Electrochemical quantification of biomarker myeloperoxidase

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Abstract

Point of care testing (PoCT) devices permit precise and rapid detection of disease-related biomarkers contributing to an early disease diagnosis and administration of an appropriate treatment. The enzyme myeloperoxidase (MPO) is a relevant biomarker for infection and inflammation events assessment, however its direct electrochemical quantification is hindered by the limited accessibility to the iron atom in its active center. Herein, such hindrance of the MPO biomolecule is overcome using the redox mediator 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The charge involved in the electrochemical reduction of the MPO-oxidized ABTS is correlated with the concentration of MPO. The use of ABTS allowed for the electrochemical assessment of a wide range of MPO concentrations (10-1000 nM) including those reported for wound infections, chronic obstructive pulmonary disease and early adverse cardiac events. The developed electroanalytical approach is rapid and inexpensive, and thus suitable for implementation in PoCT devices.

1. Introduction

Early diagnosis of infections or internal injuries are key for an accurate treatment.[1] However, conventional diagnostic protocols are time consuming and require trained personnel to use complex instruments. The development of analytical methods for disease-related biomarkers implementable into point of care testing (PoCT) devices will contribute to cost-effective, precise and rapid diagnosis and appropriate treatment. Moreover, it will facilitate the implementation of affordable screening programs among the population with a high risk for developing these diseases.

Myeloperoxidase (MPO) is a heme peroxidase enzyme released by activated neutrophils that catalyzes the oxidation of chloride ions to hypochlorous acid (HClO) using hydrogen peroxide as co-substrate.[2] MPO have been detected in wounds (200 to 1100 nM)[3] with bacterial infection, where HClO acts as a strong bactericidal agent, being specially abundant in non-healing chronic wounds with persistent bacterial colonization.[4,5] MPO has also been linked to other diseases related to inflammatory processes. Chronic obstructive pulmonary disease (COPD), characterized by chronic lung inflammation leading to progressive and irreversible airflow obstruction with periodic acute episodes of worsening exacerbations, can be early diagnosed by the presence in sputum of MPO (115-772 nM), interleukin-8 and leukotriene B4.[6] MPO is also overproduced in case of eroded arterial lesions due to neutrophil infiltration and activation into the inflamed and fissured blood vessels[7] thus, high levels of MPO in plasma (20-40 nM) are indicative of adverse cardiac events in patients with chest pain.[8] Consequently, MPO has been established as a relevant biomarker for assessment of infection, COPD and early adverse cardiac events. The rapid detection of MPO in body fluids will result in a drastic reduction of the morbidity and mortality caused by the abovementioned diseases and the related healthcare and economic burden.[9]

An ideal PoCT should be low-cost and easily operated such as an electrochemical readout system.[10] Electrochemical detection approaches are an attractive technology for microfluidic devices due to their inherent small size, low cost, high sensitivity, and portability for on-site analysis. Electrochemistry is a versatile and fast technique that has been widely used for a variety of applications such as permeability of membranes,[11,12] presence of metals in nanoparticles[13,14] and synthesis of metal nanoparticles.[15,16] In particular, electrochemistry has been successfully used for biomolecules, to assess their position in membranes,[17,18] quantify their presence in body fluids[19,20] and determine enzyme redox potentials.[21,22] PoCT devices based on electrochemistry of biomolecules have been presented for several purposes such as a non-invasive glucose level detector,[23] protein[24] and uric acid[25] detection. PoCT devices have also been developed for the MPO detection, but the tedious sample and electrode preparation, and the use of complex protocols requiring incubation with magnetic beads[26] and antibodies[27] limit their use.

