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Novel DNA photocleavage properties of $[Cr(NN)_3]^{3+}$ complexes

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ABSTRACT

The aim of this study was to investigate the ability of chromium(III) tris-diimine complexes, $[Cr(NN)_3]^{3+}$, to induce DNA photodamage and to examine its capability to impair the survival of irradiated bacteria with the purpose of using these compounds as photocleavage reagents.
$[Cr(NN)_3]^{3+}$ complexes, where NN stands for the ligands: phen (1,10-phenanthroline), 5-Mephen, 4,7-diMephen, 3,4,7,8-tetraMephen, 5-Phphen and 5-Clphen were used for this purpose. Their properties of DNA photocleavage were investigated by electrophoretic studies and assays of the photosensitization of transformed bacteria. The results clearly indicate that $[Cr(NN)_3]^{3+}$ complexes promote the photocleavage of plasmid DNA with varied degrees of efficiency after 12 h of irradiation. The combination of DNA, $[Cr(NN)_3]^{3+}$ and light proved to be required to induce the breakdown of DNA. Irradiated plasmid DNA-$[Cr(NN)_3]^{3+}$ association is also capable of impairing the transforming capacity of bacteria. These results provide evidence which confirms the responsibility and essential role of the excited state of $[Cr(NN)_3]^{3+}$ for inducing damage. Moreover, assays of the photosensitization of transformed bacterial suspensions suggest that *Escherichia coli* may be photoinactivated by irradiation in the presence of $[Cr(NN)_3]^{3+}$ complexes.

Metal complexes, which are able to bind DNA, have been extensively studied over the last years, centering the interest on the understanding of the photophysics and photochemistry imposed through interaction with DNA [1–4]. Moreover, the interaction of transition metal polypyridyl complexes with DNA has also drawn considerable attention to the development of compounds with photocleavage properties [5]. Tris(polypyridyl) Cr(III) species have been proposed as photocleavage agents mainly due to their long-lived room temperature emission and the strong oxidizing power of the excited state [6]. A particularly promising extension of these studies has been to study the effect of $[Cr(NN)_3]^{3+}$ ions (where NN is 1,10-phenanthroline or 2,2′-bipyridine) on different biological models. It is known that these metal complexes associate to DNA with small binding constants $\leq 4\times10^3\ M^{-1}$ and the binding to DNA appears to occur mainly by a surface interaction over the minor groove [7–9]. In addition, Schaeper et al. [10] demonstrated that several chromium(III) tris-diimine complexes enantioselectively bind to calf-thymus DNA, suggesting that this interaction is of increased interest in the characterization of these complexes as potential DNA photocleavage agents.

Another concern to be addressed consist in whether $[Cr(NN)_3]^{3+}$ system induces a permanent oxidative damage to DNA working as photooxidant agent.

Recently, we reported that $[Cr(phen)_2(dppz)]^{3+}$ (Bis(1,10-phenanthroline)dipyrido[3,2-a:2′,3′-c]-phenazine chromium(III)) was able to bind plasmid DNA via intercalation inducing DNA damage after reaching its excited state, in the absence of O_2, probably via a Type I mechanism (direct photooxidation) [11].

In this communication we report the photocleavage properties of $[Cr(NN)_3]^{3+}$ complexes to plasmid DNA (DNAp), where NN stands for the ligands: phen (1,10-phenanthroline), 5-Mephen, 4,7-diMephen, 3,4,7,8-tetraMephen, 5-Phphen and 5-Clphen with the purpose of

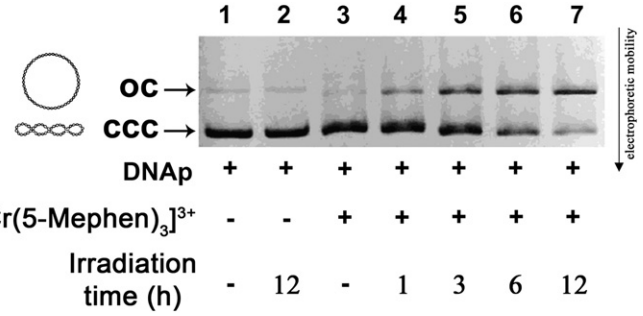

Fig. 1. Agarose gel (1%) electrophoresis showing the DNA photocleavage induced by 2.4×10^{-4} $[Cr(5\text{-Mephen})_3]^{3+}$ at different irradiation times at 6 °C in the presence of air-saturated atmosphere in 100 mM of TRIS buffer (pH 7.4): lane 1, control DNAp non-irradiated; lane 2, DNAp irradiated for 12 h in the absence of $[Cr(5\text{-Mephen})_3]^{3+}$; lane 3, DNAp + $[Cr(5\text{-Mephen})_3]^{3+}$ non-irradiated; lanes 4 to 7, DNAp + $[Cr(5\text{-Mephen})_3]^{3+}$ with irradiation times of 1, 3, 6 and 12 h, respectively.

