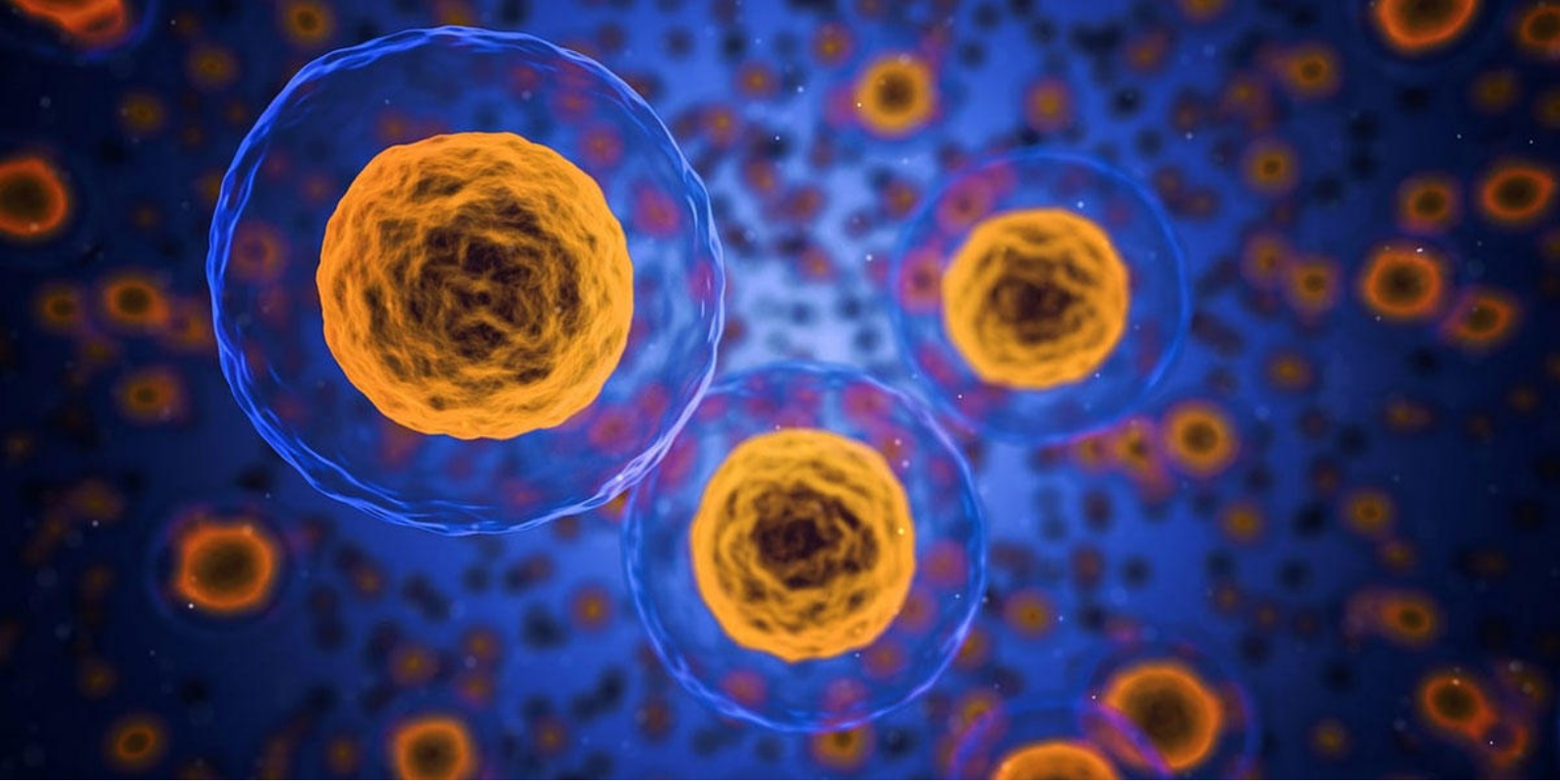


Application Note AN-FLU-002

Understanding the mechanism of a bioassay indicator by fluorescence

Analysis of Alamar Blue during its electrochemical processes with fluorescence spectroelectrochemistry

Alamar Blue, also known as resazurin, is widely used as a redox indicator in cell viability and cytotoxicity assays due to its optical properties. Alamar Blue is a weakly fluorescent blue dye. Its reduced form (resorufin) is a highly fluorescent pink dye.

