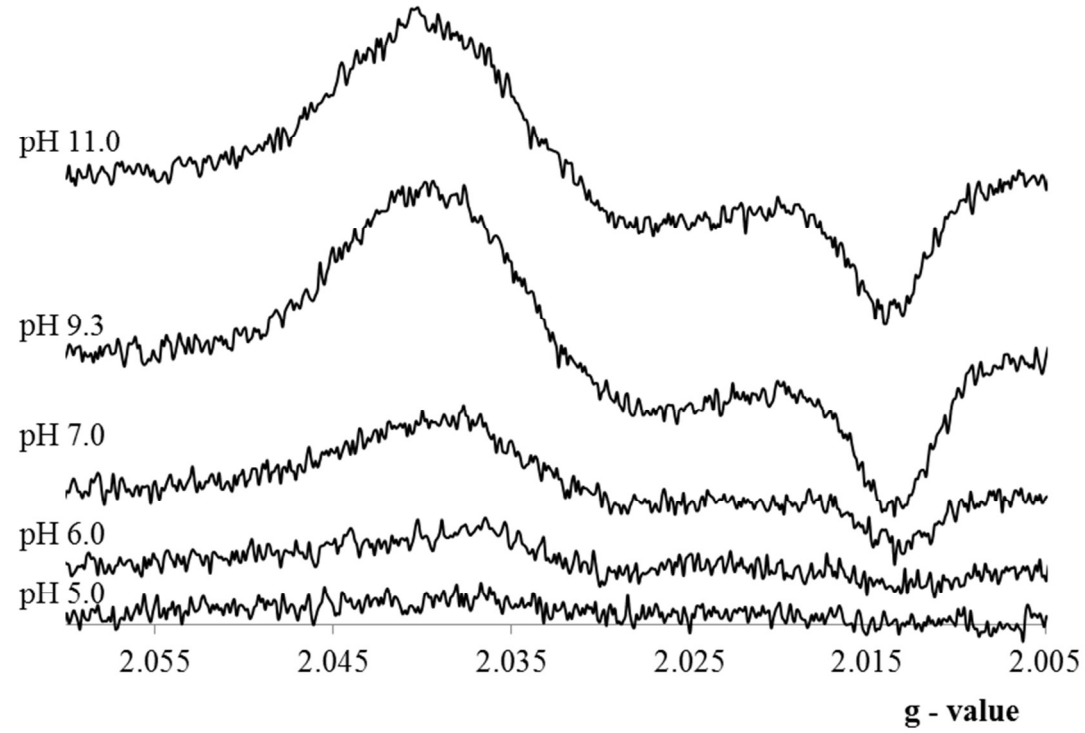


Figure 3: Formation of M-GS-DNIC after addition of Fe^{2+} (200 μM) to GSH (20 mM)/GSNO (400 μM) in different pH after 20 min. time; with the increasing pH the paramagnetic M-form of DNIC prevails over the B-form as seen in the EPR. The relative intensity integer was counted as a double integer of the baseline-corrected EPR signal and then given as a % of the value for pH 11.