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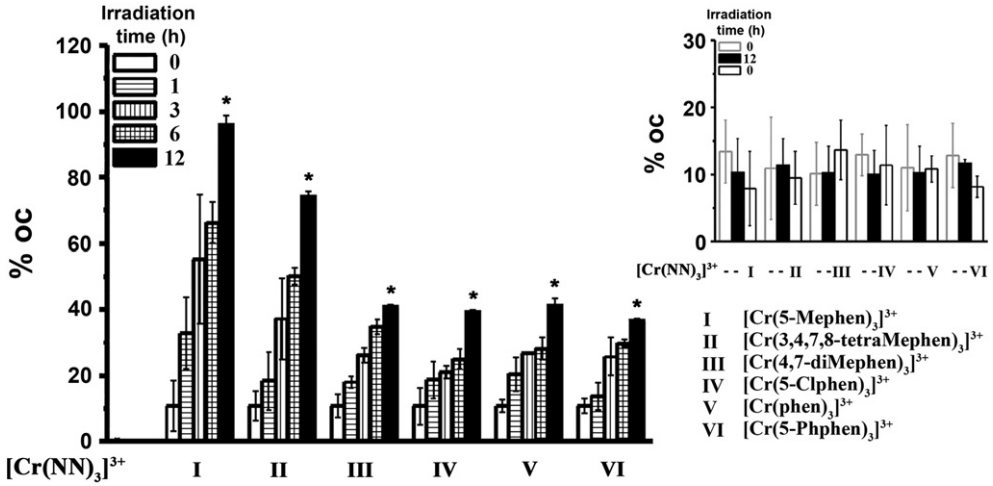

Fig. 2. Estimate of % **oc** formed on visible irradiation of DNAp in the presence of $[Cr(NN)_3]^{3+}$ complexes, with (□) 0, (▤) 1 h, (▥) 3 h, (▪) 6 h and (■) 12 h of irradiation at 457 nm. Values are normalized with respect to $[Cr(phen)_3]^{3+}$. Values represent an average of three different experiments ± standard deviation of the mean. *$p<0.05$. Inset: Unnormalized data for controls for each Cr(III) complex.

determining their relative efficiency and whether this efficiency to cleave DNA relates to the reduction potential of the excited state. These properties were analyzed by electrophoretic mobility and assays of the photosensitization of plasmid DNA and transformed bacteria [12].

The series of complexes was screened for examining their effectiveness to break single-strand DNA by photosensitization using 457 nm light. The result was measured as the conversion of the supercoiled covalently closed circular (**ccc**) form of DNAp to its open circular (**oc**) form, as a function of irradiation time. Fig. 1 illustrates the rate of the appearance of the **oc** form when the complex used was $[Cr(5\text{-Mephen})_3]^{3+}$. As observed from lanes 3 to 7, the Cr(III) complex caused a decrease in the amount of the **ccc** form, whereas the **oc** form increased with the irradiation time. From the controls in lanes 1–3 and Fig. 2 (inset), it is shown that the mere presence of light at the longest irradiation time or the Cr(III) complex alone did not modify DNAp mobility and that the joint effect of both agents (Cr(III) complex and light) was necessary to disrupt **ccc** form of DNAp. As summarized in Fig. 2, it can be observed that all complexes were able to induce at least 50% DNA photodamage at the longer time of irradiation. Nevertheless, a more detailed examination of Fig. 2 reveals that, after 12 h of irradiation, the relative effectiveness of these cleavers is $[Cr(5\text{-Mephen})_3]^{3+}>[Cr(3,4,7,8\text{-tetraMephen})_3]^{3+}>[Cr(4,7\text{-diMephen})_3]^{3+}\cong[Cr(5\text{-Clphen})_3]^{3+}\cong[Cr(phen)_3]^{3+}>[Cr(5\text{-Phphen})_3]^{3+}$.

