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On the Tunability of Toxicity for Viologen-Derivatives as Anolyte for Neutral Aqueous Organic Redox Flow Batteries

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Viologen-derivatives are the most widely used redox organic molecules for neutral pH negative electrolyte of redox flow batteries. However, the long-established toxicity of the herbicide methyl-viologen raises concern for deployment of viologen-derivatives at large scale in flow batteries. Herein, we demonstrate the radically different cytotoxicity and toxicology of a series of viologen-derivatives in in vitro assays using model organisms representative of human and environmental exposure, namely human lung carcinoma epithelial cell line (A549) and the yeast Saccharomyces cerevisiae. The results show that safe viologen derivatives can be molecularly engineered, representing a promising family of negolyte materials for neutral redox flow batteries.

Introduction

The unavoidable shift from fossil fuels to renewable sources for the generation of electricity will require the implementation of efficient and cost-effective energy storage solutions (ESSs) in the electric grid due to the intermittency of wind and solar radiation.[1] Among the various ESSs, redox flow batteries (RFBs) are considered promising candidates. In contrast to other types of batteries, energy and power can be scaled independently for RFBs, which is a great asset for stationary energy storage.[2] The all-vanadium RFB represents the state-of-the art, having been commercially deployed at MWh scale.[3] However, all redox active species are based on a critical raw material, namely Vanadium.[4] In addition, the poor environmental friendliness of the electrolytes also raises concerns.[5] Consequently, much attention is being paid to the development of redox active species based on organic molecules to replace Vanadium by carbon-based molecules that would avoid supply chain dependencies for RFBs.[6] Environmental friendliness must be taken into consideration when developing any new technology, which can also improve another key performance indicator with respect to all-vanadium RFBs. From this perspective, neutral pH electrolytes are preferential for developing redox organic molecules for the next generation of RFBs. Thus, 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), ferrocene, viologen and anthraquinone derivatives are examples of redox organic molecules that were reported to deliver promising electrochemical performances.[2,7] Furthermore, redox organic molecules have intrinsic advantages with respect to their metal-based counterparts, namely tunability of properties such as redox potential and solubility by molecular engineering.[8] Indeed, introduction of simple functional groups in the core structure of these families has proven substantial improvement in their solubility or redox potential.[9] Surprisingly, little attention has been paid to the environmental friendliness of these redox organic compounds. While moving away from corrosive acidic electrolytes used in all-vanadium RFBs has been identified as a potential benefit from an environmental perspective, concerns have also been raised about the environmental impacts of the state-of-the-art redox organic molecules.[10] The family of viologen-derivatives is the best example. Their electrochemical performance in terms of solubility, redox potential, kinetics, and stability make viologen-derivatives the most widely used redox organic molecules for neutral pH negative electrolyte of RFBs.[11] However, the most accessible viologen-derivative, i.e., methyl viologen (1,1′-bis-(methyl)-4,4′-bipyridinium dichloride, also known as paraquat), has been used as broad-spectrum herbicide for long time. As a matter of fact, toxicity of methyl viologen is known since the 1960s,[12] which has raised much criticism for deployment of viologen-derivatives at large scale. Thus, search for other redox organic molecules for neutral pH negative electrolyte has continued assuming that the use of other friendlier core

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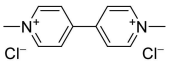
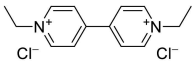
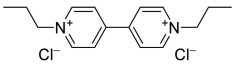
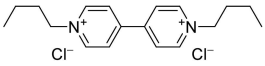
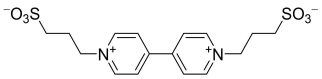
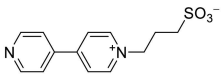
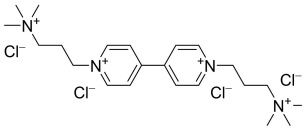
structure would facilitate upscaling from a safety and environmental perspective. However, it is also known that small modifications in the chemical structure of organic molecules can lead to radical changes in physiological activity, e.g., pharmaceutical field.[13] This raises the question of whether toxicity of viologen-derivative (and thus other families of redox organic molecules) can be also tuned as it occurs for other properties such as solubility and redox potential. Herein, a series of viologen-derivatives including non-commercial high-performing-anolyte viologen-derivatives are synthesized and electrochemically characterized. Then, their potential cytotoxic and toxicological effects for humans and unicellular fungi are investigated to address this critical question.