Fluorescence spectroelectrochemistry offers researchers a powerful method to follow and control redox reactions. This technique allows the ability to study different processes, such as electron transfer or the presence of intermediate products generated during a chemical reaction.

This Application Note describes how the characteristic fluorescence of Alamar Blue is monitored during electrochemical processes in order to obtain the complete knowledge of this system.

INSTRUMENTATION AND SOFTWARE

Spectroelectrochemical monitoring of resazurin was performed with SPELEC, a fully integrated instrument for spectroelectrochemistry that combines both the electrochemical (bipotentionstat/galvanostat) and spectroscopic components (UV-Vis light source and detector) in a portable system. Even though SPELEC contains a light source with a wavelength range of 200 nm to 900 nm, a 395 nm LED (LEDVIS395) was used as the excitation light source. This LED has a well-defined range of light emission, avoiding any contribution of light in wavelengths where Alamar Blue fluoresces.

The rest of the setup consisted of a reflection probe (RPROBE-VIS-UV), a spectroelectrochemical reflection cell for Thin-Layer Flow-Cell Screen-printed electrodes (TLFCL-REFLECELL) and the components included in the fluorescence kit (FLKIT). The FLKIT contains two short optical fibers (600 µm), two optical filters (one of which filters wavelengths between 230–500 nm, and the other filters wavelengths between 300–750 nm), and two holders for the filters. The total setup is shown in Figure 1.

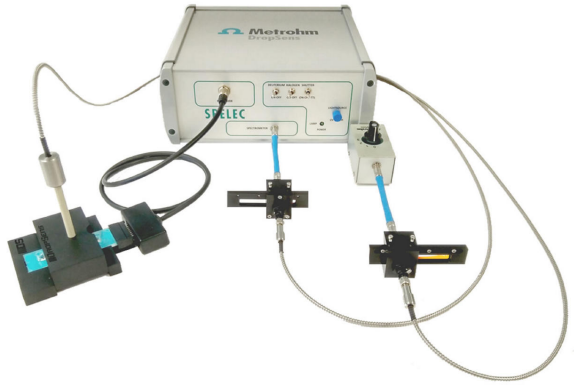


Figure 1. Fluorescence spectroelectrochemistry setup used for the study of Alamar Blue.

The Thin-Layer Flow-Cell Integrated Screen-Printed Circular Carbon Electrode (TLFCL110-CIR) consists of a flat ceramic card containing the electrochemical cell with three-electrode system (4 mm diameter circular carbon working electrode, a carbon counter electrode, and a silver pseudo-reference electrode, Figure 2). This electrodic platform is covered by a slide with a channel that limits the thickness of the solution to 400 µm.

SPELEC was controlled with DropView SPELEC, a dedicated software that provides spectroelectrochemical information and includes tools to perform adequate treatment and analysis of the collected data. All hardware and software used for this study is compiled in Table 1.

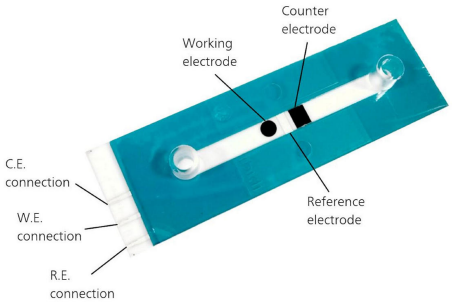


Figure 2. Thin-Layer Flow-Cell Integrated Screen-Printed Circular Carbon Electrode (TLFCL110-CIR) with the described three electrode system.