These complexes oxidize the guanine nucleobase and this effect correlates well with the reduction potential of the excited state, suggesting that the process occurs via an electron transfer mechanism (Type I photooxidation) rising a radical cation of guanine, $G^{\bullet+}$ [7]. This $G^{\bullet+}$ as a part of DNA structure created by the effect of light and the Cr(III) complex undergoes a series of reactions leading to the breakdown of DNA strand as final steps. However, no direct correlation is found between the oxidizing power of metal complex[2] and its ability to cleave DNA. Although the reduction potential of the excited state of $[Cr(5\text{-Clphen})_3]^{3+}$ has a higher value than that of the other Cr(III) complexes studied, the photocleavage efficiency is similar to that of $[Cr(4,7\text{-diMephen})_3]^{3+}$ with lower reduction potential. The reasons for this are not clearly outlined yet, nevertheless, it could be attributed to side photoreactions, such as processes of photodegradation, as proposed for ruthenium(II) complexes presenting a mechanism of photooxidation Type I [13].

To show whether the damage generated in DNAp by irradiation in the presence of $[Cr(NN)_3]^{3+}$ induces modifications in the viability of living cells, we have developed an assay that relates the DNA integrity to the number of transformed bacterial colonies [12c]. Thus, the XL1Blue strain of *Escherichia coli* (*E. coli*), unable to grow in an ampicillin agar plate, is transformed by the uptake of a plasmid DNA (pBS) carrying an ampicillin resistance cassette. The number of transformed colonies is proportional to the integrity of the DNAp. Competent *E. coli* were transformed with irradiated and non-irradiated DNAp in the presence of different $[Cr(NN)_3]^{3+}$ complexes and the corresponding controls. As expected, the number of colonies decreased when the bacteria incorporated the irreversibly photodamaged plasmid (Fig. 3) with significant inhibition rates of 11%, 19% and 37% for $[Cr(phen)_3]^{3+}$, $[Cr(5\text{-Phphen})_3]^{3+}$ and $[Cr(5\text{-Clphen})_3]^{3+}$, respectively, at short times of light treatment, such as 1 h. The results suggest that $[Cr(5\text{-Clphen})_3]^{3+}$ is a bacterial photoinhibitor slightly more efficient than the other Cr(III) complexes studied.

In both cases, the apparent lack of correlation between electrophoretic and bacterial studies is attributed to the way the readout is carried out. It is possible that the method of revealed of DNA-Cr(III) complexes within agarose gels is being affected by the interaction of ethidium bromide with DNA, displayed as bands of lesser intensity, as was observed for $[Cr(phen)_2(dppz)]^{3+}$ and to a lesser extent for $[Cr(phen)_3]^{3+}$ [11]. This would imply that the quantification of ccc vs. oc DNA is underestimated.

We also performed an alternative approach to assessing the effects of photoirradiation on bacterial cells in the presence of $[Cr(NN)_3]^{3+}$ [12d]. For these experiments, we first transformed *E. coli* XL1Blue bacteria cells with pBS plasmid in the presence of Cr(III) complex solutions, which was followed by irradiation. Fig. 4 shows the percentage colony formation units (% CFU) after 1 h of irradiation in the presence of $[Cr(5\text{-Mephen})_3]^{3+}$, $[Cr(phen)_3]^{3+}$and $[Cr(5\text{-Phphen})_3]^{3+}$. The controls were run and the bacterial growth was not significantly altered after 1 h of irradiation, in the absence of Cr(III) complex. A substantial cellular death is obtained only in the presence of light and Cr(III) complex; an inhibition higher than 50% was reached at shorter times of light treatment, this difference being statistically significant ($p<0.05$) respect to corresponding control.

The controls showed that $[Cr(NN)_3]^{3+}$ coordinated with tris-diimine ligands do not present any biological toxicity at least at the

[2] The reduction potentials of the excited state for $[Cr(3,4,7,8\text{-tetraMephen})_3]^{3+}$, $[Cr(4,7\text{-diMephen})_3]^{3+}$, $[Cr(5\text{-Mephen})_3]^{3+}$, $[Cr(phen)_3]^{3+}$, $[Cr(5\text{-Phphen})_3]^{3+}$ and $[Cr(5\text{-Clphen})_3]^{3+}$ are 1.11, 1.24, 1.40, 1.42, 1.49 and 1.53 V, respectively [6].