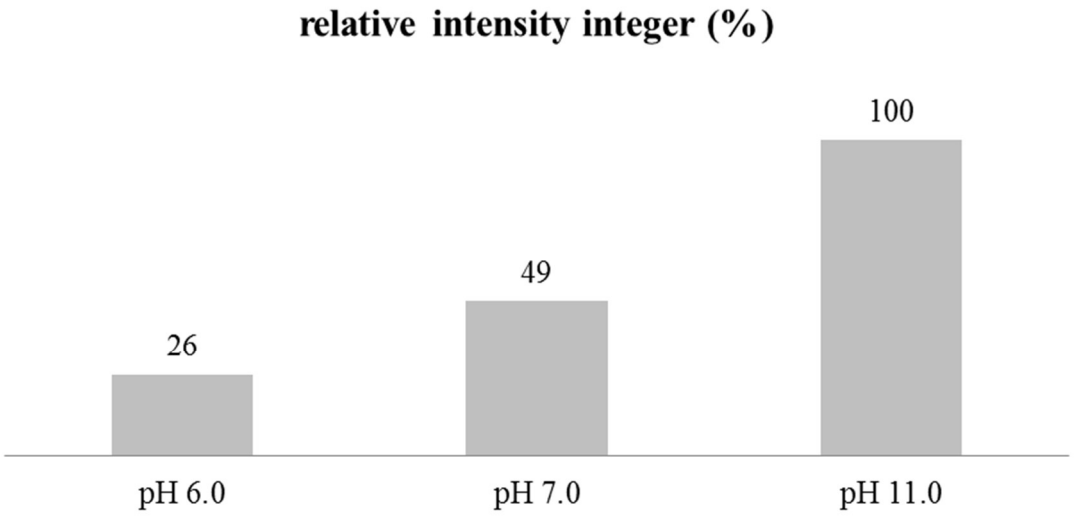
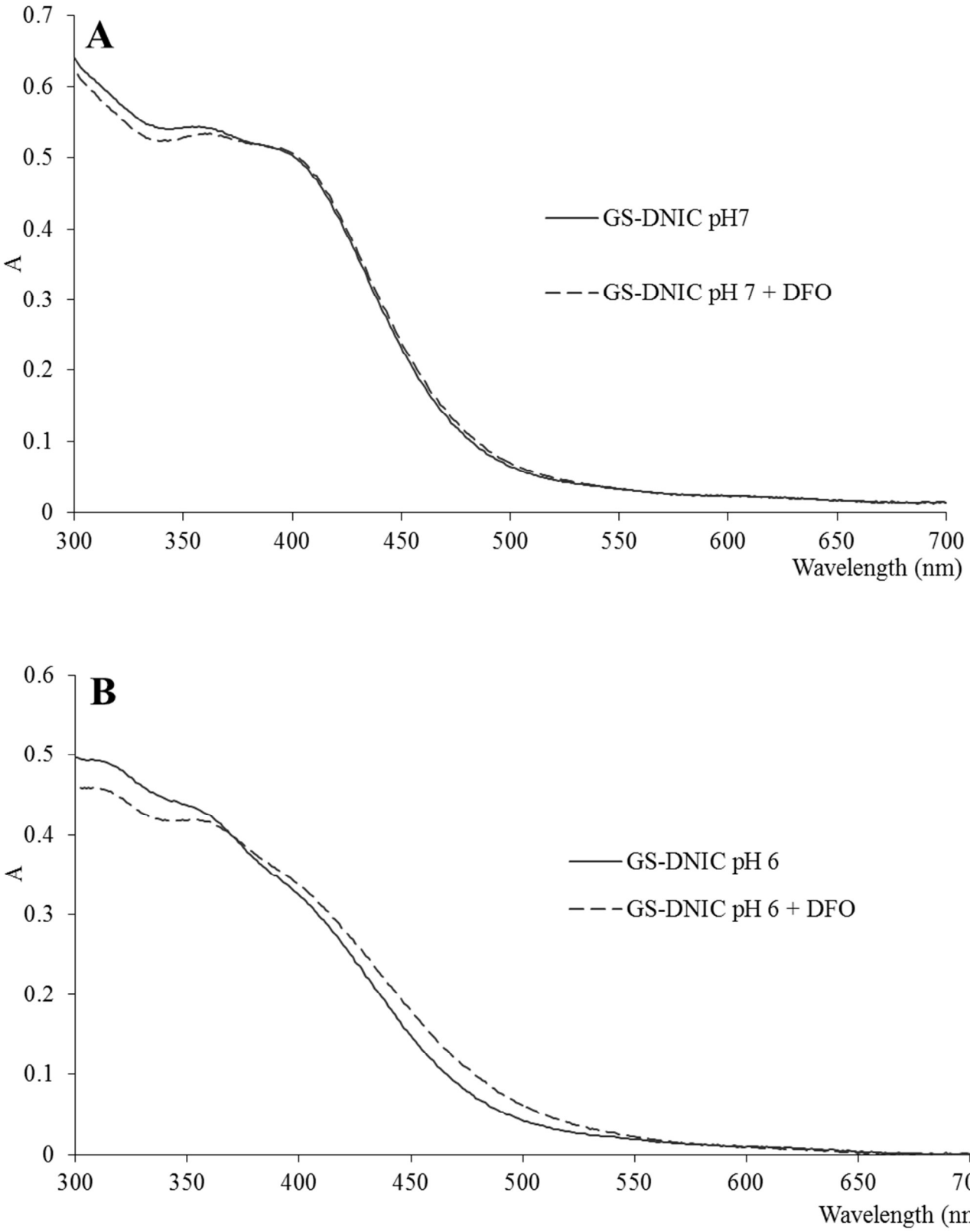


Figure 4: Formation of M-GS-DNIC after addition of Fe^{2+} (1 μM) to GSH (7 mM)/GSNO (700 μM) in different pH after 20 min. time; with the increasing pH the paramagnetic M-form of DNIC prevails over the B-form as seen in the EPR.

Stability of the tested solutions up to 2 h has been examined by EPR and is attached as a supplementary material (Supplementary Figures: 1, 2, 3, 4). The apparent stability of GS-DNIC in the presence of high excess of S-nitrosoglutathione and reduced glutathione is likely due to a steady-state equilibrium coming from simultaneous decomposition and re-synthesis of the complex [63]. The both preparations of DNICs, viz. pH 6 and pH 7, were tested for stability towards an addition of ten times molar excess of deferoxamine (DFO). Surprisingly, this selective and strong iron chelating agent was shown by EPR and UV-VIS (Figure 5) to have a minor effect on DNICs concentration. On the contrary to the previously studied DNIC containing histidine as an auxiliary ligand [62], the presently studied DNIC with glutathione was decomposed only to a small extent upon 20 min. treatment with DFO (see Figures 3a, 3b and 3c). We have observed previously [45, 81, 82] that the DNICs formed in biological samples are also inert to treatment with DFO, and we assume the effect is due to the slow kinetics of ligand exchange.



