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Achieving biocompatible SABRE: An *in vitro* cytotoxicity study

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Abstract: Production of a biocompatible hyperpolarized bolus by Signal Amplification By Reversible Exchange (SABRE) could open the door to simple clinical diagnosis *via* magnetic resonance imaging. Essential to successful progression to pre-clinical/clinical applications is the determination of the toxicology profile of the SABRE reaction mixture. Here we exemplify the cytotoxicity of the SABRE approach using *in vitro* cell assay. We conclude that the main cause of observed toxicity is due to the SABRE catalyst. Thus we develop two catalyst removal methods, one involving deactivation and ion-exchange chromatography and the second biphasic catalysis. These routes produce a bolus suitable for future *in vivo* study.

Introduction

Clinical Magnetic Resonance Imaging (MRI) is at the forefront of disease diagnosis. It utilizes strong magnetic fields, radio waves and field gradients to give detailed images of the human anatomy. However, due to MRI's inherent low sensitivity, almost all clinical applications detect water due to its high concentration in the body. Hyperpolarization methods turn typically weak MRI responses into strong signals and thus open the door to new diagnostic techniques. For example, Dynamic Nuclear Polarization (DNP) has been used to create the signal strength necessary to track the *in vivo* metabolism of pyruvate in prostate tumors^[1] whilst Spin Exchange Optical Pumping (SEOP) of noble gases has allowed the diagnosis of pulmonary diseases.^[2]

An alternative low cost approach to hyperpolarization utilizes parahydrogen (*p*-H₂) to create a non-Boltzmann nuclear spin distribution without changing of the identity molecule of interest. This technique is known as Signal Amplification by Reversible Exchange (SABRE) and is shown schematically in Figure 1.^[3] It

can enhance the signals detected by MRI and Nuclear Magnetic Resonance (NMR) spectroscopy across a wide range of nuclei such as ¹H, ¹³C, ¹⁵N and others.^[4] It works by harnessing the latent polarization of *p*-H₂ through binding to a metal catalyst, typically [Ir(H)₂(Sub)₃(IMes)]Cl,^[5] as hydride ligands. Simultaneous binding of the substrate allows spontaneous transfer of polarization through the scalar coupling network at low magnetic fields.^[6] Subsequent substrate dissociation from the catalytic complex allows the build-up of hyperpolarized substrate in solution.

A recent study showed the effect of ²H-labelling a series of nicotinamide and methyl nicotinate molecules can simultaneously improve their SABRE-enhanced NMR and MRI detection and reduce magnetic relaxation. The optimal substrate in this study was methyl-4,6-*d*₂-nicotinate (**d**₂-**MN**) which gave up to 50% polarization in conjunction with *T*₁ relaxation values approaching 2 minutes.^[7] This makes **d**₂-**MN** an ideal candidate for *in vivo* detection. However, despite some applications of methyl nicotinate (**MN**) in cosmetic and veterinary pharmaceuticals, the effect of selective deuteration has not been studied.

The isotopic labelling of drug molecules is a well-established route to modify their safety and/or efficacy.^[8] Deuteration primarily affects the biological fate of drugs that are metabolized through a pathway that involves hydrogen-carbon bond breaking as significant rate changes occur due to the kinetic isotope effect. The metabolic pathway of nicotinic acid derivatives typically proceeds *via* formation of the *N*-oxide, though routes involving 6-hydroxy species are also known. For this reason the toxicity of **d**₂-**MN** in comparison to **MN** is determined here using *in vitro* cytotoxicity analysis.

Another potential source of toxicity that arises from the SABRE technique is the iridium catalyst. Whilst some data on the adverse effects of iridium salts has been reported^[9], little information is known about organo-iridium complexes^[10]. Therefore we present a thorough investigation into the potential toxicity of the metal catalyst in conjunction with solvent and substrate effects. We determine the biocompatibility of the SABRE reaction by performing *in vitro* cytotoxicity analysis on human cell lines and present a method for depletion of the catalyst from solution in order to create a biocompatible bolus that could be progressed to *in vivo* measurement.

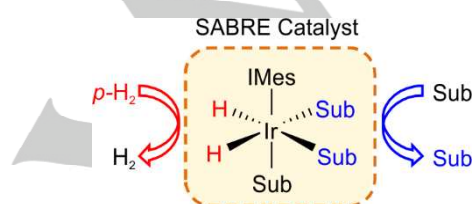


Figure 1: Schematic representation of the SABRE effect

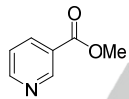
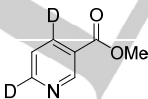
Results and Discussion

Evaluating the cytotoxicity of SABRE substrate

Methyl-4,6- d_2 -nicotinate (d_2 -MN) has been reported to give the highest ^1H polarization levels by SABRE to date.^[7] From biological perspective, MN is widely used as rubefacient in cosmetics due to its percutaneous penetration properties upon topical application and moreover known for its vasodilatory effects at lower doses and inflammatory response at higher doses.^[11] Given the substantial SABRE enhancement levels and biological applicability, d_2 -MN is considered an ideal candidate for probing on *in vitro/in vivo* tests for SABRE detection. However, whether d_2 -MN retains the characteristics of MN is still unclear. In this study, we utilize d_2 -MN as a model substrate to ascertain the broader SABRE toxicology profile.