Table 1. Hardware and software equipment overview.

Equipment	Article number
Instrument	SPELEC
Reflection probe	RPROBE-VIS-UV
Excitation light	LEDVIS395
Fluorescence kit	FLKIT
Spectroelectrochemical Reflection Flow-Cell for Thin-Layer Flow-Cell SPE	TLCFL-REFLECELL
Cable Thin-Layer Flow-Cell SPE	CAST-TLFCL
Thin-Layer Flow-Cell SPE	TLFCL110-CIR
Software	DropView SPELEC

FLUORESCENCE SPECTROELECTROCHEMISTRY OF ALAMAR BLUE

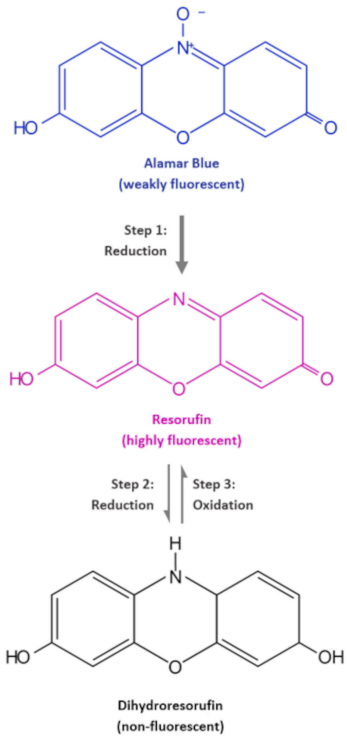


Figure 3 Redox mechanism scheme of Alamar Blue to dihydroresorufin.

Alamar Blue is a weakly fluorescent molecule that can be irreversibly reduced to resorufin, a highly fluorescent pink dye. However, when resorufin is exposed to stronger reduction conditions, it is reversibly converted to dihydroresorufin, a colorless species that does not fluoresce. The oxidation-reduction reactions are shown in Figure 3.

Electrochemical reduction of 0.001 mol/L Alamar Blue was performed in an aqueous solution of 0.1 mol/L KCl. Fluorescence emission was recorded simultaneously along with the electrochemical signal, collecting information about the electrochemical processes that took place on the electrode surface during the whole experiment.

In order to obtain as much information as possible about this system, a multi-pulse chronoamperometry experiment was performed where three different amperometric pulses were applied. In the first step, a potential of -0.45 V was applied for 300 s to reduce Alamar Blue to resorufin. During the second step, a potential of -1.00 V was applied for 300 s to reduce resorufin to dihydroresorufin. Finally, a potential of -0.10 V was applied for 300 s to oxidize the dihydroresorufin generated in the previous step back to resorufin.

CONTROLLED REDUCTION OF ALAMAR BLUE

In the first step of the multi-pulse chronoamperometry experiment described in the previous section, the irreversible reduction of Alamar Blue was carried out by applying -0.45 V for 300 s. The fluorescence emission was recorded together with the electrochemical signal, acquiring spectra every 0.75 s. Figure 4a shows the electrochemical signal (grey line), and the fluorescence spectra recorded during the measurement are shown in Figure 4b. An emission band centered at 590 nm is clearly differentiated here. According to the spectroscopic properties of resorufin, this emission band is characteristic for this molecule.

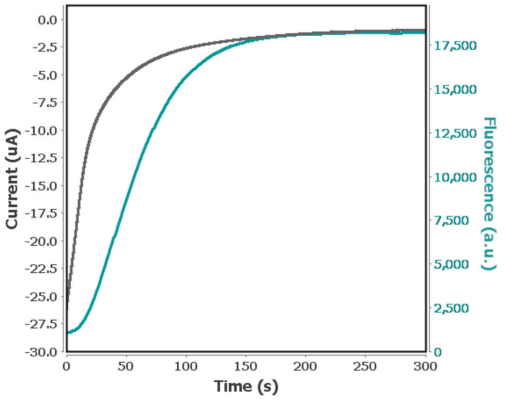


Figure 4a First step potential of a multi-pulse chronoamperometry experiment applying -0.45 V for 300 s in a solution of 0.001 mol/L Alamar Blue in 0.1 mol/L KCl (grey line) and evolution of the fluorescence spectra at 590 nm over time (green line).