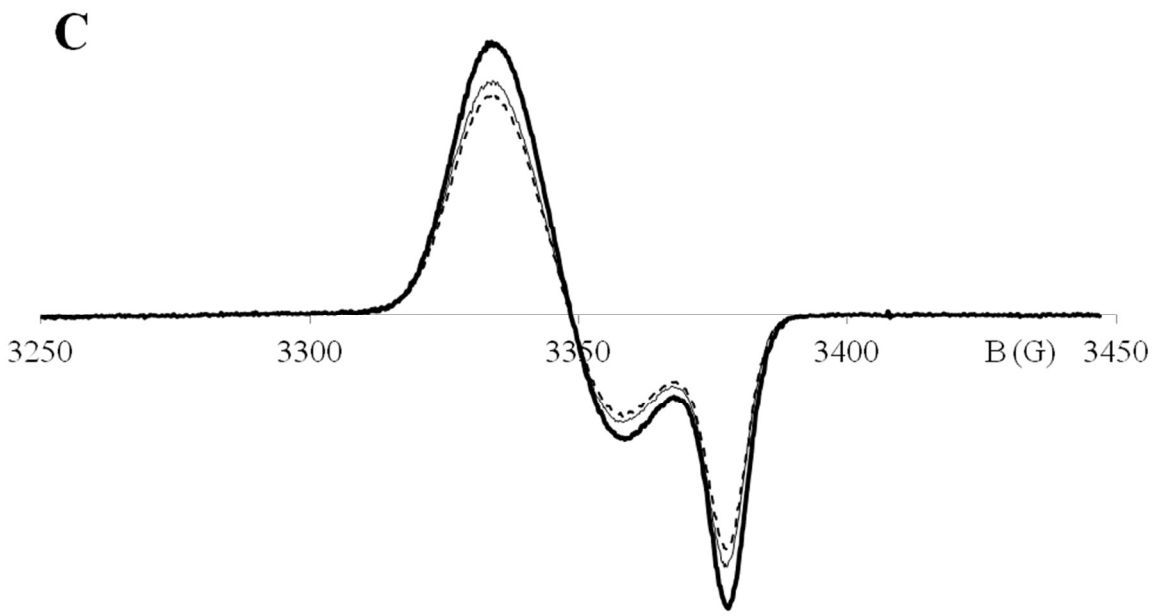


FIGURE 5. The influence of 2 mM DFO on the 170 μM GS-DNIC in 10 mM phosphate buffer. The changes observed in (a) pH 7, (b) pH 6 by UV/VIS depicture the influence of an excess of DFO on the total DNICs pool. The decomposition of the EPR-visible mononucleated paramagnetic DNIC followed similar pattern (c): DNIC in pH6 immediately (bold line), 10 min. (thin line), 20 min. (dotted line) after DFO addition.

DNA complexing measurements: Circular dichroism method was used to determine the mode of interactions between the DNA and the studied complex. The obtained ECD spectra with increasing amounts of dinitrosyl diglutathionyl iron complex are presented in Figure 6 and 7. The right band of the DNA spectrum was monotonously decreased with the increase of the DNIC concentration. This effect was completely suppressed in pH 7 (Figure 7).

The influence of increasing amounts of aquated Fe^{2+} ions on DNA was investigated by ECD spectroscopy in our previous work [83]. The mentioned results revealed identical character of changes induced by the hydrated iron ions on the DNA helix structure, as it was found in the case of the presently studied complex. The effect was also suppressed by increasing pH and ionic strength.