In order to establish the effect of selective deuteration on toxicity we began by determining the IC_{50} (half maximal inhibitory concentration) of MN and d_2 -MN. For this, well established cancer cell lines of human origin were treated with either MN or d_2 -MN at varying concentrations for up to 48 h and the viability was assessed by the MTT method.^[12] The results obtained for each cell lines is shown in Table 1. The IC_{50} for MN was found to range between 12.6–33.3 mM across the cell lines and interestingly, despite the deuterium labelling, a comparison of the IC_{50} values indicated that the toxicity levels of d_2 -MN are similar to MN. Importantly, both substrates have millimolar (mM) IC_{50} values (Table 1) and are within the concentration range used in a typical SABRE reaction (mM). It is noted here that these results are based on the solubility of the substrates directly in, the high volume of, the cell culture medium and it is still unclear how the effect would be under conditions of SABRE, where different solvents are used. Nevertheless, these data provide a reference IC_{50} value for optimizing SABRE for further analysis. From this analysis, we conclude that deuteration of the methyl nicotinate has no quantifiable effect in modulating toxicity across the cell lines studied here.

Table 1. Comparing the IC_{50} of MN vs. d_2 -MN in human cancer cell lines.

	IC_{50} (mM)	
		
	Methyl Nicotinate (MN)	Methyl-4,6- d_2 -Nicotinate (d_2 -MN)
A549	13.6	17.4
MCF7	33.3	25.2
HeLa	21.9	14.9
MDA-MB231	12.6	12.3

Effect of SABRE solvents on cell viability

SABRE induced polarization levels are typically highest in alcohol solvents such as methanol- d_4 or ethanol- d_6 .^[7, 13] However some applications can produce hyperpolarized substrates in aqueous solution, though enhancement levels are typically reduced.^[14] In order to create a biocompatible hyperpolarized

bolus with high polarization levels it has been suggested that the hyperpolarization step should be carried out in ethanol- d_6 prior to dilution with D_2O .

To assess the toxicity of these solvent mixtures we performed an appropriate cell viability assay on A549 and MCF7 cells which were treated with various dilutions of ethanol- d_6 in D_2O . As shown in Figure 2, viability of both the cell lines were significantly reduced when ethanol- d_6 (100%) was added to cell culture medium and treated for a short time (6 h). Conversely, over the same time point (6 h), 50% ethanol- d_6 in D_2O (1:1) showed no change in cell viability. Extending the treatment durations to 24 h, however, significantly reduced the viability (Figure 2 B). Whereas, we found that treatment of cells in a 30% ethanol- d_6 in D_2O (30:70) solution did not show toxicity to cells over long treatment times when compared to other deuterated solvents mixtures (Figure 2 B and see SI Figure S1). Together the results indicated that a significant reduction in cell viability is evident when the solvent contained ethanol- d_6 concentrations higher than or equals to 50%. The behavior of analogous protio solvent mixtures is similar (see SI Figure S2). From these *in vitro* cytotoxicity analysis on SABRE solvents we conclude that to achieve an optimal biocompatibility it is important to consider the duration of exposure on cells in culture (or *in vivo*) when performing SABRE using higher (>30%) ethanol- d_6 solvent.

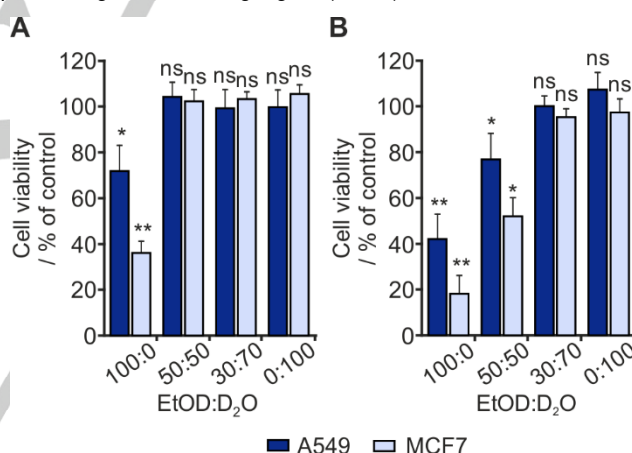


Figure 2. Effect of deuterated solvent mixture on cell viability: MTT cell viability performed on the indicated cell lines after (A) 6 h and (B) 24 h of treatment with deuterated solvents at different ratios. The final solvent volume in the cell culture medium was 10%. EtOD - ethanol- d_6 and D_2O - deuterium oxide. Data represent mean \pm SD. * $P < 0.05$, ** $P < 0.005$ and ns - Not significant vs. the untreated control (100% viable): one-way ANOVA.

