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ORIGINAL PAPER

Reduced Fluoresceinamine for Peroxynitrite Quantification in the Presence of Nitric Oxide

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Abstract A new fluorescent analytical methodology for the quantification of peroxynitrite ($ONOO^-$) in the presence of nitric oxide (NO) was developed. The quantification of $ONOO^-$ is based in the oxidation of the non-fluorescent reduced fluoresceinamine to a high fluorescent oxidized fluoresceinamine in reaction conditions where the interference of NO is minimized. Screening factorial experimental designs and optimization Box-Behnken experimental design methodologies were used in order to optimize the detection of $ONOO^-$ in the presence of NO. The factors analysed were: reduced fluoresceinamine concentration (C_{Fl}); cobalt chloride concentration (C_{CoCl2}); presence of oxygen (O_2); and, the pH (pH). The concentration of sodium hydroxide (C_{NaOH}) needed to diluted the initially solution of $ONOO^-$ was also evaluated. An optimum region for $ONOO^-$ quantification where the influence of NO is minimal was identified - C_{Fl} from 0.50 to 1.56 mM, C_{CoCl2} from 0 to 1.252×10^{-2} M, pH from 6 to 8 and C_{NaOH} 0.10 M. Better results were found in the presence of NO at pH 7.4, C_{Fl} 0.5 mM, without oxygen, without cobalt chloride and with a previous dilution of peroxynitrite solution with C_{NaOH} 0.1 M. This methodology shows a linear range from 0.25 to 40 μM with a limit of detection of 0.08 μM. The bioanalytical methodology was successfully applied in the $ONOO^-$ quantification of fortified serum and macrophage samples.

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Introduction

Peroxynitrite anion ($ONOO^-$) and nitric oxide (NO) are the principal reactive nitrogen species (RNS). Like other reactive oxygen and nitrogen species the $ONOO^-$ is involved in several physiological or pathological processes [1]. Also due to is reactivity, short half-life, limited diffusion, lower concentration, rapid diffusion, antioxidant mechanisms, participation in several oxidation and nitration reactions and possible interferences is quantification is not straightforward [1–6]. The $ONOO^-$ anion is in equilibrium with is conjugated acid the peroxynitrous acid (ONOOH, pK_a 6.8). At a pH values lower than 7.4 the $ONOO^-$ anion is rapidly converted by protonation in ONOOH with posterior decomposition to nitrate, but it is relatively stable in basic solution. The $ONOO^-$ anion is a strong oxidizing and nitrating agent and it oxidant capacity depends of the chemical composition of the medium. In a biological medium the $ONOO^-$ is mainly formed by the reaction of the superoxide radical (O_2^-) with the NO generated by the nitric oxide synthase (NOS) [2–4].

The $ONOO^-$ detection is done mainly through the nitration of tyrosine and posterior detection of the 3-nitrotyrosine by immunochemical or chromatographic techniques [3, 5–7] or through the oxidation of fluorescent probes as the 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123 [3, 6, 8, 9] or chemiluminescent probes as the luminol and coelenterazine [3, 6]. Other fluorescent probes based in the same principle and also used in is detection are fluorescein [10–14], rhodamine [15] or boron-dipyrromethene (BODIPY) [16, 17] derivatives. Recently the $ONOO^-$ detection was done

by HPLC-UV and GC-MS [18]. Beside these methodologies the detection of $ONOO^-$ was also done by electron paramagnetic resonance (EPR) [3], amperometry [19, 20] and electrophoresis [21, 22]. Of the fluorescent probes indicated for the $ONOO^-$ detection only the fluorescein hydrazide [10] were used for is quantification. Beside this method other fluorescence methods for $ONOO^-$ quantification are based in the oxidation of folic acid [23] or L-tyrosine [24] or NADH fluorescence quenching [25].