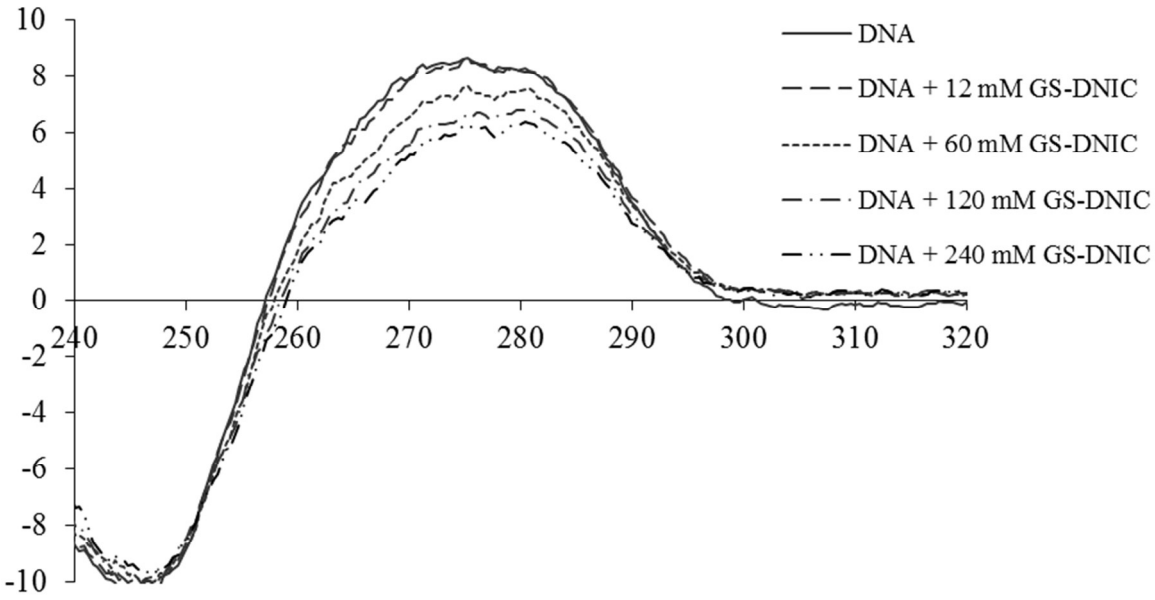


Figure 6: ECD spectra of DNA (200 μM) in the presence of increasing amounts of GS-DNIC in the presence of excess glutathione (20 mM,) in 10 mM phosphate buffer, pH 6; GS-DNIC concentrations: 12 μM; 60 μM; 120 μM; 240 μM.

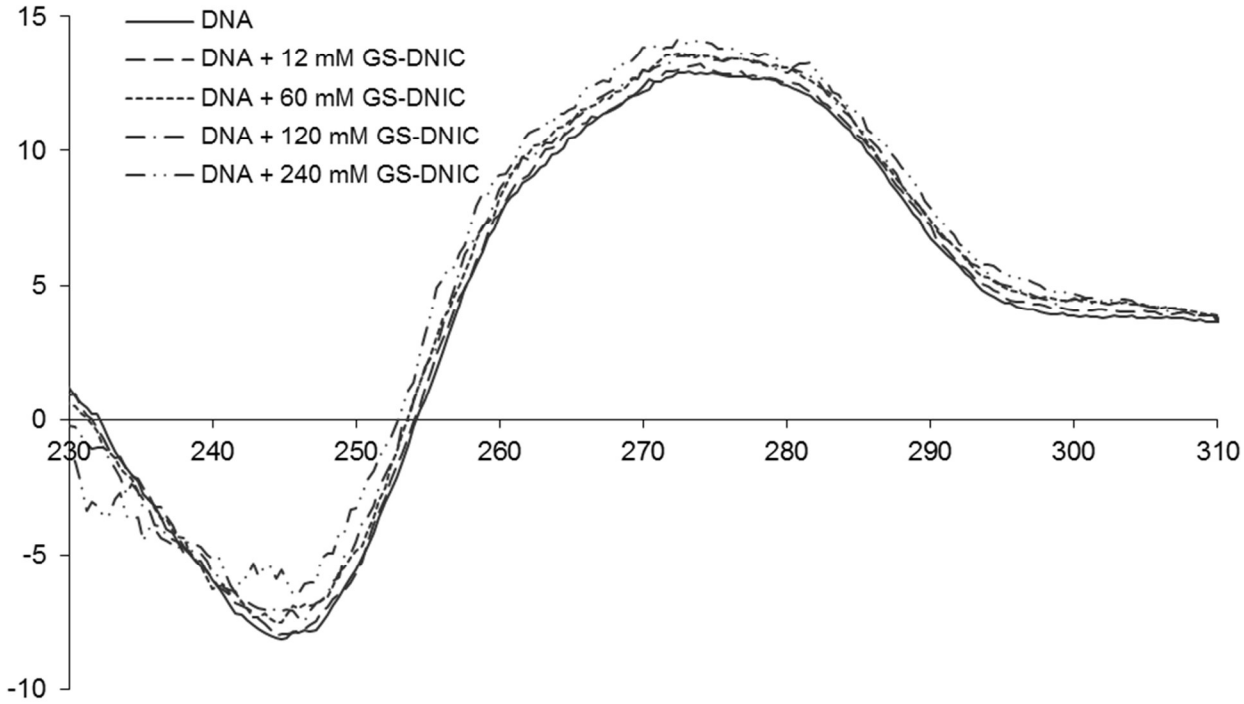
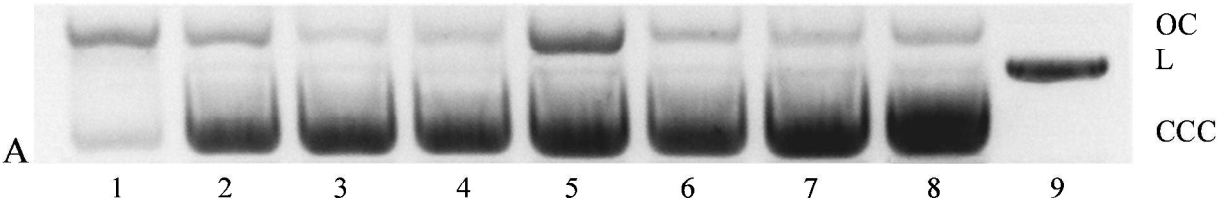