As the optimal solvent mixture for biocompatibility determined here is 30% ethanol- d_6 in D_2O , we wished to quantify the effects of using this solvent directly for the SABRE polarization of d_2 -MN. Thus, we prepared a sample containing 5 mM $[\text{IrCl}(\text{COD})(\text{IMes})]$, 20 mM d_2 -MN in ethanol- d_6 in D_2O (30:70). After exposure to 3 bar $p\text{-H}_2$ at 298 K in a 60 G field we found that a 105 ± 22 fold total signal enhancement is observed at 9.4 T. The corresponding ^1H NMR spectra are shown in the supplementary information (see Figure S3). This is a significant reduction in polarization when compared to using 100% ethanol- d_6 as solvent and polarization under of 3 bar $p\text{-H}_2$ gave a signal enhancement of over 2800-fold.^[7] As we have previously shown, further optimization may be achieved through using a higher pressure of $p\text{-H}_2$ and isotopically labelled catalysts and work is ongoing to improve the polarization levels.

Evaluating the cytotoxicity of substrate in solvent

Having found that 30% ethanol- d_6 in D_2O does not induce toxicity to cells *in vitro*, we then performed viability assay for d_2 -MN dissolved in this solvents mixture. Again here, we compared d_2 -MN with MN to exclude any toxic effect that arise from the selective deuteration in d_2 -MN when dissolved in alcohol solvents. Importantly, d_2 -MN shows good solubility in this solvent composition and it did not reduce the viability of A549 and MCF7 cells when treated for up to 6 h (Figure 3 A and 3 B, respectively). However, longer treatment times at concentrations higher than 5 mM, are shown to affect the viability of both cell lines (Figure 3 C and 3 D). Surprisingly, as shown in Figure 3 D, when compared to MN, d_2 -MN induced a significant reduction in the viability of MCF7 cells at this concentration (5 mM) when treated for long time (24 h). This further indicates that the deuteration might affect the toxicity effects of the substrate either by itself or that toxicity is more pronounced as an additive effect when mixed in alcohol solvent at this long time (24 h) exposure. Together, our data suggest that the *in vitro* cytotoxicity of d_2 -MN in an ethanol- d_6 : D_2O solvent mixture depends on the duration of exposure on cells in culture. It is worth mentioning that the aim of our cytotoxicity assessment is to allow us to understand the transient effect these compounds or solvents play under the

stated conditions. We are aware that in an *in vivo* setting biocompatibility would be dependent on physiological status and the pharmacokinetics of the organism and the mechanism of action of the treated material. Nonetheless, the SABRE approach requires the contrast agent to stay in the body for a comparatively short time prior to excretion as relaxation limits utility and further reduces toxicity concerns.

Evaluating the biocompatibility of SABRE reaction mixture

Given that the cytotoxic dosage of d_2 -MN in the solvent ethanol- d_6 : D_2O (30:70) is well above the amounts used for a typical SABRE reaction (considering only less than or equal to 10% will be taken as a bolus for treatment) we sought to investigate the effect of the SABRE reaction mixture on A549 and MCF7 cell lines.

For this, we prepared a typical SABRE solution containing 5 mM of $[IrCl(COD)(IMes)]$ together with 20 mM of d_2 -MN in ethanol- d_6 : D_2O (30:70) and activated it with 3 bar H_2 . We exposed the cells to various bolus volumes (1.25, 2.5, 5 and 10%) of the activated mixture and assessed the viability of the cells at different time periods by MTT assay. As illustrated in Figure 4, treatment with the SABRE reaction mixture over a short period of time (1 h) did not reduce the viability of A549 and MCF7 cells when lowest volume (e.g. 1.25%) was added to cell culture medium. However, cells that were treated with 10% bolus of the SABRE reaction mixture showed less viability at the same time point.

To distinguish the cytotoxic effect of the substrate and catalyst in the mixture we prepared in parallel analogous solutions containing various concentrations of either d_2 -MN or $[IrCl(COD)(IMes)]$ alone. Unfortunately, $[IrCl(COD)(IMes)]$ is less soluble in ethanol- d_6 : D_2O (30:70) and moreover cannot form an active catalyst without the presence of a substrate when activated with H_2 . Under these conditions, a precipitate formed (see SI Figure S4) and cytotoxicity assessment using this emulsion to treat cells is not expected to provide comparable results. Nevertheless, we treated the cells with a solution of the d_2 -MN alone and no adverse effect was observed even at treatments over 24 h on both cell lines. This indicates that the deleterious effect of the SABRE reaction mixture on cells could be due to the presence of the catalyst and not the substrate (see SI Figure S5). In contrast, the cells treated with various bolus volumes of the $[IrCl(COD)(IMes)]$, prepared by homogeneous mixing of the precipitate showed loss of cell viability at higher volumes and short time points (see SI Figure S6). Together these data indicate that the SABRE reaction mixture induces a reduction in cell viability at higher volumes and this is likely due to the presence of activated catalyst.