The reduced fluoresceinamine has been recently proposed as a NO fluorescent probe [26]. In this report the selectivity of reduced fluoresceinamine to the hydrogen peroxide (H_2O_2), O_2^-, nitrite and several other inorganic substances were assessed. Posterior evaluations shows that could also been used as an $ONOO^-$ probe. As for other selected $ONOO^-$ oxidizable fluorescent probes the reduced fluoresceinamine should be readily oxidized by $ONOO^-$ but not by the NO or O_2^- [3]. In this paper the possibility of $ONOO^-$ quantification by the reduced fluoresceinamine in the presence of NO was evaluated. Response surface experimental design methodologies were used in order to establish the more adequate conditions to the $ONOO^-$ quantification by reduced fluoresceinamine in the presence of NO. The optimized methodology for $ONOO^-$ quantification was used in serum and macrophage samples analysis.

Experimental

Reagents

Fluoresceinamine (5 and 6 isomers mixture, ≥90 %), hydrogen peroxide (30 %), sodium nitrite and zinc powder were obtained from Sigma-Aldrich Química S.A. (Spain). Sodium hydroxide, hydrogen chloride, cobalt (II) chloride, tris (hydroxymethyl)-aminomethan-hydrochlorid and trisodium citrate were purchased from Merck, Darmstadt (Germany). Mili-Q water with resistivity of 18.2 MΩ/cm at 25 °C was used in all experiments. The blood macrophages cells from murine mouse (RAW 264.7 cell line murine) were also obtained from Sigma-Aldrich Química S.A. (Spain).

Solutions

All the solutions prepared from a solid powder were done by rigorous weighting to the required final concentration. Tris buffer solution pH 7.4 was made by tris(hydroxymethyl)-aminomethan-hydrochlorid 50 mM and trisodium citrate 20 mM. The pH was adjusted with sodium hydroxide. Tris buffer solutions of higher pH values were also adjusted with sodium hydroxide and of lower pH values with hydrogen chloride. Fluoresceinamine, 0.25, 0.50, 1.13, 1.50 and 2.00 mM, and cobalt chloride, 11.31 and 22.62 mM, solutions were prepared by dissolution of the powder in hydrogen chloride 0.1 M. The chemical reduction of fluoresceinamine was made as previous described [26] by addition of 1.4 mL of the fluoresceinamine solution, 14.0 mL of hydrogen chloride 0.1 M and 0.5 g of zinc powder in a 15 mL Falcon tube. The Falcon tube was posterior stirred until the fluorescence and the yellow colour disappeared. After centrifugation 5.0 mL of the obtained reduced fluoresceinamine solution were diluted with 10 mL of Tris buffer solution pH 7.4 and 4.7 mL sodium hydroxide 0.1 M. Diluted solutions of the reduced fluoresceinamine, 4.91, 9.81, 22.09, 29.45 and 39.27 μM, and cobalt chloride, 0.48 and 0.96 mM, solutions were done in the fluorescent cell.

Saturated NO solutions (1.9 mM) were prepared from a gas bottle with deoxygenated water after bubbling argon during 15 min. The peroxynitrite solutions were prepared in a refrigerated beaker under constant stirring by mixing 100 mL of $NaNO_2$ 600 mM, 100 mL of H_2O_2 600 mM in HCl 0.6 M and 100 mL of NaOH 3.6 M. The solution will turn yellow indicating the formation of peroxynitrite. The agitation is maintained until no O_2 is formed. Standard and serum fortified $ONOO^-$ and NO solutions were done in deoxygenated NaOH 0.1 M by rigorous dilution to the desired concentrations. For the serum a lower (19×) and a higher dilution (94×) were done. Macrophages fortified $ONOO^-$ suspensions were done in a saline Tris buffer solution. The macrophages initially in the recommended culture medium (Dulbecco's Modified Eagle Medium + L-Glutamine, 2 mM + Fetal Bovine Serum, 10 %) were centrifuged and posterior suspended in the saline Tris buffer solution.