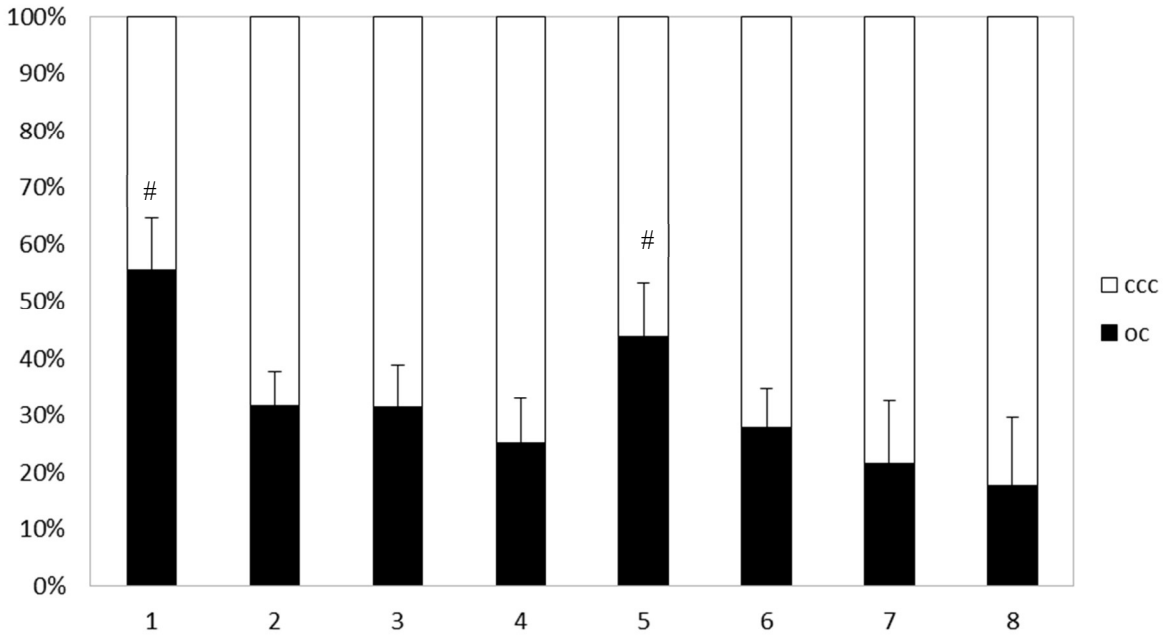
Figure 7: ECD spectra of DNA (200 μM) in the presence of increasing amounts of GS-DNIC in the presence of excess glutathione (20 mM,) and GSNO (2mM) in 10 mM phosphate buffer, pH 7; GS-DNIC concentrations: 12 μM; 60 μM; 120 μM; 240 μM.

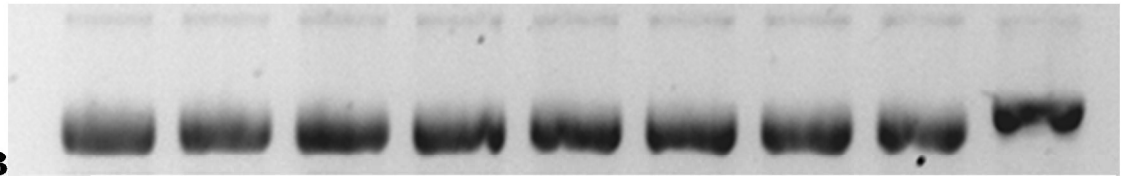
Plasmid nicking assay

An induction of plasmid DNA strand breaks by Fe^{2+} was observed at pH 6, as indicated by higher fluorescence of bands assigned to the OC form of the plasmid (Figure 8A versus B) in a manner dependent on the concentration of ROS (H_2O_2) (compare even and odd lines in figure 8A). This effect was less pronounced, yet still visible, in case of iron ions in the presence of an excess of GSH (compare Figure 8, lines 1 and 2 to lines 5 and 6). Simultaneously, the formation of the DNICs completely attenuated the observed effect (compare to lines 3 and 4, Figure 8). For the samples incubated with aquated iron ions the damage was induced to the greatest extent, what is consistent with our earlier results for aquated iron [62]; about 55% of the plasmid was found in its OC form, and the remaining 45% in the CCC form. At the same time, statistically unimportant induction of strand breaks was observed in the sample containing 1 μM Fe trapped in the form of GS-DNIC (line 3 and 4). At pH 7 DNA breaks formed both by the treatment with GS-DNIC and by the treatment with Fe^{2+} were much less pronounced, not exceeding 20% of plasmid content for the milimolar iron concentrations (Figure 8). It is noteworthy, that the attenuation of ROS effect on plasmid DNA damage was observed when Fe ions were in the form of DNIC but could not be observed in the presence of GSH alone.