Thus, we conclude that in order to achieve biocompatibility the amount of activated catalyst has to be either reduced or eliminated.

Method of catalyst deactivation and removal

We have therefore developed a protocol to remove the SABRE catalyst from solution. The addition of a chelating ligand to the SABRE reaction prevents reversible exchange of the substrate, deactivating the SABRE process without affecting the polarization levels whilst extending T_1 relaxation times.^[15] We postulated that the addition of bathophenanthrolinedisulfonic

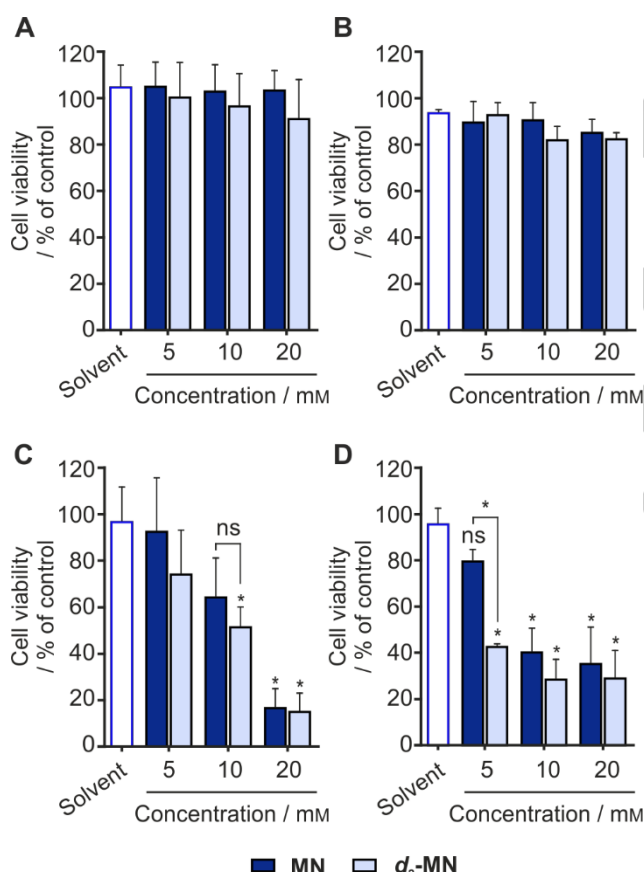


Figure 3. Cytotoxicity of alcohol solubilized methyl nicotinate: MTT cell viability data showing (A and C) A549 and (B and D) MCF7 cells treated for (upper panel) 6 and (lower panel) 24 h with different dilutions of MN and d_2 -MN solubilized in 30% ethanol- d_6 in D_2O . The final solvent volume in the cell culture medium was 10%. Data are presented as mean + SD and are from 3 independent experiments (n=3). Statistically significant differences from untreated control group (or from protio form of MN) is shown. *P<0.05 and ns - Not significant vs. the untreated control group; one-way ANOVA.

acid disodium salt (BPS) would see it irreversibly bind to the iridium center whilst giving an opportunity to remove the

