This scientific report is presented in correlation with the milestones M4 and M6 and with the activities T9, T10 and T13 from the workplan according to the Additional act no 3/2015.

(M4) Quantifying the reaction between ONOO'and Oxymyoglobin in soluble fraction of muscle

Decoloration, rancidity and modification of muscle aliments flavour (fresh or even those treated with nitrite) are the consequences of redox activities that take place because the oxidative species are „scavenged” by different forms of myoglobin (the principle hemoprotein found in cardiac skeletal muscles) [1]. Peroxynitrite, formed in biological tissues by the rapid reaction between nitric oxide (NO) and superoxide (O$_2^-$), is a powerful oxidant, that promotes lipid peroxidation, DNA’s and produces cellular injuries in biological tissues. Some decomposition reactions of nitric oxide (NO) and peroxynitrite (ONOOH/ONOO'), promoted especially by deoxymyoglobin (MbFe$^{2+}$) and oxymyoglobin (MbFe$^{3+}$O$_2$ or redMb), with the formation of other myoglobin species (eg. Methmyoglobin/MbFe$^{3+}$OH$_2$/merMb and/or ferrylmyoglobin/ferrylMb), are presented in Scheme 1 adapted from ref[2].

(T9) The reaction of Oxymyoglobin with ONOO- in buffered solutions and monitoring ONOO- with μsensors

For oxymyoglobin synthesis, myoglobin was treated with sodium borohydride (NaBH$_4$). The reaction and the formation of oxymyoglobin was monitored by UV-Vis spectroscopy (Figure 1). In order to diminish the detection time and to create a simplified procedure (this tasks are necessary due to peroxynitrite instability in buffered solution and even more in soluble meat fractions), a single line flow injection analysis system (FIA) was created and applied for the analysis described in this report. Moreover, the PSSNC sensors (peroxynitrite sensitive selective catalytic films, consisting in a cobalt phthalocyanine film deposed over the surface of a screen printed carbon electrode), were optimized for the reduction of this molecule, for the electrochemical detection in buffered solutions and in meat extract fractions.
Initially, the metMb, was used to follow the reaction with peroxynitrite. The fingerprint of metMb in chronoamperometry (the presence of the double peak) can be very useful to identify furthermore this compound and to distinguish it from redMb or ferrylMb. Using a ratio of metMb/PON of 1/5 (suitable for the peroxynitrite to oxidize metMb), one can observe in chronoamperogram from Figure 2, that incubating 400 µM PON with 80 µM metMb, the measured current correspondes to the quantification limit of PON with the electrode used (LOQ=12.5 µM).

Figure 2. The amperometric response of the SPCE/CoPc (E=0.1 V) for 80 µM metMb, 80 µM metMb incubated with 400 µM PON and 30, respectively, 20 µM PON.

The measured currents for metMb (using different concentrations), are at the limit ratio of 3:1 (for signal/noise ratio). Different concentrations of PON were incubated with metMb 20 µM. Figure 3A presents the amperometric response at different interval times of incubation and a decrease of signal can be observed, after 3 minutes of incubation the current will remain stable due to the total (like in the case of incubation with 200 µM PON) or the partial (like in the case of incubation with 100 µM PON) consumption of PON (Figure 3B).

Figure 3. The influence of time over the incubation of 20 µM metMb with 200 µM PON (A) and the equivalent currents registered for different time periods of incubation of 20 µM metMb with 200, respectively 100 µM PON, in PON concentrations (B).

The UV-Vis technique was also used to monitorize the reaction of redMb with PON. After the reduction of metMb, the concentrations of the redMb were evaluated using the absorbance at 417, 542, and/or 580 nm [ε417=128 mM⁻¹ cm⁻¹, ε542=13.9 mM⁻¹ cm⁻¹ and ε580=14.4 mM⁻¹ cm⁻¹] like in ref. [4]. During incubation of 170 µM peroxynitrit with 107 µM metMb, at pH 12, metMb could not
observed (maximum absorbance at 502 nm, $\varepsilon_{502}=10.2$ mM$^{-1}$cm$^{-1}$[5]). On the other hand, at pH 9 (PBS), we can clearly observe how redMb (identified at 542 and 580 nm), is transformed, during 3 minutes, into metMb, and using the extinction molar coefficients for 502 nm, we confirmed that the stoichiometry redMb / metMb is 1:1, because 10 µM redMb are oxidized with 10 µM metMb. This results are in accordance with the electrochemical methods.