Results and Discussion

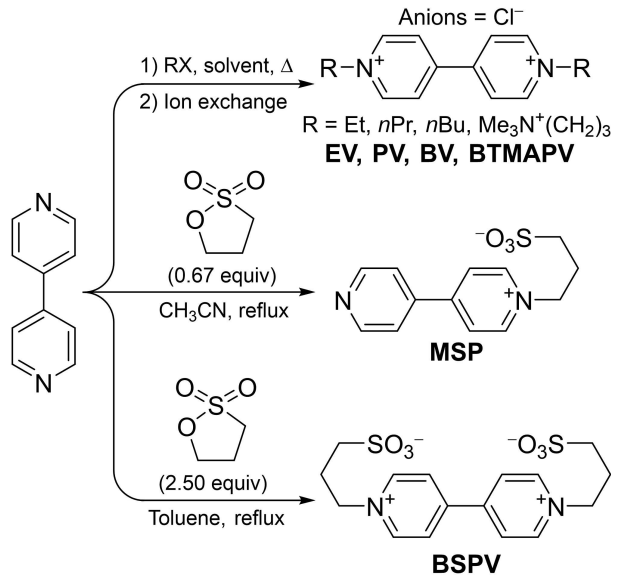
Synthesis of the viologen-derivatives

Since there are many viologen-derivatives (4,4-bipyridine derivative) investigated for aqueous organic redox flow batteries (AORFBs), a representative selection was required. The choice of viologen-derivatives to be explored was done according to their relevance as neutral pH anolyte in RFB. On the one hand, a series of *N*-alkylated homologues to methyl viologen (MV) with different alkyl chain lengths (ethyl, propyl, and butyl) were explored (Table 1). In addition, the toxicity of bis-substituted sulfonatepropyl and trimethylammoniopropyl viologen-derivatives, namely 1,1-bis(3-sulfonatopropyl)-4,4-bipyridinium (BSPV) and 1,1′-bis[3-(trimethylammonio)propyl)]-4,4′-bipyridinium tetra-chloride (BTMAPV) was investigated, as they are the most commonly used anolytes in aqueous organic RFB at neutral pH, achieving high cycle stability.[14] Finally, the toxicological study has been extended to the mono-*N*-alkylated 4,4-bipyridine derivative, 3-([4,4′-bipyridin]-1-ium-1-yl)propane-1-sulfonate (MSP), which is a degradation product of BSPV derived from the nucleophilic attack of hydroxide anions generated by oxygen reduction at the anolyte,[15] as well as an intermediate product in the synthesis of its corresponding viologen-derivative. The synthetic routes for accessing the viologen derivatives employed in this study are schematically described in Scheme 1, with the exception of MV, which is commercially available.

Table 1. Chemical structure and redox potentials of investigated viologen derivatives.

Viologen derivative	Chemical structure abbreviation	$E_{1/2}$ [V vs. Ag/AgCl]
1,1′-Bis(methyl)-4,4′-bipyridinium dichloride	 MV	−0.593
1,1′-Bis(ethyl)-4,4′-bipyridinium dichloride	 EV	−0.598
1,1′-Bis(propyl)-4,4′-bipyridinium dichloride	 PV	−0.620
1,1′-Bis(butyl)-4,4′-bipyridinium dichloride	 BV	−0.615
1,1′-Bis(3-sulfonatopropyl)-4,4′-bipyridinium	 BSPV	−0.532
3-([4,4′-bipyridin]-1-ium-1-yl)propane-1-sulfonate	 MSP	Pseudo-reversible[a]
1,1′-Bis[3-(trimethyl ammonio) propyl)]-4,4′-bipyridinium tetrachloride	 BTMAPV	−0.528

[a] Separation between cathodic and anodic peak is large as shown in Figure S1.



Scheme 1. Synthesis of viologen derivatives.

As shown in Scheme 1, 4,4′-bipyridine was *N*-alkylated twice with selected haloalkanes to afford the corresponding viologen derivatives EV, PV, BV and BTMAPV in high yields (85–90%).[16] Subsequently, ion exchange chromatographies were performed in order to obtain the corresponding chloride salts. In the case of sulfonatepropyl derivatives MSP and BSPV, they were achieved in a straightforward manner by reaction of 4,4′-bipyridine with commercially available 1,3-propane sultone in the adequate proportions and reaction conditions[15] (see Section S1 of the Supporting Information for the detailed synthetic routes and NMR characterization of all viologen-derivatives).