The direct electrochemistry of MPO in solution is hindered due to the difficult access of the electrons from and to the heme group iron atom placed at the bottom of a deep crevice of the MPO structure.[28] Therefore, we propose the use of a redox mediator to facilitate the electron transfer, as has been previously reported for polymerization processes using other enzymes such horseradish peroxidases[29] or lignin oxidases (laccases).[30] In this context, we selected the mediator 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) for the electrochemical detection of MPO. Our approach consists in the electrochemical reduction of the MPO-oxidized ABTS generating a measurable current (Fig 1) correlated to the MPO concentration in the sample. The current work establishes an electroanalytical approach based on the use of ABTS to quantify the presence of MPO

in liquid samples, which due to its simplicity, low cost and minimal set up requirements could be easily implemented in PoCT devices.

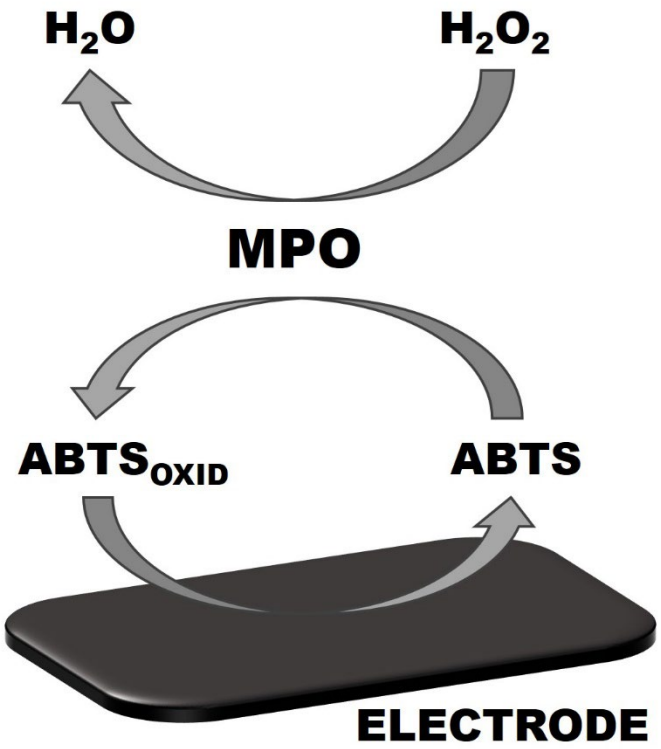

Figure 1. Scheme of the electrochemical quantification of MPO using redox mediator ABTS

2. Materials and Methods

2.1. Materials

Human myeloperoxidase (MPO) > 98 % purity was purchased from Planta Natural Products (ref: 700-03-001) as lyophilized powder, ABTS (98 %) and hydrogen peroxide were purchased from Sigma-Aldrich, and phosphate buffer saline (PBS) tablets were purchased from Fischer scientific. Disposable screen-printed gold electrodes (ref: DRP-C220AT) were purchased from Metrohm DropSens having working and auxiliary gold electrodes and a silver reference electrode and water was ultrapure MilliQ® (18.2 MΩ·cm).

2.2. Electrochemical cell

The screen-printed electrodes were rinsed successively with ethanol and water prior drying with N_2 and placing in a home-made electrochemical cell of 200 µL that uses an o-ring to avoid liquid leakage. The electrochemical experiments were performed using a µAutolab Potentiostat (Ecochemie, NL) in a conventional three-electrode configuration.

2.3 Electrochemical experiments

Fresh ABTS solution was prepared dissolving the appropriate ABTS powder in PBS solution (pH 7.4) in milliQ grade water. Electrochemically oxidized (EC) ABTS stock solution was obtained by applying a constant potential (0.7 V) to fresh ABTS solution in the electrochemical cell until no color and current changes were registered. Fresh and EC-oxidized ABTS solutions at the desired concentrations were obtained by the appropriate dilution in PBS of the corresponding stock solutions. Lyophilized MPO was reconstituted in PBS and the appropriate amount of MPO stock was added to a PBS solution containing fresh ABTS (1 mM) and hydrogen peroxide (1 mM) to initiate an oxidation reaction incubated at room temperature (23 ± 1 ºC) for 5 min to ensure the oxidation of ABTS by