resultant species via ion-exchange chromatography. This procedure is shown

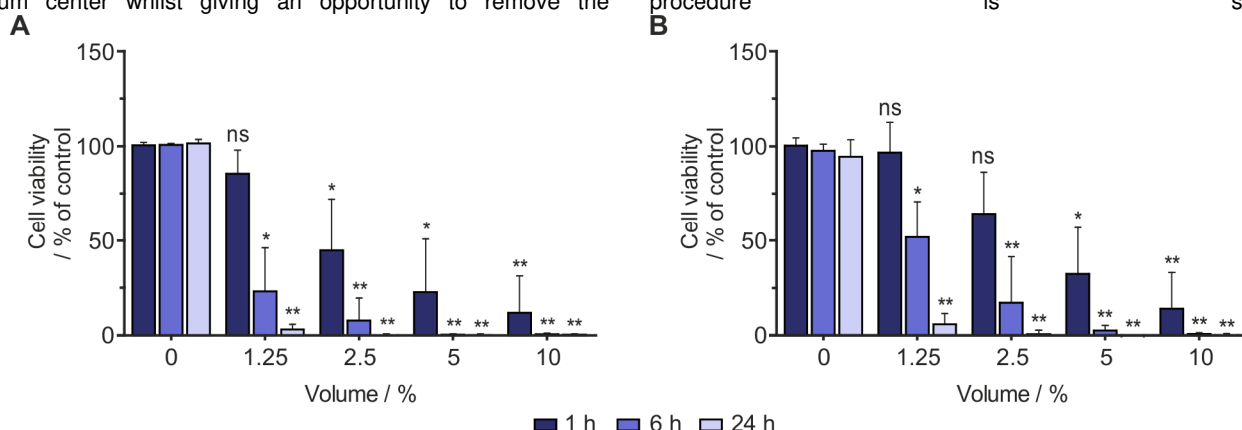


Figure 4. Evaluating the biocompatibility of SABRE reaction mixture: (A) A549 and (B) MCF7 cells were treated with different bolus volumes (0, 1.25, 2.5, 5 and 10 %) of SABRE reaction mixture and cell viability was assessed 1, 6 and 24 h thereafter by MTT assay. Data are presented as mean + SD and are from 3 independent experiments (n=3). *P<0.05, **P<0.005 and ns - Not significant vs. the untreated control (100% viable); one-way ANOVA.

schematically in Figure 5. First, we prepared the activated SABRE reaction mixture in ethanol- d_6 : D_2O solution prior to the addition of a solution of 2.0 eq. of BPS in D_2O . Following the reaction by 1H NMR spectroscopy reveals the immediate formation of a new hydride species at $\delta -19.6$ which we attribute to $[Ir(Imes)(BPS)(d_2-MN)(H)_2]Cl$ and confirm by LC-MS (see SI Figure S7). After filtration through DEAE-Sephadex® with D_2O as eluent less than 2% of the catalyst remains in solution and high mass recovery of d_2-MN , which can be delivered in the biocompatible ethanol- d_6 : D_2O solvent mixtures. This protocol is

therefore efficient at removing the SABRE catalyst from solution.

Importantly, the hyperpolarized SABRE signal is still visible after the deactivation and depletion steps. The total signal gains were 74 ± 21 fold which represents a 30% reduction in signal when compared to the standard SABRE sample in ethanol- d_6 : D_2O (30:70). 1H NMR spectra are shown in the supplementary information (see SI Figure S7). As the deactivation and depletion process takes a minimum of 12 seconds longer than a standard sample measurement, we attribute the loss to relaxation effects.

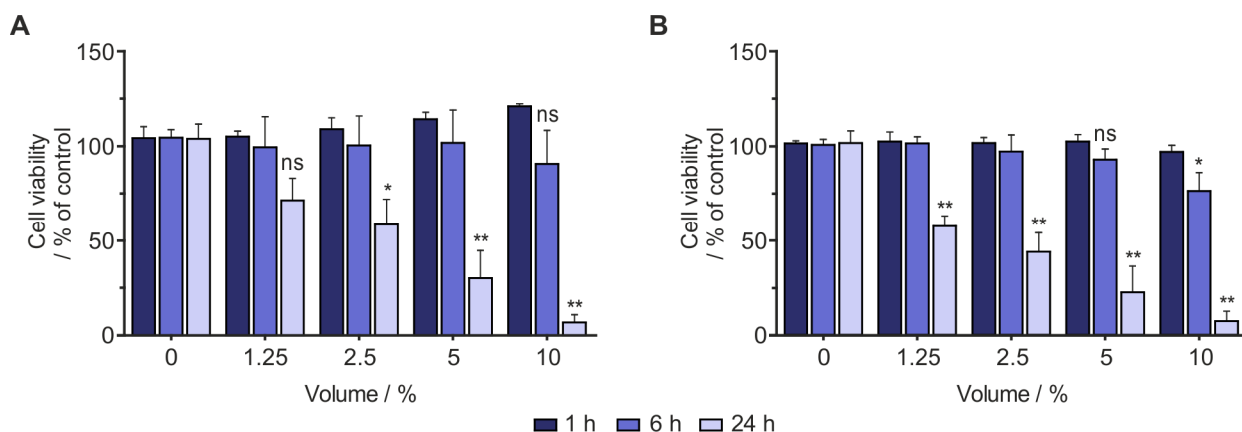


Figure 6. Achieving biocompatible SABRE by deactivating the catalyst: MTT viability assay showing (A) A549 and (B) MCF7 cells treated with various volumes of catalyst depleted SABRE reaction mixture for 1, 6 and 24 h. Data are presented as mean + SD and are from 3 independent experiments (n=3). *P<0.05, **P<0.005 and ns - Not significant vs. the untreated control (100% viable); one-way ANOVA.