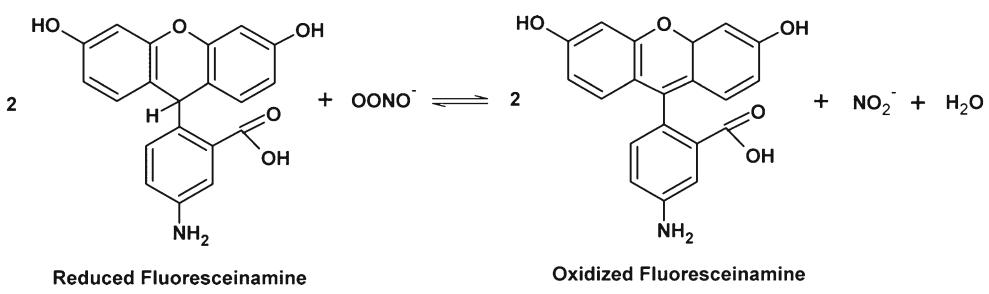


Fig. 1 Proposed oxidation reaction equation of the reduced fluoresceinamine by $ONOO^-$ (reduced fluoresceinamine chemical structure is adapted from ref. [26])

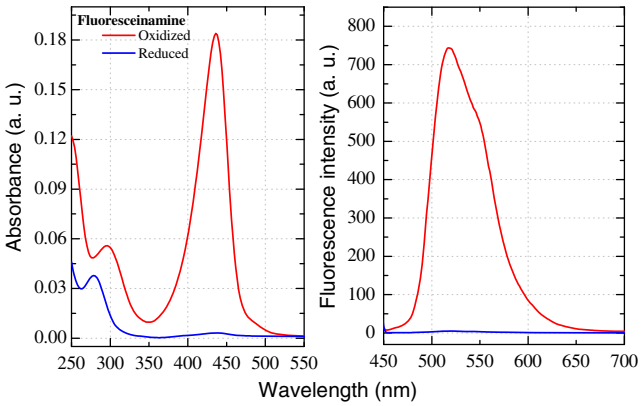


Fig. 2 UV-visible absorption and fluorescence emission spectra at excitation wavelength of 440 nm of the reduced and oxidized fluoresceinamine 10 μM

Instrumentation

The detection was done, in the experimental design optimization and in the standard solutions quantification, by an USB4000 and, in the samples quantification, by a QE65000 charge-coupled device using a 450 nm LED and a sampling compartment (CUV-ALL-UV 4-way) from Ocean Optics. Also two 1.0 mm core diameter fiber optics (P1000-2-UV–VIS) from Ocean Optics were used. One fiber to guide the light from the source to the sampling compartment and the other to guide the emitted light to the detector. The reaction time profiles were obtained collecting the signal at a wavelength of 518 nm, every 10 s with an integration time of 300 ms.

The Absorbance and the Fluorescence emission spectra were collected respectively in a Jasco V-530 UV-Visible spectrophotometer and in a Jasco FP-6200 spectrofluorimeter. The absorption spectra were obtained in a wavelength range from 250 to 600 nm with a 2 nm interval, slit with 2 nm and wavelength scan rate medium; the Fluorescence emission spectra were obtained in a wavelength range from 220 to 730 nm with a 1 nm interval, slit with 5 nm, sensitivity response medium, response time fast and wavelength scan rate 1000 nm/min.

The evaluations in the experimental design optimization, the quantification and the Absorbance and Fluorescence spectra were obtained in a standard 1 cm fluorescence quartz cell.

Experimental Design Optimization

In order to define the optimum regions, namely for $ONOO^-$ maximum and for NO minimum fluorescence intensity, an evaluation of the significance of all the factors and of the possible interactions between the factors by screening fractional and full factorial designs, followed by an optimisation Box-Behnken design with the most significant factors was done [27, 28]. The set of experimental design variables evaluated was: (i) reduced fluoresceinamine concentration (C_{Fl}), (ii) presence of oxygen (O_2) (iii) cobalt chloride concentration (C_{CoCl2}), (iv) pH (pH) and (v) sodium hydroxide concentration (C_{NaOH}). The response variable analysed was the maximum fluorescence intensity for $ONOO^-$ and the fluorescence intensity after 50″ for NO.