Figure 4a also shows the evolution of the fluorescence band centered at 590 nm with time (green line). Operando measurements allow users to obtain spectroscopic information during the entire measurement. The fluorescence signal increases from the start of the experiment and then remains constant from 200 s until the end of the experiment due to the complete reduction of Alamar Blue to resorufin. A potential of -0.45 V is enough to reduce Alamar Blue to resorufin, but not enough to reduce the resorufin to dihydroresorufin.

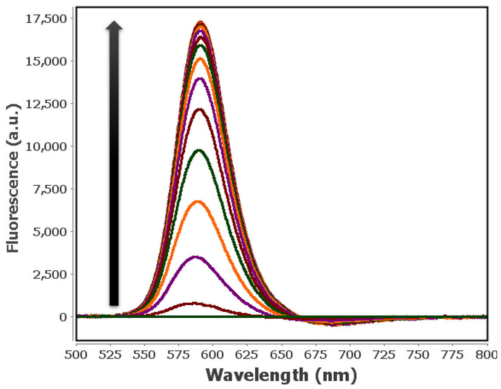


Figure 4b Fluorescence spectra recorded during the first potential step of the three-part experiment.

REDUCTION OF RESORUFIN TO DIHYDRORESORUFIN

In order to reduce the resorufin (generated in the previous step) to dihydroresorufin, the next potential pulse was performed by applying -1.00 V for 300 s. Figure 5a displays the electrochemical signal (grey line) as well as the evolution of the fluorescence band at 590 nm with time (green line). Figure 5b shows the fluorescence spectra recorded during this second step.

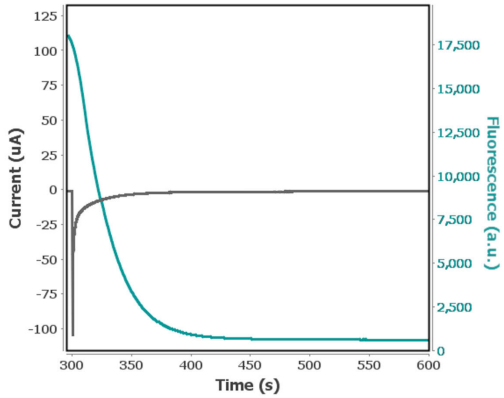


Figure 5a Second step potential of a multi-pulse chronoamperometry experiment applying -1.00 V for 300 s in a solution of 0.001 mol/L Alamar Blue in 0.1 mol/L KCl (grey line) and evolution of the fluorescence spectra at 590 nm over time (green line)

Although the intensity of the emission band at 590 nm was completely stable at the end of the first step (Figure 4a, green line), the second step demonstrates that this emission band decreases abruptly for 100 s when a potential of -1.00 V is applied. There was no fluorescence detected during the rest of the experiment, demonstrating the successful reduction of resorufin (highly fluorescent) to dihydroresorufin, a non-fluorescent molecule (Figure 3).

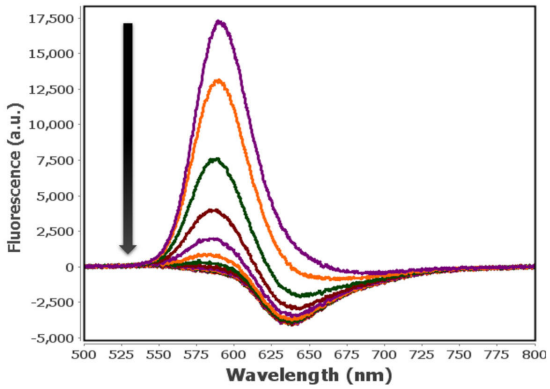


Figure 5b Fluorescence spectra recorded during the second potential step of the three-part experiment.