Electrochemical characterization of the viologen-derivatives

Electrochemical characterization of the different viologen-derivatives in 1 M KCl was carried out by cyclic voltammetry in a three-electrode cell, using glassy carbon as working electrode (Experimental Section). In all cases, the potential scan was limited to the first reduction process, thus cyclic voltammograms (CV) showed a pair of redox peaks corresponding to the reversible one electron transfer reaction to the viologen dication V^{2+} to form the radical cation $V^{\bullet+}$ (Figures 1A and 1B).

The series of *N*-alkylated viologen derivatives exhibited very similar CV (Figure 1A), with redox potential values very close to that of reference MV (Table 1), indicating negligible electronic or steric effect of the alkyl chain length (methyl-, ethyl-, propyl-, butyl) on the viologen redox properties. In the case of the bis-sulfonatepropyl- and trimethylammoniopropyl-functionalized viologens, BSPV and BTMAPV, the reduction potentials are slightly less negative than that of MV (Figure 1B and Table 1), which is consistent with the values reported in literature.[17] Overall, changes in the length of alkyl chain did not result in significant potential shift, while sulfonatepropyl- and trimethylammoniopropyl- substituents led to a small but undesired shift to more positive redox potential values. Despite the potential shift, sulfonatepropyl- and trimethylammoniopropyl-functionalized viologens are the most widely used anolytes due to their superior solubility and cycle stability.[14]

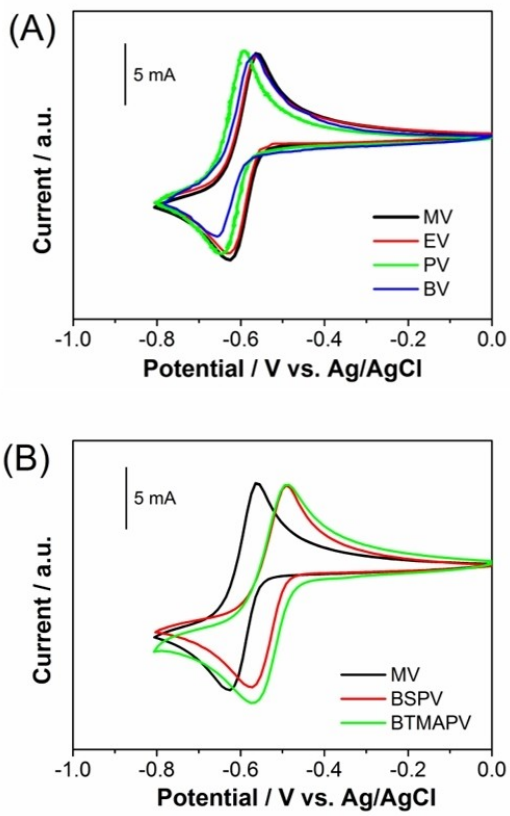


Figure 1. Cyclic voltammograms for the first reduction process of 25 mM viologen derivatives in 1 M KCl at 10 mV s^{-1} with a glassy carbon electrode: (A) N-alkylated viologens; (B) MV, BSPV and BTMAPV.

Cytotoxicity of the viologen-derivatives

The potential cytotoxic effect of the different viologen-derivatives was assessed in human lung carcinoma epithelial cell line (A549), using MV as benchmark due to its well-known toxicity. The viability of this human cell line after 24 h exposure to solutions of the different viologens was determined by the neutral red uptake assay, which is based on the ability of viable cells to incorporate and retain the neutral red dye in their lysosomes (details on the toxicology assays and statistical analysis are given in the Experimental Section). The concentration of viologen-derivatives in the toxicity assessment was optimized to enhance the interpretability of results. Thus, the highest tested concentration (20 mM) was selected after conducting preliminary screening assays, which established that benchmark MV at this concentration caused a critical impact on the viability of A549 cells (close to 100% of dead cells). In addition, a lower concentration (5 mM) was also explored, as

representative of exposure conditions where MV induced severe viability reduction but not total cell elimination. Figure 2 compares the results obtained for the different viologen-derivatives at the two concentrations.