The significance of the main effects and the variable interactions in screening fractional and full factorial designs were evaluated using higher orders interactions as comparison. When center samples are used a curvature checking of the response is done by the evaluation of the significance of the curvature trough a curvature test and of the main effects and the variable interactions. The two tests for the evaluation of the significance of the main effects and the variable interactions are presented respectively as HOIE (Higher Order Interactions Effects) and "Center" tests [29]. The evaluation of the significance of the main effects, the variable interactions, the global linear model, global quadratic model, quadratic effects and shape of the response surface in Box-Behnken optimisation design is done by analysis of variance (ANOVA) through the F-ratio and respective p value. Also the value of multiple correlation coefficients of the response variables is evaluated [28].

Results and Discussion

Figure 1 shows a hypothesis for the oxidation reaction equation based on the probably chemical structure of the

Table 1 Levels of design variables under investigation in the experimental designs in the evaluation of the reaction of reduced fluoresceinamine with $ONOO^-$ and NO 10 μM

Design variables	Abbreviation	Cube levels		Central level
		Low	High	
Reduced fluoresceinamine (mM)	C_{Fl}	0.25	2	1.13
Oxygen	O_2	with	without	–
Cobalt chloride (mM)	C_{CoCl2}	without	22.62	11.31
pH	pH	6	8	7
Sodium hydroxide (M)	C_{NaOH}	1.00×10^{-6}	0.10	0.05

Table 2 Results obtained by a fractional a) and a full factorial b) experimental designs in the evaluation of the reaction of reduced fluoresceinamine with $ONOO^-$ 10 μM

a)

Fractional factorial experimental design

	($R_{Multiple}$=0.947)	
Main / Interactions	HOIE	b
	F-ratio (p-value)	
C_{Fl}	74.47 (0.00)	91.70
O_2	1.96 (0.19)	−14.89
C_{CoCl2}	5.58 (0.04)	−25.10
pH	0.41 (0.54)	6.83
C_{NaOH}	4.06 (0.07)	21.42

b)

Full factorial experimental designs

	pH 7.4, with oxygen ($R_{Multiple}$=0.989, CT=6.47×10^{-3})			pH 7.4, without oxygen ($R_{Multiple}$=0.995, CT=3.23×10^{-4})		
Main / Interactions	HOIE F-ratio (p-value)	Center F-ratio (p-value)	b	HOIE F-ratio (p-value)	Center F-ratio (p-value)	b
C_{Fl}	24.05 (0.13)	103.47 (9.50×10^{-2})	123.37	50.61 (0.09)	2.04×10^{3} (5.00×10^{-4})	101.99
C_{CoCl2}	0.24 (0.71)	1.04 (0.42)	−12.34	4.58 (0.28)	184.10 (5.40×10^{-3})	−30.68
C_{NaOH}	11.81 (0.18)	50.83 (0.02)	86.47	28.66 (0.12)	1.15×10^{3} (9.00×10^{-4})	76.75
$C_{Fl}\times C_{CoCl2}$	3.54 (0.18)	15.23 (0.06)	47.33	5.01 (0.27)	201.53 (4.90×10^{-3})	32.10
$C_{Fl}\times C_{NaOH}$	2.45 (0.36)	10.53 (0.08)	39.35	2.17 (0.38)	87.17 (0.01)	21.11
$C_{CoCl2}\times C_{NaOH}$	1.16 (0.48)	4.99 (0.16)	27.08	0.51 (0.60)	20.68 (0.05)	10.28

$R_{Multiple}$ multiple correlation, *CT* probability value (p) for a 5 % significance level of a curvature test, *Main* main effects, *Interactions* interactions effects, *HOIE* High Order Interactions Effects test, *F-ratio* Fisher ratio, *Center* Center test, *b* beta-regression coefficient, *p-value* probability value (p) for a 5 % significance level

reduced fluoresceinamine [26] and Fig. 2 shows the absorbance and fluorescence emission spectra of the oxidized and reduced emission fluoresceinamine. As shown a negligible absorbance and emission of fluorescence is observed for reduced fluoresceinamine. For the oxidized and reduced fluoresceinamine a maximum absorbance of 0.18 and 0.003 and a maximum fluorescence intensity of 744 and 5 were obtained. Both species have a maximum absorbance at 436 nm and a maximum fluorescence emission at 516 nm when excited at 440 nm. Also a shoulder at 550 nm is observed in the fluorescence emission spectrum which is more evident in the oxidized fluoresceinamine.