Final conc.	1	2	3	4	5	6	7	8	9
GSH	-	-	7 mM	7 mM	7 mM	7 mM	7 mM	-	-
GSNO	-	-	700 µM	700 µM	-	-	700 µM	-	-
Fe	1 µM	1 µM	1 µM	1 µM	1 µM	1 µM	-	-	-
H_2O_2	1 mM	100 µM	1 mM	100 µM	1 mM	100 µM	-	-	-
pUC19	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng linear





B

	1	2	3	4	5	6	7	8	9
Final conc.	1	2	3	4	5	6	7	8	9
GSH	-	-	7 mM	7 mM	7 mM	7 mM	7 mM	-	-
GSNO	-	-	700 μM	700 μM	-	-	700 μM	-	-
Fe	1 μM	1 μM	1 μM	1 μM	1 μM	1 μM	-	-	-
H_2O_2	1 mM	100 μM	1 mM	100 μM	1 mM	100 μM	-	-	-
pUC19	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng	450 ng linear

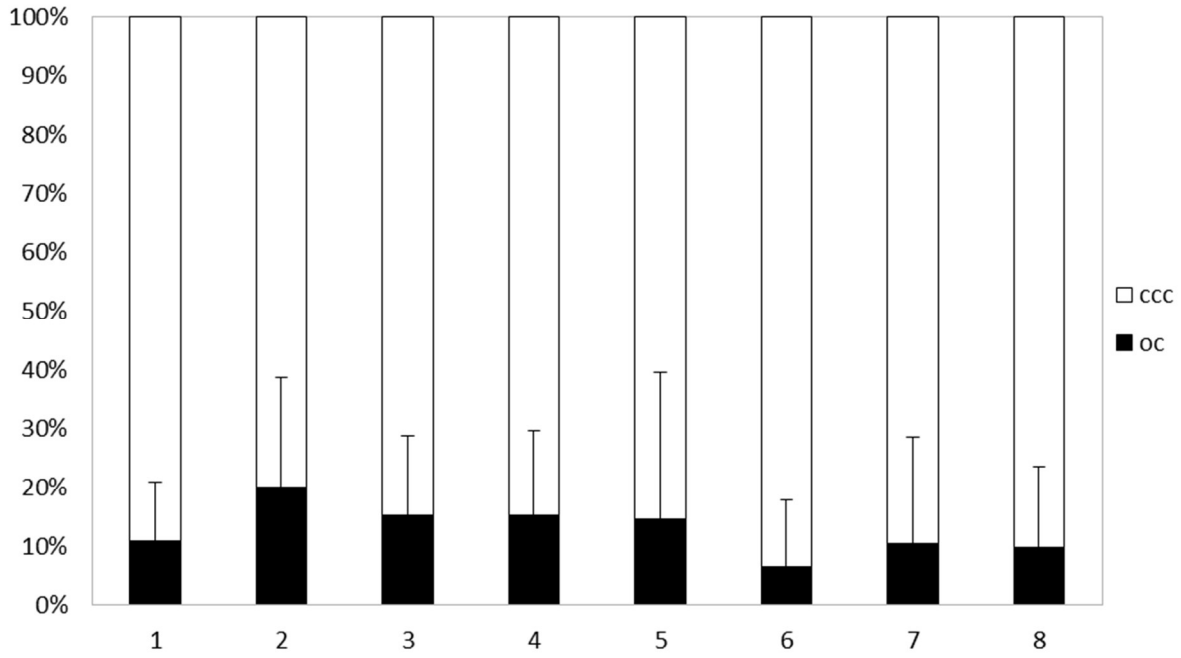


FIGURE 8. Nicking of the plasmid DNA by GS-DNIC, versus the effect of iron. Panel A: pH 6.2, B: pH 7.2. Gel images: pUC19 plasmid (400 ng) was treated with the following solutions: Fe (aq) + 1000 μM H_2O_2 (Line 1); Fe (aq) + 100 μM H_2O_2 (Line 2); DNIC + 1000 μM H_2O_2 (Line 3); DNIC + 100 μM H_2O_2 (Line 4); (Fe)GSH + 1000 μM H_2O_2 (Line 5); (Fe)GSH + 100 μM H_2O_2 (Line 6); GSNO (Line 7); non-treated (Line 8), respectively. The mobility of the plasmid cleaved with Sma1 endonuclease (linear form) is shown in Line 9.
Intensities of bands 1-8 correspond to the intensity graphs. Filled box – open circular nicked form (OC); empty box– covalently closed circular -supercoiled form (CCC). Hash denotes statistically

significant difference *vs* DNIC @ 1000 μM H_2O_2**.** The p-values are: 0.00187 for $Fe_{(aq)}$ *vs* DNIC, and 0.0275 for Fe(GSH) *vs* DNIC.

DISCUSSION

Despite the fact that the interactions of LMW DNIC with proteins have been widely characterized, little is known about the direct interactions of LMW DNIC with other important biological macromolecules, such as DNA. To gain insight into the possible genotoxic effects of DNIC we investigated the influence of GS-DNIC formation on iron-induced DNA damage.