Cells exposed to all *N*-alkylated viologens (MV, EV, PV, and BV) presented a critical decrease in their viability, being this effect statistically significant at both concentrations when compared to non-exposed cells. At the lower concentration (5 mM), cell viability upon exposure to with *N*-alkylated viologens was ≈15–30%, while viability decreased to almost 0% in cells exposed to 20 mM. No remarkable differences were noted between cells incubated with MV and *N*-substituted viologen-derivatives with longer alkyl chains (<10% at 5 mM), in line with the similar toxicity reported for several *N*-alkylated viologen-derivatives in rats.[18] Likewise, the viologen bis-functionalized with trimethylammoniopropyl groups (BTMAPV) caused a statistically significant reduction in the viability of this cell line, albeit to a lesser extent than alkylated viologens, with ≈20% and ≈68% viability reduction when exposed to 5 mM and 20 mM of BTMAPV, respectively.

On the contrary, the two derivatives functionalized with one and two sulfonatepropyl groups (MSP and BSPV) proved to be safe, with cell viability at 5 mM comparable to that of the control (unexposed cells). At 20 mM, only BSPV induced a slight but statistically significant decrease in the viability of this cell line (≈15%), which is anyhow considerably lower than that of 100% caused by the benchmark MV at the same concentration.

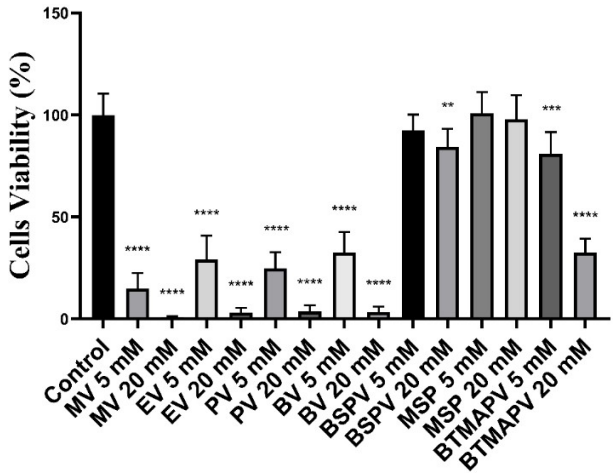


Figure 2. Viability of A549 cells (Neutral red assay) treated with MV and other viologen-derivatives at 5 and 20 mM. Results are expressed as % of control (untreated cells). Data represent the mean (± standard deviation, SD) of at least 6 biological replicates obtained in two independent experiments. Differences were established using a One-way ANOVA followed by Dunnet post hoc test to compare every mean with the control and considered significant at $P \leq 0.05$. ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Toxicity of viologen-derivatives in the yeast *S. cerevisiae*

The toxicological potential of the different viologen-derivatives in unicellular fungi was also assessed, selecting the yeast *S. cerevisiae* as model organism representative of environmental exposure. Yeasts can be found in a broad range of environments. Particularly, *S. cerevisiae* has been isolated from soils and water samples, hence being a suitable model for ecotoxicity evaluations.[19] Indeed, yeast assays have been proposed as a useful preliminary screening methodology to evaluate the toxicity level of environmental samples polluted with pesticides.[20] The viability of this microorganism, as an indicator of the potential environmental impact of the viologens, was evaluated applying the drop plate method. It consists in adding drops of different dilutions of a *S. cerevisiae* culture over standard YPD (yeast extract 1%, peptone 1%, dextrose 2%) agar plates containing 2 mM of the different viologen derivatives with the aim of analyzing yeast colony growth after incubation (the detailed protocol is reported in the Experimental Section). Since both organisms (A549 cells and *S. cerevisiae*) belong to different biological kingdoms, they present substantial differences in terms of structure, complexity and organization that make them to respond differently to the stressors. Thus, the concentration of viologen-derivatives in the toxicity assessment for the yeast S. cerevisiae was again optimized to enhance the interpretability of results. As in the case of the cell line, test viologen concentration was selected in a pre-screening assay as the threshold concentration of benchmark MV affecting critically the viability of *S. cerevisiae*. As displayed in photographs in Figure 3, *S. cerevisiae* cells exposed for 48 h to alkylated viologen-derivatives EV, PV and BV presented a slightly slower colony growth than that observed in the control (untreated cells) as revealed by the colony size at higher dilutions. The results point at a mild toxicity of these longer-chain alkylated viologen-derivatives, which contrasts with the acute toxicity observed for MV. In turn, BTMPAV also caused a critical impact in the viability of *S. cerevisiae*, which was even more pronounced than that noted for MV, as it inhibited colony