The levels of the factors (Table 1) and the concentration of NO and $ONOO^-$ (10 μM) evaluated in the experimental design methodologies were defined attending to a previous evaluation and to a practical constraints namely the solubility of fluoresceinamine and precipitation of fluoresceinamine at C_{NaOH} higher than 0.1 M. The presented concentrations correspond to the solutions prepared from

Table 3 Results obtained by a fractional factorial experimental design in the evaluation of the reaction of reduced fluoresceinamine with NO 10 μM

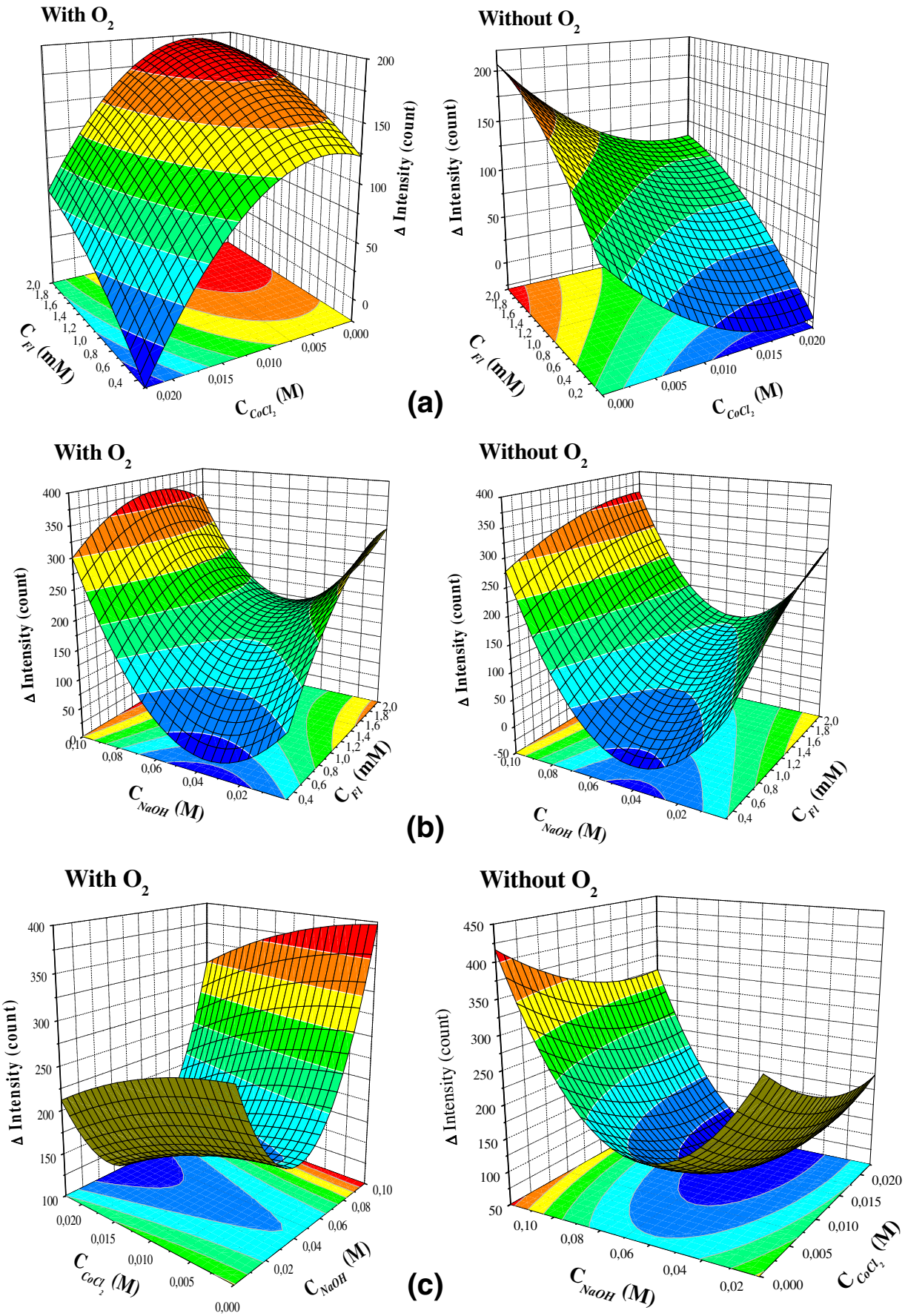
Fractional factorial experimental design