(T10) Monitoring the concentration of Myoglobin and Metmyoglobin during the reaction described above

The meat extract is was obtained from manz meat (veal under the age of 2) in a similar way described in reference[6]. This extracts were used fresh for UV-Vis and electrochemical measurements. Initially dilutions of the extract (PBS pH 9) were analysed with the electrochemical method described above. For different injected dilutions the current were measured. Quantification of the concentration of redMb was realised with measurements at 580 nm ($\varepsilon_{580}=14.4$ mM$^{-1}$cm$^{-1}$). The concentration of the stock meat extract was determined to be 480 µM, which corresponds to cca. 20 mg of myoglobin for 1 g of meat, similar to the data described in literature. Moreover, if we use the absorption at 525 nm (representing the isobestic point for the absorption in visible range for the 3 forms of myoglobin) and a molar extinction coefficient of 7.6 mM$^{-1}$cm$^{-1}$, we obtain a value of 485 µM of myoglobin for this stock solution. A more complex method for determining the content of myoglobin was described by Krzywicki, and improved by Tang, in 2004 [7]. The decrease of PON concentration cannot be monitored in UV due to the overlap of absorption of proteins in general(280 nm) with the absorption of PON at 302 nm. Following spectrophotometrical, in UV domain, the reaction of between the myoglobin found in meat extracts and peroxynitrite, one can observe, in time, the appearance of metMb (absorption at 502 nm, $\varepsilon_{502}=10.2$ mM$^{-1}$cm$^{-1}$, respectively 610 nm), and of deoxiMb (absorption at 520 nm) [7]. The similarity of absorption spectra, especially in UV zone, corresponding to redMb and meat extracts after incubation with PON, prove, once again that PON is decomposed during the irreversible oxidation of redMb.

The amperometric FIA method was used for the meat extract dilutions incubated with PON. When a mixture of 100 µM PON incubated with the meat extract diluted 20 times, the decrease of the current was observed, sustaining the idea that PON was consumed due to the reaction with the myoglobin content for meat (Figure 4A).Comparing the amperometric response for the equivalent myoglobin content from meat extract (20 µM) with the amperometric response of the same concentration of myoglobin in buffered solutions, it can be observed that in the real sample the current is much low, probably due to the complexity of the biological medium (and due the eventual presence of other molecules capable to decompose PON, (Figure 4B).

![Figure 4](image_url)

(A) Amperometric response, obtained with the FIA system, with the electrode SPCE/CoPc for 100 µM PON and the incubation of PON with the meat extract diluted 20 times. (B) Comparison of the chronoamperogram described at (A) with the one measured for 20 µM redMb incubated with 100 µM PON, in buffered solutions.
The electrochemical FIA method developed in this project, which is based on the use of the electrode SPCE/CoPc, allows the quantification of the reaction products in the case of the reactions described above.

**Results planned for 2015:** 1 work presented at an international conference/symposium; 1 article published in an international journal; 1 request for an invention patent.

**Results achieved 2015:** 1 ISI paper; 1 manuscript prepared for publication; 1 book chapter published; 3 book chapters; 2 works presented at international conferences; 1 patent application

**ISI papers**

**Book chapters:**

**Participations at international conferences:**
1. I.S. Hosu, M. Badea-Doni, Flow injection system for the electrochemical detection of peroxynitrite with a cobalt phthalocyanine modified screen printed carbon electrode, Flow Analysis XIII, 5-10 iulie 2015, Praga, Cehia

**Patents:**
1. PROCEDEU DE OBTINERE ŞI DE UTILIZARE A UNUI MATERIAL ELECTROSENZITIV PENTRU DETERMINAREA SELECTIVĂ A PEROXINITRITULUI
   Mihaela Doni, Ioana Silvia Hosu, Maria-Luiza Jecu, Florin Oancea
   Patent application OSIM no.: A2015/00929 / 27.11.2015

**References**

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