117 the MPO. Cyclic voltammograms (CV) were performed at 10 $mV \cdot s^{-1}$ and room
118 temperature, scanning towards negative potentials. Chronoamperometric experiments
119 were performed at 0.2 V and room temperature during 5 min.
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Results and Discussion

ABTS has been used as a substrate for MPO quantification in spectrophotometric techniques,[31] however, its suitability as a redox mediator for electrochemical quantification of MPO has not been demonstrated yet. The CV of ABTS (Fig 2) revealed a reversible redox behavior observing both the oxidation and reduction peaks without evidences of undesired reactions on the electrode surface. Therefore, the observed reversibility indicated the suitability of this molecule to be electrochemically detected after its oxidation by MPO.

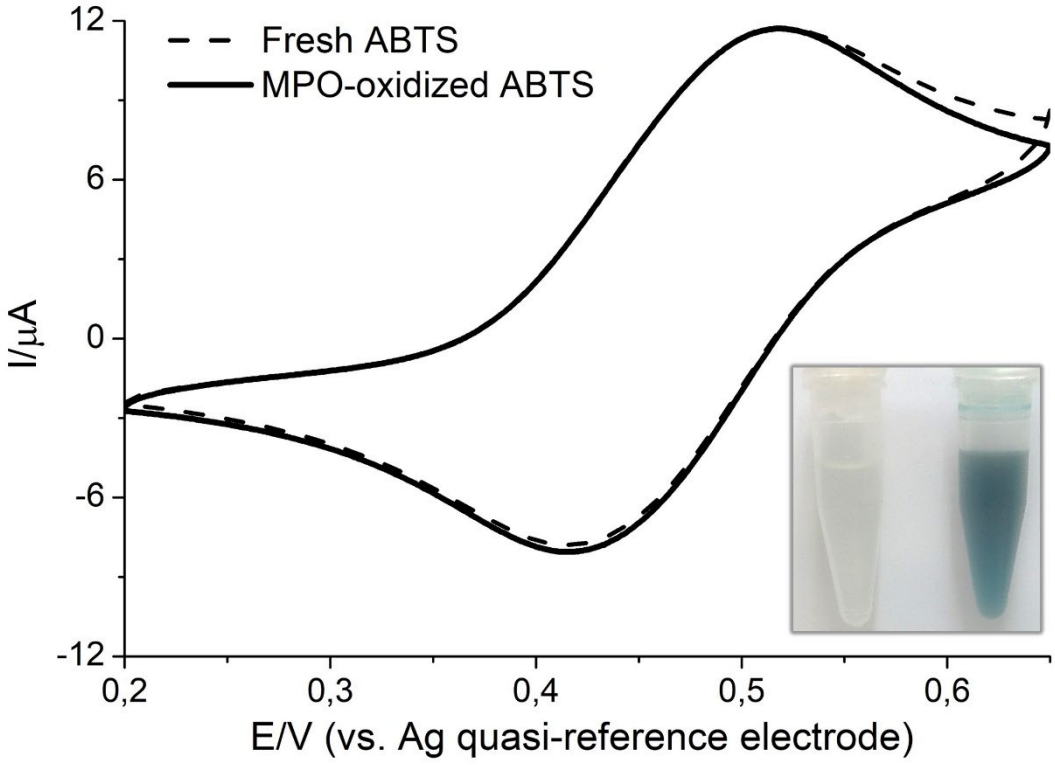
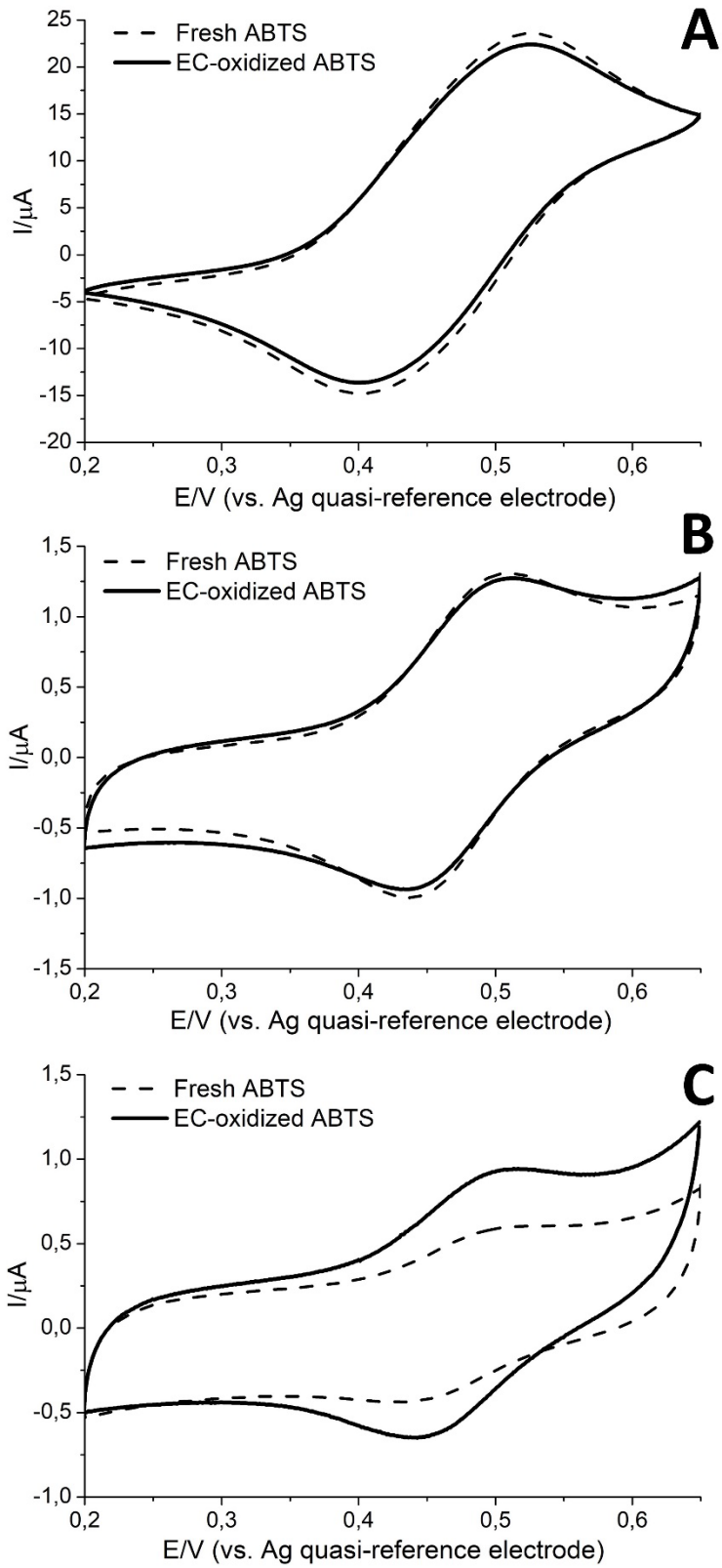