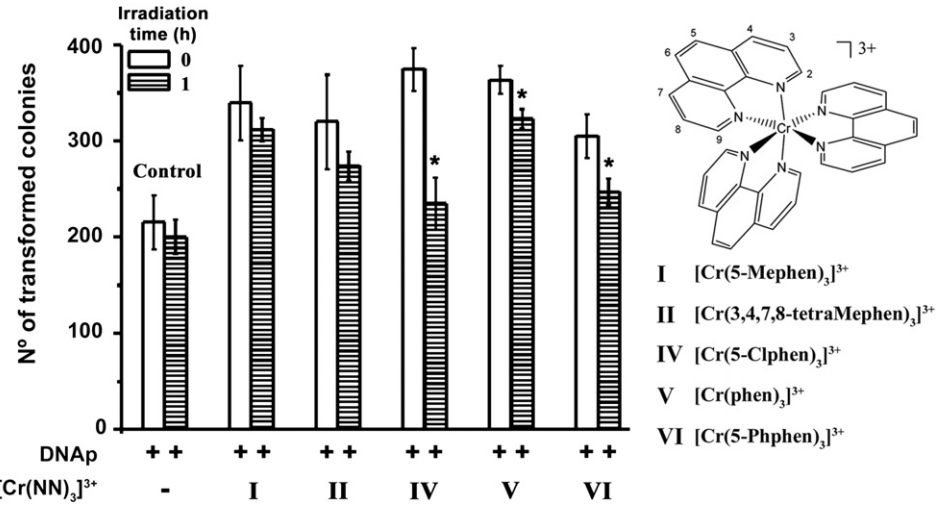

Fig. 3. Plot showing (□) (controls) the number of transformed colonies with DNAp non-irradiated in the presence and absence of $[Cr(NN)_3]^{3+}$ complexes and (▤) the number of colonies transformed with DNAp irradiated for 1 h in the presence and absence of $[Cr(NN)_3]^{3+}$ complexes. $^*p<0.05$.

concentrations used, since light (control, ▤) and Cr(III) complex (□) alone have not been able to induce bacterial inactivation (Fig. 4 inset). Thus, $[Cr(NN)_3]^{3+}$ caused inhibition of bacterial growth only in the presence of light. Effect of concentration and temperature will be the subject of future research.

The results reported suggest that $[Cr(NN)_3]^{3+}$ complexes, bound non-covalently to DNA, promote the photocleavage of DNA under irradiation at $\lambda = 457$ nm where the complexes are the only absorbing species [8]. These results indicate that the excited state of $[Cr(NN)_3]^{3+}$ is responsible for single strand breakdown in plasmid DNA. Unlike $[Cr(phen)_2(dppz)]^{3+}$, these complexes are not of the intercalator type, which is consistent with their weaker mode of action. However, they could be considered as photocleavage reagents. These compounds would offer an alternative mode of action to the process since they present a mechanism of photooxidation Type I (absence of O_2) rather than that their homologous Ruthenium complexes which acting under the Type II mechanism, via the action of the singlet oxygen [5]. Further research is still required to obtain more information on the biological effects of these agents.

Abbreviations

phen	1,10-phenanthroline
5-Mephen	5-methyl-1,10-phenanthroline
4,7-diMephen	4,7-dimethyl-1,10-phenanthroline
3,4,7,8-tetraMephen	3,4,7,8-tetramethyl-1,10-phenanthroline
5-Phphen	5-phenyl-1,10-phenanthroline
5-Clphen	5-chloride-1,10-phenanthroline
dppz	dipyrido[3,2-a:2′3′-c]-phenazine
DNAp	plasmid DNA
ccc	covalently closed circular
oc	open circular
lin	linear
LED	Light Emitted Diode