In the circumstances of oxidative stress, pools of reactive oxygen and nitrogen species induce the liberation of the transition metals, mostly iron from their cellular storage molecules and active centres of proteins were they act as enzymatic cofactors [84]. Fenton reactions that consume NO (via reaction with superoxide anion) can also oxidize nitrite to NO_2 to further increase oxidation and nitration [85]. Noteworthy, as shown by cell fractionation, most of the dinitrosyl iron formed during oxidative stress is again trapped in the form of inert nitrosylated complexes with proteins and protein-lipid aggregates [43, 49, 86]. Forming of high-molecular DNICs with proteins, both outside the nucleus and in the close vicinity of the DNA strand, decreases the amount of radicals produced in Fenton reaction [43–45]. Taken together literature data encourage to look for a genoprotective effect of chelatable iron trapped in the form of DNICs. Herein we presented results supporting our hypothesis that formation of DNIC could prevent DNA from radical-induced strand breakage.

Among biological ligands available as substrates of DNIC formation, thiols seem to be the most relevant [1, 21, 24, 49, 53]. Boese et al. [14] conducted the electrophoresis of cysteinate DNIC at isoelectric pH of L-cysteine and observed that DNIC moved towards the anode, indicating that this complex carries a net negative charge, as consistent with the current conclusions drawn from spectroscopic studies and theoretical calculations for similar complexes [87, 88]. Therefore DNIC formation should prevent iron from interacting with the DNA strand. On the other hand, as reviewed in [16], the free electron present in the paramagnetic Fe-N-O moiety

is highly delocalized increasing the covalent trans-molecular character of the bonding (see also [89]); thus in DNICs covalent bonding is formed between the iron atom and the two NO groups (see also Enemark and Feltham's theory of the electronic structure of Me–N–O groups [90]). The iron becomes formally negatively charged, and the two nitrosyl groups take on the nature of nitrosyl cations (NO^+). Depending on the electron-donating properties of the two auxiliary ligands, a partial back-donating effect results in the transfer of electron density to antibonding π^* orbitals of NO groups. To address the question, if the formation of thiolate iron nitrosyls can play a protective role against the Fenton-type activity of iron during oxidative stress we incubated the supercoiled form of plasmid circular DNA with dinitrosyl iron complex of glutathione together with hydrogen peroxide. Glutathione is abundant in the cellular milieu and is one of the most effective low-mass scavengers of cellular nitrosylated iron. This ligand forms DNIC which is not disrupted by the selective and 'canonical' labile iron chelator, DFO. GS-DNIC in milimolar concentrations and in the presence of an excess of glutathione was shown to be stable for several hours [63], moreover (as presented) in these conditions GS-DNIC appears inert towards ligand exchange with DFO. Because of its chemical stability and peptide nature GSH can be a relatively good model for the analysis of protein-DNICs formation and its effect on the labile iron genotoxicity.

The widely known feature of mononucleated high-spin DNICs is that they can exist in equilibrium with their dimeric analogues, Roussin's red esters [80, 91] (see Equation 1). Preparation of dinitrosyl diglutathionyl iron complex in the solution containing stoichiometric amounts of GSH, readily shifts the equilibrium towards its EPR-silent bi-nucleated form. Along with increasing the excess of thiol, bi-nucleated DNICs (B-DNICs) are converted into a mononuclear EPR-active form of DNIC (M-DNIC). The equilibrium strongly depends also on pH as shown by Borodulin et al. [80]. Moreover, stoichiometric preparations of GS-DNIC were

found very sensitive to any traces of reactive oxygen species, while the preparations containing an additional pool of unbound GSH were stabilized by the anti-oxidative effect of the latter. The preparations containing an excess GSH were much more stable against DNIC disruption by DFO. Finally and importantly, having in regard the biological ratio of cellular GSH (0.5- 10 mM, [70]) to the cellular labile iron (lower μM range, [71]) and NO levels during NO burst (several up to tens of micromoles [72–74]), we found it reasonable to perform the experiments in the high excess of GSH and NO to iron [89, 92–94].

According to Lo Bello et al [63] in the conditions of GS-DNIC synthesis similar to applied here about 90% of the ferrous ions are present as GS-DNIC, being in an equilibrium with a small but considerable amount of the free Fe^{2+}. As mentioned, GS-DNIC is very slowly disrupted by DFO (Figure 5). At the same time the formation of DNIC doesn't occur, if iron ions are chelated by DFO first [45, 81, 82];. This shows that the GS-DNIC complex is inert to substitution with DFO. Yet, the apparent thermodynamic stability of the DFO-Fe complex along with rapid binding of DFO to free iron ions allows treating DFO as a scavenger for the free iron that is slowly and successively liberated from GS-DNIC. DFO forms strongly bound Fe^{2+} and Fe^{3+} complexes ($K = 4.0 \times 10^{30}$ for Fe^{3+}-DFO(aq) and $K = 1.6 \times 10^{7}$ for Fe^{2+}-DFO(aq) [95]). It is worth to mention, that in steady-state equilibrium resulting from simultaneous decomposition and re-synthesis of DNICs [63], both iron (II) and iron (III) ions can occur, as the dynamic equilibrium between DNICs, their dimeric RRE forms and mononitrosylated iron is regulated by the series of one-electron redox-type reactions [91, 96].