Fig 2. Cyclic voltammograms of fresh ABTS and MPO-oxidized ABTS (MPO: 250nM, H_2O_2: 1mM, ABTS: 1mM). Inset: Fresh ABTS (left) and MPO-oxidized ABTS (right) solutions.

Despite MPO oxidizes ABTS, achieving the characteristic color change from transparent to intensive green[32] (Fig. 2), the differences in the cyclic voltammograms were not conclusive. The obtained electrochemical reduction scan produced a reduction current of equal magnitude than the control without enzyme. The presence of the reduction current peak may be related to the oxidation of ABTS during the electrochemical scan that starts in an oxidative potential. This process would occur at the surface of the electrode independently of the solution context, thus, obtaining similar electrochemical response for both fresh ABTS (control) and the sample with MPO-oxidized ABTS. To further confirm this phenomenon, dilutions from EC-oxidized ABTS stock and from fresh ABTS stock solutions were prepared and analyzed using cyclic voltammetry (Fig. 3). Similar CVs were obtained for both fresh ABTS and EC-oxidized ABTS at high concentrations (Fig. 3 A and B), while at low concentration, the EC-oxidized ABTS presented higher intensity current peaks than the fresh ABTS (Fig. 3C). This observation confirms that ABTS is electrochemically oxidized at high potentials during the reduction scan. The reduction current observed for ABTS has two contributions - from the initial EC-oxidized ABTS dilution and from the *in situ* ABTS oxidized during the reduction scan. Upon dilution of the ABTS samples, the contribution of the *in situ* ABTS oxidized is minor than the contribution from the initial EC-oxidized ABTS. A similar behavior is expected in case of using MPO to oxidize ABTS instead of EC. Therefore, cyclic voltammetry can only be applied to a low and narrow range of MPO concentrations and is not suitable for a PoCT electrochemical device.



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160 **Fig 3**: Cyclic voltammograms of EC-oxidized and fresh ABTS solutions at several ABTS

161 concentrations: A) 500 µM, B) 125 µM and C) 25 µM

Alternatively, a chronoamperometric electrochemical method was employed, applying a constant reduction potential of 0.2V, according to the performed CVs (Fig. 3). This potential is out of the ABTS oxidation zone, thus decreasing the presence of EC-oxidized ABTS and its consequent contribution to the overall ABTS electrochemical reduction current. To assess the suitability of this method, the aforementioned reduction potential was applied to different dilutions of EC-oxidized ABTS and freshly prepared ABTS (Fig. 4). The registered reduction currents analysis for both the fresh ABTS (Fig. 4A) and EC-oxidized ABTS (Fig. 4B) revealed a linear relationship according to the concentration of each redox specie (Fig. 4C). The large current difference between EC-oxidized ABTS and fresh ABTS coupled to the linear relationship indicate the suitability of the chronoamperometric method with the redox mediator ABTS for the electrochemical quantification of MPO.

To validate this method, freshly prepared ABTS was oxidized by MPO in concentrations found in exudates of infected wounds,[3] in blood from patients with coronary plaque erosion[8] and in sputum from patients with COPD.[6,33] The oxidation of fresh ABTS by different MPO concentrations resulted in green colored solutions electrochemically reduced at 0.2V (Fig 5A). Upon increasing the applied MPO concentration, a higher reduction charge was required to reduce the MPO-oxidized ABTS establishing a clear parabolic relationship (Fig. 5B). Therefore, the chronoamperometry of the redox mediator ABTS has been demonstrated as a promising electrochemical method for quantitative assessment of clinically relevant MPO concentration.

The simplicity and minimal electrode setup requirements of the developed approach pave the way for its implementation into PoCT devices for MPO detection. In these forthcoming sensors, the presented MPO electrochemical quantification can be combined with sample preparation steps according to the nature of the sample (blood, sputum,

187 wound fluid) to avoid the risk of interfering substances. For instance, immunocapture
188 with magnetic beads can separate MPO from other substances and concentrate it at the
189 electrode surface[34] or paper-based microfluidics for sample fractionation.[35]