Cytotoxicity assessment of catalyst depleted SABRE mixture

The cytotoxicity of the catalyst depleted samples on cells was then evaluated by taking different volumes (1.25, 2.5, 5 and 10 %) of the reconstituted mixture in ethanol- d_6 : D_2O (30:70) and by following the treatment conditions in the same manner as performed with the non-quenched SABRE reaction mixture. Pleasingly, 10% of the bolus containing the catalyst depleted SABRE reaction mixture did not alter the viability of A549 cells for up to 6 h of treatment (Figure 6 A). Similarly, MCF7 cell lines

showed no changes in cell viability when treated with higher volumes (10 %) of the catalyst depleted SABRE mixture for up to 1 h and for up to 6 h with lower volumes (≤ 5 %, Figure 6 B). However, longer treatments (24 h) showed significant decrease at both lower and higher volumes in both cell lines (Figure 6 A and 6 B). It is noted that when extrapolating to an *in vivo* model, it is unlikely to show the same long-term toxicity due to higher metabolic activity and detoxification mechanisms. Together these data indicate that the deactivation and removal of the catalyst could overcome the adverse effect of SABRE reaction mixture when treating live cells under the conditions used here.

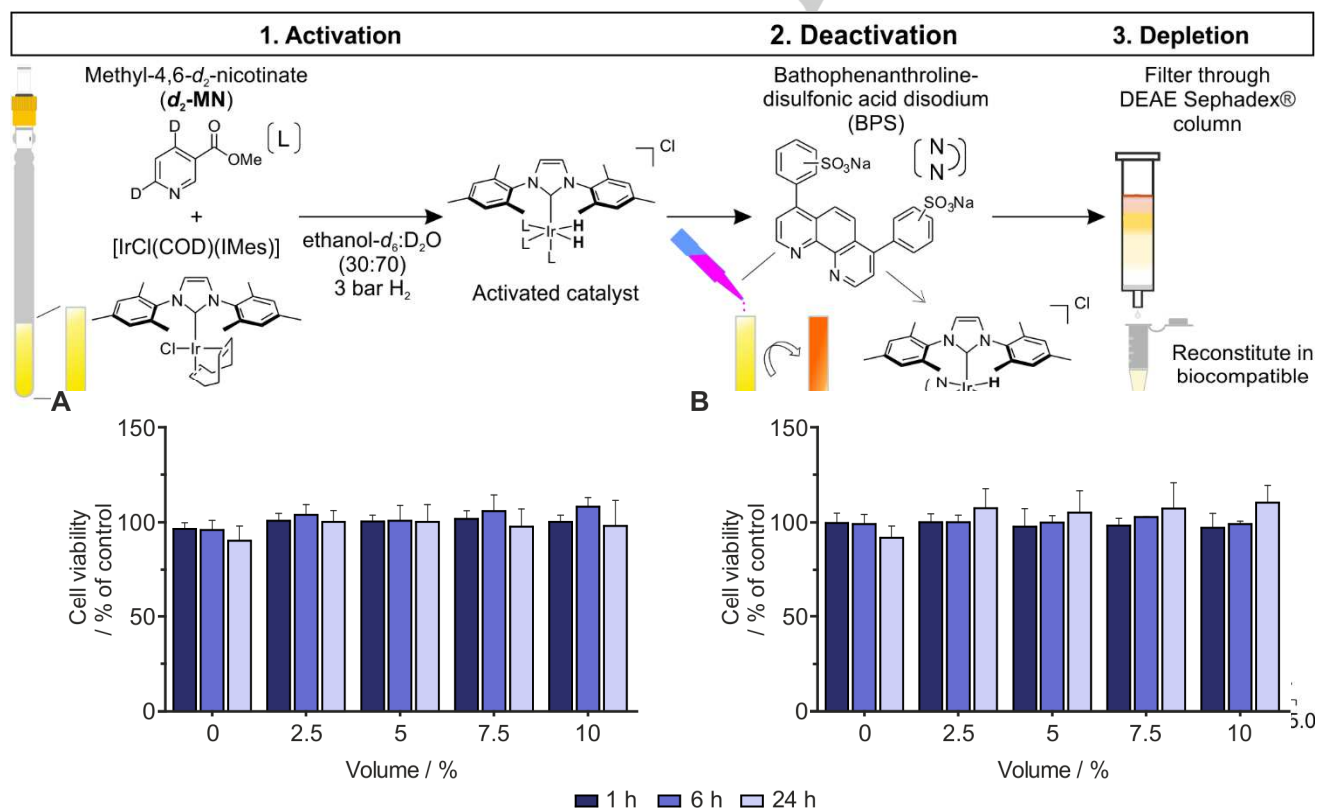


Figure 7. Evaluating the biocompatibility of biphasic SABRE reaction mixture: MTT viability assay showing (A) A549 and (B) MCF7 cells treated with various volumes (0, 2.5, 5, 7.5 and 10%) of the bolus from the aqueous fraction of a SABRE reaction in biphasic solvents for 1, 6 and 24 h. Data are presented as mean + SD and are from 3 independent experiments (n=3).