REOXIDATION OF DIHYDRORESORUFIN TO RESORUFIN

Finally, a third potential step was applied with the goal of reoxidizing the dihydroresorufin generated in the second step back to resorufin. Figure 6a shows the electrochemical signal (grey line) and the spectra evolution of the fluorescence band centered at 590 nm (green line) when -0.10 V was applied for 300 s. This graphic shows that the optical signal reaches the fluorescence values obtained in the first pulse of the chronoamperometry experiment, shown in Figure 4a.

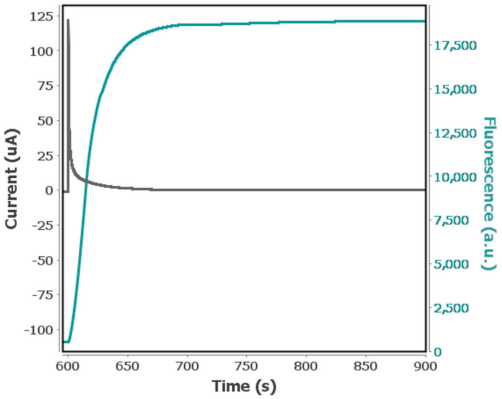


Figure 6a Third step potential of a multi-pulse chronoamperometry experiment applying -0.10 V for 300 s in a solution of 0.001 mol/L Alamar Blue in 0.1 mol/L KCl (grey line) and evolution of the fluorescence spectra at 590 nm over time (green line).

The spectra evolution displayed in Figure 6b confirms the increase of the fluorescence band centered at 590 nm. Dihydroresorufin is completely oxidized back to resorufin because the fluorescence maximum observed in the first step of the experiment (Figure 4b) is also obtained here in the third step. Therefore, the reversibility of the redox process between resorufin and dihydroresorufin is clearly demonstrated.

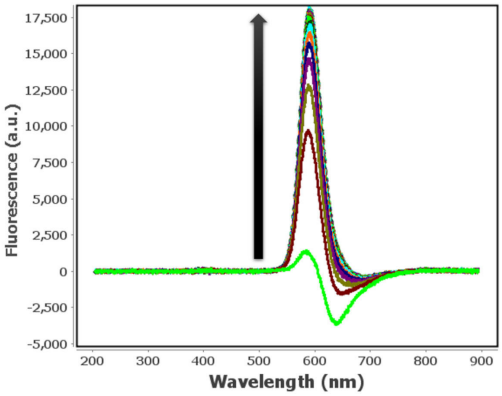


Figure 6b Fluorescence spectra recorded during the third potential step of the three-part experiment.

CONCLUSION

Spectroelectrochemistry is a powerful technique that provides information of a different nature by combining the advantages of electrochemistry and spectroscopy. In this study, the fluorescence signal associated with Alamar Blue has been analyzed with varying potential. The reduction of this molecule at a potential of -0.45 V to generate resorufin produces an enhanced fluorescence signal. Further reduction of resorufin to dihydroresorufin takes place at -1.00 V, decreasing the optical signal due to the properties of the reduced species. Finally, the reoxidation of dihydroresorufin back to resorufin displays an increase in the fluorescence signal and shows the excellent reversibility of this electrochemical process. Understanding this mechanism opens up new possibilities for the development of new applications for Alamar Blue, such as its use as a cell viability indicator.