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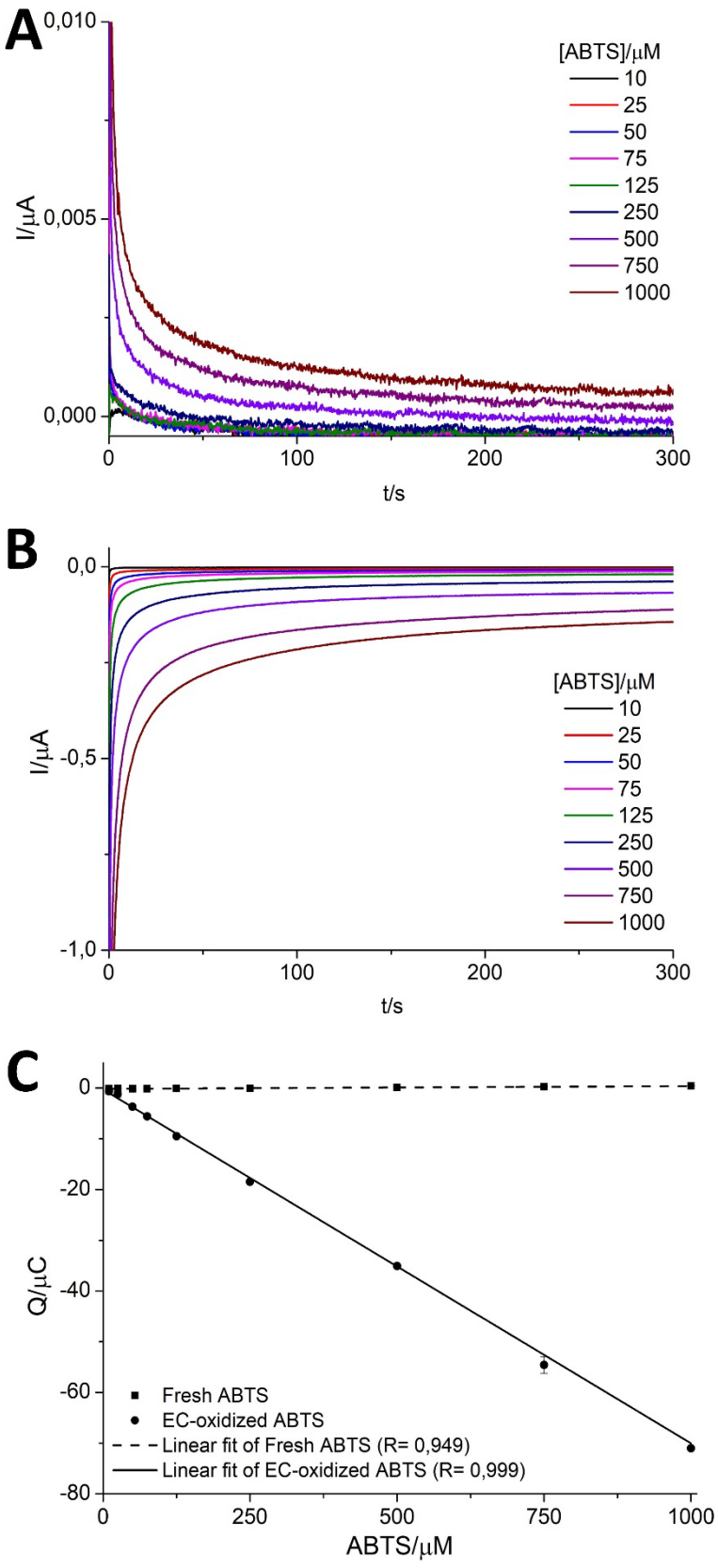