Achieving biocompatible SABRE by Biphasic catalysis

We hypothesized that using the recently reported biphasic approach to SABRE catalysis could be a more rapid and facile way to deplete the solution of the iridium catalyst.^[16] In this method the SABRE catalyst is located in a chloroform or dichloromethane (DCM) phase and minimally in the aqueous phase. Conversely, the hyperpolarized substrate is distributed between the two. For toxicity assessment on cells, the aqueous phase was isolated and various bolus volumes (2.5, 5.0, 7.5 and 10%) were added to the cell culture medium. We performed the appropriate viability assay at different time points as illustrated in the previous sections. Treatment with the phase separated

SABRE mixture did not reduce the viability of either A549 or MCF7 cells at any of the time points tested (Figure 7). The minimal cytotoxic effect seen here is similar to the effect seen when treated with the substrate alone (see SI Figure S9). This result indicates that the cytotoxicity associated with the SABRE reaction mixture is negated by this method. While we are able to produce a biocompatible bolus by this phase separation method, the polarization level achieved by the biphasic catalysis is approximately 2000-fold for the same substrate (**d₂-MN**), (see SI Figure S10). Nevertheless, we conclude that polarization in the biphasic mixture is higher than that observed in an ethanol-*d*₆:D₂O solution under 3 bar *p*-H₂ and at 298 K.

Conclusions

To conclude, we have shown through *in vitro* cytotoxicity studies that it is possible to create a biocompatible SABRE bolus for future *in vivo* detection. This is an important step towards the pre-clinical/clinical disease state diagnosis using the SABRE technology. We have exemplified our toxicity study for methyl-4,6-*d*₂-nicotinate (**d₂-MN**) which shows strong hyperpolarization levels and long magnetic lifetimes.^[7] By determination of the IC₅₀ values across a number of human cancer cell lines we have shown that deuteration of the substrate did not show significant change in toxicology when compared to its protio analogue. This will provide important information for the development of SABRE contrast agents in the future. We will also look to determine the effect of other common isotopic labelling strategies (e.g. ¹³C and ¹⁵N) as we further expand our substrate profile. For example, we are interested in the tuberculosis drug, isoniazid^[17], and molecules that can sustain long lived singlet states^[4f, 18].

The solvent composition was shown to have a substantial effect on cell survival rate in an *in vitro* assay. 100% ethanol-*d*₆ produced adverse effects on the cells over time periods of up to 6 h. We overcame this by dilution with D₂O and found that a ethanol-*d*₆:D₂O (30:70) showed no cell death over 24 h. Again solvent deuteration had no effect over their protio analogues.

We have shown that the iridium catalyst is the largest contributor in reducing the viability of cells in the SABRE mixture. Therefore, we have developed simple and robust method to remove it from solution by ion-exchange chromatography. To achieve this we add bathophenanthrolinedisulfonic acid disodium salt (BPS) and subsequently flush the solution through DEAE-Sephadex®. The eluent from this procedure showed minimal adverse effects on a number of cell lines for up to 6 hours exposure and retained 70% of the initial SABRE induced polarization. Furthermore, the recently reported biphasic approach to SABRE catalysis also leads to bolus biocompatibility after separation of the aqueous phase.

We are currently working towards developing an automated delivery method that includes all of the proposed deactivation, depletion or separation methods to exclude the catalyst that can be used in a clinical setting. Additionally, as the SABRE catalyst is the main source of toxicity, we are considering synthetic strategies to reduce toxicity through further changes to the catalyst.

Experimental Section

Chemicals and reagents. All chemicals were purchased from Sigma-Aldrich, Fisher or Alfa-Aesar. Deuterated solvents (ethanol-*d*₆, deuterium oxide (D₂O) and chloroform-*d* (CDCl₃)) were purchased from Sigma. The following compounds were prepared according to literature procedures: methyl 4,6-*d*₂ nicotinate^[19] and [IrCl(COD)(IMes)].^[20]

Cell culture. The human alveolar adenocarcinoma cells (A549), breast cancer cells (MCF7), cervical cancer cells (HeLa) were kindly provided by Prof. Christoph Borner, IMMZ, Freiburg, Germany. The human breast adenocarcinoma cell line MDA-MB-231 was a gift from Prof. Thomas Kaufmann, University of Bern, Switzerland. All the cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-Glutamine (2 mM) (all from Gibco, Life Technologies). The cells were maintained in a humidified atmosphere under standard conditions (37°C; 5% CO₂). The media were changed at regular intervals and upon reaching appropriate confluence (90%) the cells were passaged after brief exposure to Trypsin-EDTA (Invitrogen) solution.