($R_{Multiple}$=0.816)

Main/ interactions	HOIE F-ratio (p-value)	b
C_{Fl}	2.97 (0.18)	156.82
O_2	2.95 (0.18)	−156.24
C_{CoCl2}	3.20×10^{-2} (0.87)	16.27
pH	1.46×10^{-2} (0.91)	−10.98

$R_{Multiple}$ multiple correlation, *Main* main effects, *Interactions* interactions effects, *HOIE* High Order Interactions Effects test, *F-ratio* Fisher ratio, *b* beta-regression coefficient, *p-value* probability value (p) for a 5 % significance level

Fig. 3 Response surfaces of fluorescence intensity obtained with the optimization Box Behnken experimental design, through the reaction of reduced fluoresceinamine with $ONOO^-$ 10 μM at pH 7.4 with and without O_2, of (**a**) reduced fluoresceinamine concentration vs. cobalt chloride concentration (**b**) reduced fluoresceinamine concentration vs. sodium hydroxide concentration and (**c**) cobalt chloride concentration vs. sodium hydroxide concentration ▶


With O2
Without O2
Δ Intensity (count)
C Fl (mM)
C CoCl2 (M)
(a)
With O2
Without O2
Δ Intensity (count)
C NaOH (M)
C Fl (mM)
(b)
With O2
Without O2
Δ Intensity (count)
C CoCl2 (M)
C NaOH (M)
(c)

the solid powder. In order to define the more adequate reaction conditions for the $ONOO^-$ quantification in the presence of NO the same levels of the factors were evaluated by the screening factorial and optimisation Box-Behnken experimental designs.

Screening Factorial Experimental Designs

For the $ONOO^-$ and NO initial screening 2^{5-1} fractional factorial designs were performed with sixteen experiments each - four continuous design variables and one category, each one with two levels and one response variable. With this design the two variable interactions are confounded with the three variable interactions. Also for the $ONOO^-$, at pH 7.4 with and without oxygen, was also done a 2^3 full factorial with eleven experiments each - three continuous design variables, each one with two levels, three center samples and one response variable.

$ONOO^-$

In Table 2a are presented the results of the HOIE test found by a fractional experimental design in the evaluation of the significance of the main effects and in Table 2b the results of the HOIE and "center" tests found by a full factorial experimental designs in the evaluation of the significance of the main effects and interactions of the reaction of reduced fluoresceinamine with $ONOO^-$ 10 μM. A screening full factorial experimental design was done to evaluate the possible interactions and the possibility of using an optimization experimental design to evaluate regions of maximum and minimum fluorescence intensity.

A multiple correlation of 0.95 for the fractional and of 0.99 for the full factorial screening experimental designs was found. By the screening fractional factorial experimental design the most relevant is that the C_{Fl} (b=+91.70) and C_{NaOH} (b=+21.42) induce a statistically significant increase and the C_{CoCl2} (b=−25.10) induces a statistically significant

Table 4 ANOVA of the system response obtained for the fluorescent intensity using a Box Behnken experimental design in the evaluation of the reaction of reduced fluoresceinamine with $ONOO^-$ 10 μM with oxygen at pH 7.4