DNA-DNIC interaction: The pH of the cell is compartmentalized and varies from 4.7 for lysosomes, *ca* 7.2 for cytosol up to 8 for mitochondria [97]. Determinations of the pH of the nucleus are scarce, and most authors assume that the nuclear pH equals that of the cytosol. That is because the nuclear envelope has an abundance of pores that are permeable to small molecules,

and as such, presents a weak diffusive barrier to H^+ [97]. In normal conditions, pH ca. 7.2, the presence of labile iron does not cause a particular threat to the double helix DNA, as the iron ions do not seem to interact with the DNA helix in the pH above 7 [83] (see Figure 7). in the circumstances of the oxidative stress, the pH of the cellular interior is considerably lower, due to the stress-induced acidosis, as shown in myocardial ischemia [98, 99]. Also in cultured rat cardiac myoblasts, 100 μM H_2O_2 induces a marked decrease in the pH [100]. Similar results were observed in cerebellar astrocytes and C6 glioma cells [101] or in rat erythrocytes [102]. As shown by Tsai et al. [101], the pH level of the cell interior during the stress-induced acidosis can fall by 0.47 units. The intracellular pH in human ventricular myocardium fell as low as by 0.6 pH units upon treatment with H_2O_2 [103]. Since the acidosis is completely inhibited by potent membrane-crossing ·OH scavengers, it was suggested to result from the production of intracellular ·OH in the Fenton reaction dependent H_2O_2 decompositon [101]. Thus, the data presented here illustrate DNIC effects both in normal conditions and in circumstances of increased ROS production (such as inflammation), within the range of physiological pH.

Plasmid nicking assay: A considerable pool of iron ions is present in the close vicinity of the cellular DNA, as a consequence of the presence of iron-dependent DNA-processing enzymes. Plasmid nicking assay allowed estimating, if the binding of iron in the form of DNIC can protect DNA from the oxidative stress-induced lesions. Indeed, in comparison to the control, free iron ions containing samples, we observed a significant reduction of DNA breakage in DNIC containing samples. We believe that this effect might have been caused by Fe binding in the form of GS-DNIC. This substantial protective effect, visualized in Figure 8, did not occur for the DNA treated with Fe^{2+} in the presence of GSH alone, thus the observed protection cannot be ascribed to the radical-scavenging effect of GSH. This implies that the formation of nitrosyliron complex

with GSH that is ubiquitously present in the cellular milieu, effects in the protection of the DNA from the toxic action of iron. In the higher pH (above 7) an attenuation of the Fenton-derived genotoxic effect of iron ions can be observed regardless of iron complexation form. This might be explained in the light of the previously reported circular dichroism results showing that in pH 7 and above there is no interaction between DNA strand and Fe^{2+} ions (regardless of the ligand effect) [62].

Noteworthy we found that in the presence of oxygen the glutathionyl complex hampers radical formation by Fenton type reactions and protects DNA from oxidative damage. Such a protective effect is surely not mediated simply by summarised effects of the DNIC components. On the other hand this effect was not observed for the histidinyl DNIC [62]. It must be reminded here, that in comparison to glutathionyl DNIC histidinyl DNIC, was much less stable in the presence of DFO, and therefore nitrosylated iron bound non-specifically (i.e. not in protein sites for metal binding) by histidine-type moieties shall be accounted to CIP. This observation is consistent with the binding affinity order of coordinated ligands toward the $\{Fe(NO)\}^9$ motif observed in the biomimetic model study ($[SPh]^-$ > $[-SC+H_3S]^-$ > $[C_3H_3- N_2]^-$ > $[OPh]^-$ > $[NO_2]^-$) [96]. The above observation may suggest a new look at the deleterious effects connected with radical generation by iron, depending on its complexing sites in cellular micro- and macromolecular ligands.

CONCLUSIONS

Herein it has been proven that the formation of GS-DNIC attenuates iron genotoxicity (Figure 8). As observed by the plasmid DNA cleavage assay, a significant reduction of DNA breakage was observed for GS-DNIC-bound iron, as compared to the DNA-cleaving effect of free iron ions (Figure 3). The presented results are in line with the observations of Gorbunov et al. [40] and C.

Lu & Koppenol [41] who have shown that iron incorporation into nitrosyl complexes attenuates iron activity in Fenton reaction. Additionally, GS-DNIC was shown by EPR to be stable in the presence of DNA.

The presented data shed light on a plausible mechanism of mutual genoprotective effect of iron ions and NO that was previously observed and reported in the literature.

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