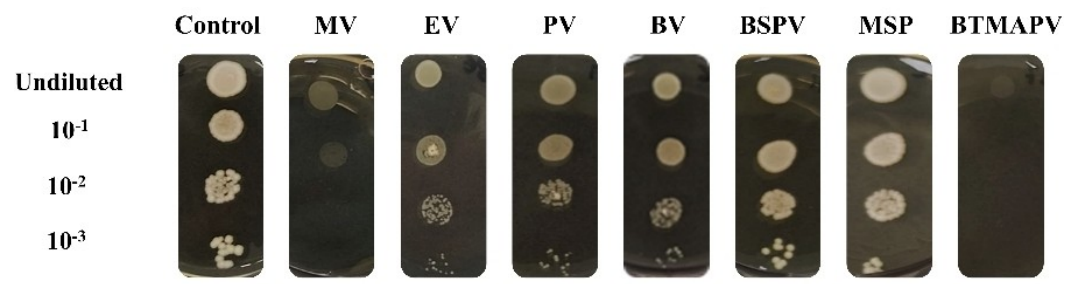


Figure 3. Viability of S. cerevisiae cells (drop plate method) exposed to 2 mM of the different viologen-derivatives.

growth at all dilutions. The high toxicity of this viologen-derivative may be ascribed to the presence of the terminal cationic trimethylammonium moiety, as the environmental toxicity of quaternary ammonium compounds at very low concentrations is well-established.[21] Interestingly, no effects were observed after 48 h exposure to derivatives functionalized with anionic sulfonatepropyl groups (MSP and BSPV), both resulting in similar yeast growth levels as in the control at all tested dilutions. That is, both toxicological assays with human cell lines and yeast indicate negligible toxicity of sulfonatepropyl- viologen-derivatives at concentrations where the reference MV exhibits acute toxicity.

Finally, toxicity of a benchmark catholyte, potassium ferrocyanide, was tested for human lung carcinoma epithelial cell line (A549) and the yeast *S. cerevisiae* (Section 3 in the Supporting Information, Figure S2 and Figure S3). In both cases, exposure to potassium ferrocyanide did not generate any harmful effect. Note that methyl viologen was tested again as benchmark. The results indicate that combination of molecular engineering of viologen-derivatives with potassium ferrocyanide open the door for the development of environmentally friendly AORFBs.

Conclusions

In conclusion, we have investigated whether toxicity is a tunable parameter of redox organic molecules. In particular, we have addressed the critical issue of whether viologen-derivatives are intrinsically toxic, which is crucial for their practical deployment as safe anolytes in neutral redox flow batteries. Despite further studies being needed to shed light into the mechanisms and being aware that toxicity is a complex topic, generally speaking we can conclude that toxicity of redox organic molecules can be tuned. The toxicological assessment of a series of commercial and synthesized viologen derivatives revealed remarkable differences in their cytotoxic and toxicological impact in model organisms representative of human and environmental exposure. On the one hand, *N*-alkylated viologens significantly reduced cell viability irrespectively of the alkyl chain length, with the reference compound MV exhibiting the most acute toxicity of the series for both model organisms. Trimethylammoniopropyl-functionalized viologen also proved highly toxic in both toxicological assays. Conversely, mono- and bis-sulfonatepropyl-functionalized derivatives showed no effect in cell viability, representing safer and more environmentally-friendly candidates for the anolyte of neutral organic redox flow batteries. The results illustrate the power of molecular engineering towards implementation of safe-by-design redox organic molecules in sustainable, efficient, and cost-effective energy storage solutions.

Experimental Section

General methods

Materials

All common reagents and solvents were purchased from Aldrich or Alfa-Aesar and used as received without further purification.

NMR measurements

NMR spectra were measured on Bruker Avance 300 MHz spectrometer. ^{1}H NMR: splitting pattern abbreviations are: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; ddd, doublets of doublets of doublets; ddt, double doublet of triplets; dt, doublet of triplets; dq, doublet of quartets; td, triplet of doublets; qd, quartet of doublets; p, pentuplet; h, sextet; hept, heptet; m, multiplet; br, broad; a, apparent; the chemical shifts are reported in ppm using residual solvent peak as reference. ^{13}C NMR spectra were recorded at 75.4 MHz or 100.6 MHz using broadband proton decoupling and chemical shifts are reported in ppm using adequate solvent peaks as internal reference (CH_3OH: 49.50) and the multiplicities were determined by DEPT experiments.