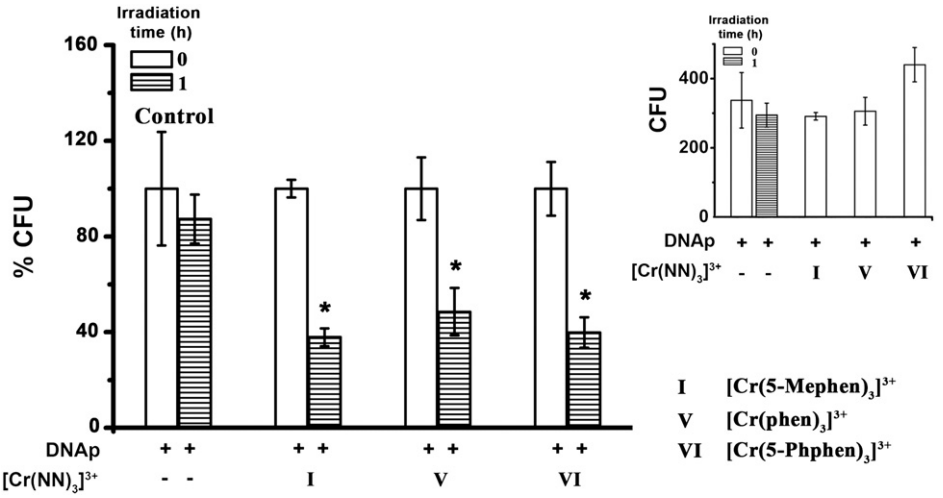

Fig. 4. Representation of the percentage of viable *E. coli* XL1Blue bacterial cells transformed with pBS plasmid: (□) control transformed bacterial suspension non-irradiated in the presence and absence of $[Cr(NN)_3]^{3+}$ (1×10^{-5} M); (▤) transformed bacterial suspension irradiated ($\lambda = 457$ nm) for 1 h in the presence and absence of $[Cr(NN)_3]^{3+}$. Values are normalized with respect to the control and represent an average of four different samples from the same experiment ± standard deviation of the mean. $^*p<0.05$. Inset: Unnormalized data for controls for each Cr(III) complex.

CFU	Colony Forming Unit
TRIS	Buffer solution (0.1 M Tris-HCl, 0.1 M NaCl, pH 7.4)
Tris	Tris(hydroxymethyl)aminomethane