FURTHER READING

1. Ibánez, D.; Izquierdo-Bote, D.; Pérez-Junquera, A.; et al. Raman and fluorescence spectroelectrochemical monitoring of resazurin-resorufin fluorogenic system, *Dyes and Pigments*. 2020, *172*, 107848. https://doi.org/10.1016/j.dyepig.2019.107848
2. O'Brien, J.; Wilson, I.; Orton, T.; et al. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, *Eur. J. Biochem.* 2000, *267*, 5421. https://doi.org/10.1046/j.1432-1327.2000.01606.x
3. Twigg, R.S.; Oxidation-Reduction Aspects of Resazurin, *Nature*. 1945, *155*, 401. https://doi.org/10.1038/155401a0

RELATED APPLICATION NOTE

AN-FLU-001 Fluorescence spectroelectrochemistry of $[Ru(bpy)_3]^{2+/3+}$ in semi-infinite diffusion regime

CONTACT

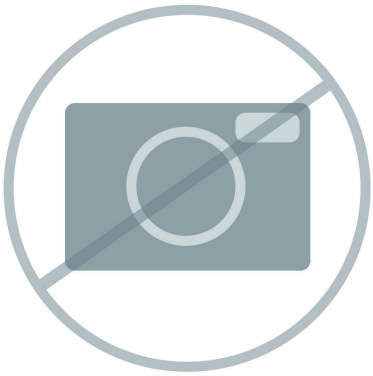
Metrohm SG
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Technocentre, #06-08
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CONFIGURATION



SPELEC UV-VIS Instrument (200-900 nm)

SPELEC is an instrument for performing spectroelectrochemical measurements. It combines in only one box a Lightsource, a Bipotentiostat/Galvanostat and a Spectrometer (UV-VIS wavelength range: 200-900 nm) and includes a dedicated spectroelectrochemical software that allows optical and electrochemical experiments synchronization.



Reflection probe VIS-UV

Reflection probe VIS-UV designed to perform reflection experiments suitable to work with our Reflection Cell for our Screen-printed electrodes or with any conventional cell.



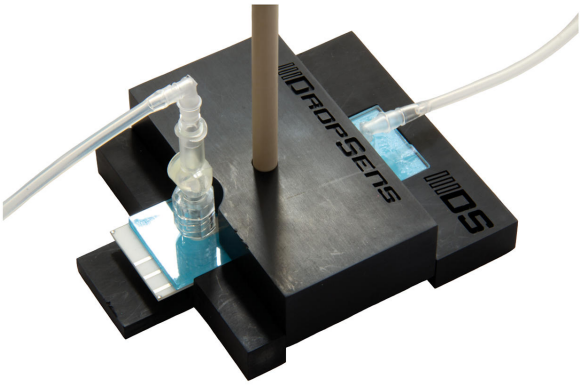
LED light-VIS 395 nm

LED light of 395 nm wavelength



Fluorescence kit

Kit for performing fluorescence experiments composed by 2 x short optical fibers (600 µm) ended in SMA 905 connections, 2 x optical filters one of 230-500 nm and other for 300-750 nm wavelength and 2 x
holders for the filters.
Complete your set up by adding the required excitation light depending on the range of interest (ref. LEDUV275, LEDVIS395 or LEDRGB).
For performing fluorescence spectroscopy experiments coupled to spectroelectrochemistry (ref. SPELEC) with SPEs acquire the suitable reflection probe (RPROBE) and reflection cell (REFLECELL).



Spectroelectrochemical Reflection Cell for Thin-Layer Flow-Cell Screen-Printed Electrodes

Cell for performing spectroelectrochemical measurements in flow conditions in combination with the TLFCL-CIR format Screen-Printed Electrodes, the suitable fittings and ref. RPROBE-VIS-UV.



Stat cable for TLFCL format SPEs

Cable connector from µStat instruments and the SPELEC line of instruments to TLFCL format SPEs



Thin-Layer Flow-Cell Integrated Screen-Printed Circular Carbon Electrode

Thin-Layer Flow-Cell Integrated Screen-Printed Circular Carbon Electrode (Aux.:C; Ref.:Ag)