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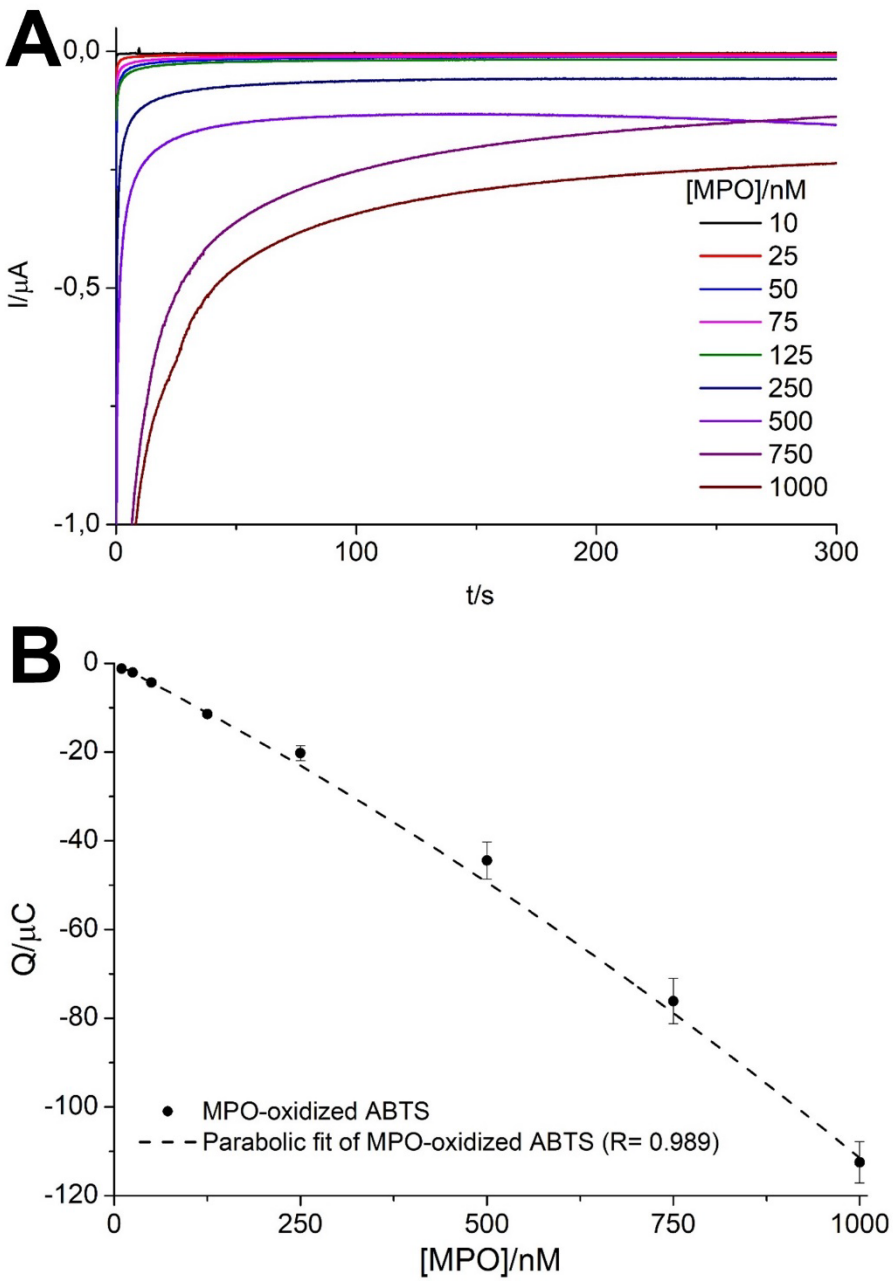
192 **Fig 4**: Chronoamperometry of A) fresh ABTS solutions, B) EC-oxidized ABTS and C)

193 reduction charge *vs.* ABTS concentration.



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195 **Fig 5**: A) Chronoamperometry of ABTS oxidized (1 mM) by different MPO
196 concentrations in the presence of H_2O_2 (1mM) and B) reduction charge *vs*. MPO
197 concentration.

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4. Conclusions

The electrochemical reduction of the MPO-oxidized ABTS has been demonstrated as an analytical method to quantify the presence of MPO. ABTS has shown its capability to be used as a redox mediator for MPO, overcoming the access limitations to the MPO iron atom. Cyclic voltammetry was discarded as an electrochemical analytical method due to the high contribution of undesired processes to the peak intensity. Contrarily, the chronoamperometry technique at 0.2 V permitted the rapid (< 10 min) quantification of disease relevant MPO levels (10-1000 nM). Therefore, the developed simple and rapid electrochemical method represents a suitable analytical technique that could be implemented in PoCT devices for assessment of bacterial infection, COPD or adverse cardiac events.

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Notes

The authors declare no conflicts of interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Abbreviations

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Chronic obstructive pulmonary disease (COPD), cyclic voltammogram (CV), electrochemically (EC), Myeloperoxidase (MPO), phosphate buffer saline (PBS), Point of care testing (PoCT)

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