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[12] Tris (1,10-phenanthroline) chromium(III), $[Cr(phen)_3]^{3+}$, tris (5-methyl-1,10-phenanthroline) chromium(III), $[Cr(5\text{-}Mephen)_3]^{3+}$, Tris (4,7-dimethyl-1,10-phenanthroline) chromium(III), $[Cr(4,7\text{-}diMephen)_3]^{3+}$, Tris (3,4,7,8-tetramethyl-1,10-phenanthroline) chromium(III), $[Cr(3,4,7,8\text{-}tetraMephen)_3]^{3+}$, tris (5-phenyl-1,10-phenanthroline) chromium(III), $[Cr(5\text{-}Phphen)_3]^{3+}$ and tris (5-chloride-1,10-phenanthroline) chromium(III), $[Cr(5\text{-}Clphen)_3]^{3+}$), as perchlorate salts, obtained from previous studies, were synthesized according to the literature [11] and recrystallized at least three times. The spectroscopic, crystallographic and time result data ensure a purity of at least 99.9%. The stock solutions $[Cr(phen)_3]^{3+}$, $[Cr(5\text{-}Mephen)_3]^{3+}$, $[Cr(4,7\text{-}diMephen)_3]^{3+}$, $[Cr(3,4,7,8\text{-}tetraMephen)_3]^{3+}$, $[Cr(5\text{-}Phphen)_3]^{3+}$ and $[Cr(5\text{-}Clphen)_3]^{3+}$ were prepared in TRIS buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 7.4) and the concentrations of the saturated solutions were calculated using molar absorptivity values of $\varepsilon_{354} = 4{,}200\ M^{-1}\ cm^{-1}$, $\varepsilon_{275} = 63{,}778\ M^{-1}\ cm^{-1}$, $\varepsilon_{380} = 3{,}388\ M^{-1}\ cm^{-1}$, $\varepsilon_{320} = 12{,}300\ M^{-1}\ cm^{-1}$, $\varepsilon_{243} = 93{,}325\ M^{-1}\ cm^{-1}$ and $\varepsilon_{275} = 61{,}660\ M^{-1}\ cm^{-1}$, respectively. All the experiments were carried out in TRIS buffer. Millipore Milli Q water (specific resistance of 18.5 $M\Omega\ cm^{-1}$) was used for preparing buffer solutions. All other chemical reagents were of analytical grade. Subcloning vector pBS (pBluescript II SK (+), Genbank accession number x52328) and *Escherichia coli* XL1-Blue Subcloning-Grade Competent Cells were purchased from Stratagene (La Jolla, CA, USA). The stock solution of pBS plasmid DNA (DNAp) was dissolved in 0.5x TE buffer (5 mM Tris-HCl, 0.5 mM EDTA pH 8 in MQ water) and kept at 4 °C. The concentration of plasmid DNA per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient (6,600 $M^{-1}\ cm^{-1}$) at 260 nm. **(a)***Plasmid and bacteria photolysis.* Test samples for photolysis purposes were prepared in sterile 0.6 ml tubes containing 0.6 μL of plasmid DNA solution (0.3 μg of pBS = pBluescript II SK(+)) with 14.4 μL of chromium(III) complex solution (240 μM). DNAp was incubated for a period of 15 min with Cr(III) complex prior to irradiation. The samples were irradiated with light of 457 nm. The source of irradiation was a white LED (type NSPW500CS, NICHIA America Corporation). The intensity of LED ($I_0 \sim 10^{-7}$ Einstein min^{-1}) was measured by actinometry using Potassium Ferrioxalate techniques. For all the photolysis experiments, the irradiation beam was achieved with the LED attached to the cap of the tubes containing the photolysis solution. Samples were kept at 6 °C under air-saturated atmosphere during irradiation, and stored in the dark before and after photolysis. The samples were loaded onto agarose gels after irradiation. Each electrophoretic run was done for each complex at all irradiation times. Suspensions of transformed bacterial cells were photolyzed in the same conditions. **(b)***Agarose Gel Electrophoresis.* Photosensitized DNAp samples were separated using horizontal 1% (w/v) agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). Electrophoresis was carried out at 100 V for 40 min to separate supercoiled covalently closed circular (**ccc**) and open circular (**oc**) forms of the plasmid DNA. After electrophoresis, DNA was visualized by soaking the gel for 15 min with an aqueous solution of ethidium bromide (0.5 μg/mL). The bands were seen in a transilluminator (254 nm) and the gel was photographed to provide a record of the distances migrated by the various DNA fragments. The ratio of ccc to oc forms was estimated by densitometry using the WCIF ImageJ program. **(c)***Bacterial transformation of photosensitized plasmids.* Transformations of *E. coli* XL1Blue strain were performed according to the manufacturer's protocol with 0.5 ng DNAp samples irradiated as indicated above. Following transformation, 100 μL bacterial suspensions were spread over the surfaces of Luria Broth agar plates in duplicate. For bacterial selection, plates contained 100 μg/mL Ampicillin (LB-Ampicillin Agar). The Petri dishes were incubated at 37 °C overnight. Transformed colonies were counted in order to assess the effect of irradiation on Cr(III)-DNA complex. The statistical analysis of the experimental data was performed using a two-way analysis of variance (ANOVA) and Tukey test. The significance level was established at $p < 0.05$. **(d)** *Photoinactivation of transformed bacterial cells.* Transformations of *E. coli* XL1Blue strain were performed with 0.5 ng DNAp and the appropriate concentration of Cr(III) complexes (1×10^{-5} M) according to the manufacturer's protocol. Following transformation, 18 μL suspension of transformed bacterial cells in the presence of $[Cr(phen)_3]^{3+}$, $[Cr(5\text{-}Mephen)_3]^{3+}$, $[Cr(5\text{-}Clphen)_3]^{3+}$ and $[Cr(5\text{-}Phphen)_3]^{3+}$ was irradiated with light of 457 nm. The irradiations were performed as stated above. The time of photolysis was 1 h. Samples were kept at 6 °C under air-saturated conditions during irradiation. After irradiation, the different suspensions for each complex to different irradiation times were diluted with 80 μL Super Optimal Broth (SOB medium) and 40 μl aliquots were spread over the surfaces of LB-Ampicillin Agar Petri dishes, in duplicate. The Petri dishes were incubated at 37 °C overnight. The colony-forming units (CFU) were counted and a statistical analysis of the experimental data was performed using a two-way analysis of variance (ANOVA) and Tukey test. The significance level was established at $p < 0.0$.
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