Treatment of cells. Immediately after Trypsin-EDTA treatment viable cells were counted in a haemocytometer by Trypan blue (Sigma) exclusion. The required number of cells (normally 10⁴ per well in a 96-well tissue culture plate (Nunc)) was seeded 24 hours (h) before treatment so that they are in exponential growth phase at the start of the experiment. Before treatment various volumes of the bolus from substrate, catalyst or the SABRE reaction mixture were diluted to a maximum of 10 µl in the same solvent from which the compounds were originally prepared. For cell assays, throughout this study (unless otherwise indicated) we kept the final amount of the solvent to be 10% in the total volume of the cell growth medium (i.e. 10 µl in 100 µl).

MTT assay. The MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay is a commonly used colorimetric method to determine *in vitro* cytotoxicity of the given compound by means of a functional mitochondrial dehydrogenase activity in living cells^[12]. In this method, MTT - tetrazolium is reduced to insoluble formazan crystals and it is directly proportional to the number living cells. Therefore, this assay represents a measure for cell viability as well.

For this assay, cells (normally 10⁴ cells per well in a 96-well plate) were taken in triplicates and treated with a range of compounds (see above) or kept as cell viability control with no compound treatment (untreated) in cell growth media. After incubation at desired time points the cell growth media was replaced with fresh media (100 µl) and

incubated with 10 μ l of MTT (5 mg/ml, dissolved in cell growth media and filter (0.22 μ m) sterilized) for 4 h in a humidified atmosphere at 37°C. The medium with MTT was then carefully aspirated and the formed formazan crystals were solubilized in 100 μ l of dimethyl sulfoxide (DMSO). The absorbance of the coloured (purple) solution was then measured at 570 nm using a microplate reader (MultiskanGO, Thermofisher). The absorbance values (averaged out of triplicates) were blanked against DMSO and the absorbance of cells exposed to cell growth medium only (i.e. untreated) was taken as 100 % viable (i.e. control). The cell viability of the compound treated samples were then calculated by normalising to the untreated control sample and are normally expressed as % of control. Each assay was repeated for a minimum of three times for statistical analysis of the data.

Evaluation of IC₅₀. For the evaluation of IC₅₀, compounds were dissolved in cell growth media and cells were treated with various concentrations of the compound (ranging from a maximum of 80 mM to a minimum of 1.25 mM) in 1/2 serial dilutions. Cell viability was analysed by MTT assay after 48 h of treatment. IC₅₀ values of **MN** and **d₂-MN** were calculated by using Graphpad Prism software.

Catalyst deactivation and removal. A solution of [IrCl(COD)(IMes)] (5 mM) and **d₂-MN** (20 mM, 4.0 eq.) in ethanol-d₆:D₂O (30:70, total volume 3.0 mL) was degassed prior to the introduction of hydrogen at a pressure of 3 bar. After 5 minutes, the sample was opened to air and a solution of bathophenanthroline disulfonic acid disodium salt hydrate (BPS) (10 mM, 2.0 eq.) in water (1.0 mL) was added. The resulting suspension was eluted through DEAE-Sephadex® (2.5 g) with water (11 mL). For treatment on cells the eluent was vacuum dried and reconstituted in 30% ethanol-d₆ in D₂O. Prior to treatment various volumes of the bolus were further diluted as indicated above.

Biphasic SABRE - Biphasic separation. A solution of [IrCl(COD)(IMes)] (5 mM) and **d₂-MN** (20 mM, 4.0 eq.) in chloroform-d (1.5 mL) and D₂O (containing 0.9% NaCl) (1.5 mL) was mixed together and was degassed prior to the introduction of hydrogen at a pressure of 3 bar. After phase separation the aqueous layer was removed and prior to treatment various volumes of the bolus were further diluted as indicated above.

Statistical analysis. All experiments were performed at least three times (n=3) and all data is presented as mean and standard deviation (SD) of the mean. The significance of the differences between treated samples and the untreated control was assessed by one-way analysis of variance (ANOVA) using GraphPad Prism Software. Statistical significance was set at P values <0.05.

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Conflict of interest

The authors declare no conflict of interest.

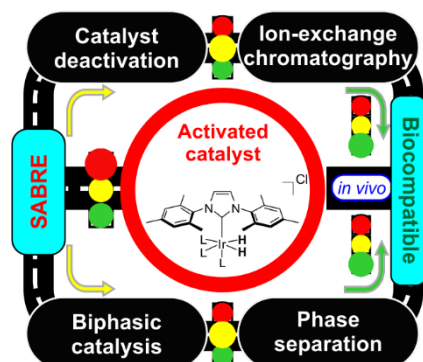
Keywords: SABRE • hyperpolarization • biocompatibility • biphasic catalysis • cytotoxicity

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It is possible to head towards the destination, *in vivo* imaging under SABRE with biocompatibility, via two routes. One requires quenching of catalyst and separation via an ion-exchange column. The second route, the biphasic catalysis, employs the aqueous phase and no catalyst is carried forward. Upon exiting a biocompatible medium is achieved.