Electrochemical characterization

Cyclic voltammetry experiments were conducted with an Autolab PGSTAT12 (Methrom-Autolab, The Netherlands) using NOVA 2.1.3 software. A three-electrode cell was set up, comprising a polished glassy carbon working electrode ($A_{electrode} = 7\ mm^2$), a Pt wire counter electrode (99% purity) and the Ag/AgCl (3 M KCl) electrode, which served as the aqueous reference electrode. The cyclic voltammetry curves were obtained using 25 mM viologen derivatives in 1 M KCl at a scan rate of 10 $mV\,s^{-1}$.

Toxicology assays

Organisms and culture conditions

The human lung carcinoma epithelial cell line (A549) was cultured in commercial Dulcobecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS) and 1% penicillin/streptomycin. Cells were kept in a thermostatic incubator under optimal growth conditions (humidified atmosphere containing 5% CO_2 and 37 °C). The Saccharomyces cerevisiae BY4741 strain was grown and maintained in standard YPD [yeast extract (1%), peptone (1%), dextrose (2%)] broth or agar at 30 °C.

Neutral Red uptake assay using A549 cell line

The viability of human lung cell line after 24 h of exposure to the different viologen- derivatives was determined by the Neutral Red uptake assay. A549 cells were seeded in 96-well plates at 3×10^4 cells per well and incubated in optimal growth conditions for 24 h. After this time, cells were washed with Dulcobecco's phosphate-buffered saline (DPBS), and 200 μL of two concentrations (20 and 5 mM) of viologen and viologen-derivatives resuspended in fresh medium supplemented with 1% of fetal bovine serum (treatment media) were added to each well. Cells treated with treatment medium alone were used as negative control (untreated cells). Afterwards, cells were incubated 24 h in presence of the different viologen-derivatives. After this time, culture medium was discarded, wells were washed with DPBS, and 100 μL of a neutral red solution were added to each well and incubated for 2.5 h at 37 °C. This

solution was prepared as follows: Neutral red powder was resuspended in DPBS to a concentration 4 mg/mL, further diluted at 1:100 in treatment media, and incubated for 24 h at 37 °C in absence of light before being used in the experiments. After this time, the solution was centrifuged to remove debris from neutral red powder. Once the incubation with neutral red was completed, the solution was discarded, cells were washed with DPBS and fixed with formaldehyde 4% for 2 min. Cells were washed again with DPBS, and 150 μL of extraction solution (50% ethanol 96°, 49% distilled water and 1% glacial acetic acid) were added to each well. After a gentle shaking step of 10 min, 100 μL of the supernatant were transferred to a new opaque 96-well plate, and fluorescence was measured employing a microplate reader (BioTek Synergy HT, excitation: 530/25; emission: 645/40). Results were expressed as percentage of control (fluorescence of non-exposed cells). Data represent the mean of two independent experiments with at least three biological replicates each.

Drop plate tests using S. cerevisiae

The effect of the viologen and viologen-derivatives over the viability of *S. cerevisiae* was studied applying the following protocol: one colony was selected in a petri dish and resuspended in YPD broth. Cells cultures in liquid media were placed on an orbital shaker at 180 rpm at 30 °C until exponential growth phase (optical density at 600 nm, $OD_{600} = 1$). Finally, cell suspension was serially diluted, and 2 μL of each dilution were transferred to YPD agar plates with 2 mM of the different compounds, which were subsequently incubated at 30 °C for 48 h.

Statistical analysis

Statistical analysis data are presented as means ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used for multiple comparisons test, followed by Dunnet post hoc test to compare every mean with the control. Statistical tests were carried out using Prism 8.0 (GraphPad Prism, GraphPad Software, Inc.). Differences were considered significant at $P \leq 0.05$.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: cytotoxicity · toxicology · viologen derivatives · anolyte · redox flow battery

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RESEARCH ARTICLE

Viologen toxicity: The long-established toxicity of the herbicide methyl-viologen raises concern for deployment of viologen-derivatives as anolyte for neutral aqueous organic redox flow batteries (AORFB) at large scale. Here we show that non-toxic viologen derivatives can be molecularly engineered, holding great promise as safe anolytes for AORFB.

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