ANOVA ($R_{Multiple}$=0.950)						
Effect	SS	d.f.	MS	F-ratio (p-value)	b	$(S.E.)_b$
Model	1.928×10^5	9	2.142×10^4	5.150 (0.043)		
Error	2.079×10^4	5	4.158×10^3			
Adjusted total	2.136×10^5	14	1.525×10^4			
Factor						
Intercept	7.380×10^4	1	7.380×10^4	17.747 (0.008)	156.845	37.231
C_{Fl}	3.329×10^4	1	3.329×10^4	8.005 (0.037)	73.722	26.056
C_{NaOH}	2.409×10^4	1	2.409×10^3	5.794 (0.061)	1.098×10^3	455.989
C_{CoCl2}	1.156×10^4	1	1.156×10^4	2.779 (0.156)	-3.360×10^3	2.016×10^3
$C_{Fl}\times C_{NaOH}$	7.702×10^3	1	7.702×10^3	1.852 (0.232)	−25.074	18.425
$C_{Fl}\times C_{CoCl2}$	180.947	1	180.947	4.351×10^{-2}(0.843)	3.843	18.425
$C_{NaOH}\times C_{CoCl2}$	7.052	1	7.052	1.696×10^{-3}(0.969)	0.759	18.425
$C_{Fl}\times C_{Fl}$	1.074×10^4	1	1.074×10^4	2.582 (0.169)	−30.816	19.177
$C_{NaOH}\times C_{NaOH}$	9.782×10^4	1	9.782×10^4	23.523 (0.005)	93.009	19.177
$C_{CoCl2}\times C_{CoCl2}$	687.729	1	687.729	0.165 (0.701)	−7.799	19.177
Model check						
Main	6.894×10^4	3	2.298×10^4			
Int.	7.890×10^3	3	2.630×10^3	0.632 (0.625)		
Int. + Squ.	1.159×10^5	3	3.864×10^4	9.293 (0.017)		
Squ.	1.159×10^5	3	3.864×10^4	9.293 (0.017)		
Error	2.079×10^4	5	4.158×10^3			
Lack of fit						
Lack of fit	1.834×10^4	3	6.113×10^3	4.985 (0.172)		
Pure error	2.452×10^3	2	1.226×10^3			
Total error	2.079×10^4	5	4.158×10^3			

$R_{Multiple}$ Multiple correlation, *SS* sum of squares, *d.f.* degrees of freedom, *MS* mean squares, *F-ratio* Fisher ratio, *b* beta-regression coefficient, $(S.D.)_b$ standard deviation of b, *Main* main effects, *Int.* interactions effects, *Squ.* square effects, *p-value* probability value (p) for a 5 % significance level

decrease in the fluorescence intensity. The other two variables, O_2 and pH, provokes respectively a non-statistically decrease (b=−14.89) and increase (b=+6.83) of the fluorescence intensity.

Attending to the previous results two full factorial experimental designs were done with and without oxygen and the pH was fixed at 7.4 (physiological pH). By these experimental designs, and such as the found by the fractional factorial experimental design, the C_{Fl} and C_{NaOH} induce an increase and the C_{CoCl2} induces a decrease in the fluorescence intensity. Opposite to that observed by the fractional factorial experimental design a non-significant statistically variation was found for the three variables. All the two-variable interactions are non-statistically significant. The most relevant conclusion with these two full factorial experimental designs is about the curvature of the response variable. The statistically significance of the curvature test (with oxygen, $p=6.47\times10^{-3}$; without oxygen, $p=3.23\times10^{-4}$) and of the "Center" test with oxygen for the C_{Fl} and C_{CoCl2} and without oxygen for all the main effects and interactions indicate that a maximum or a minimum could be found in a response surface evaluation by an optimization experimental design.

NO

In Table 3 are presented the results of the HOIE test found by a fractional experimental design in the evaluation of the significance of the main effects of the reaction of reduced fluoresceinamine with NO 10 μM. A relatively low multiple correlation of 0.82 is found. All the main effects are non-statistically significant. The C_{Fl} (b=+156.82) and C_{CoCl2} (b=+16.27) induce an increase and the O_2 (b=−156.24) and the pH (b=−10.98) induce a decrease of the fluorescence intensity. Greater variations were found for the C_{Fl} and the O_2 variables.

Table 5 ANOVA of the system response obtained for the fluorescent intensity using a Box Behnken experimental design in the evaluation of the reaction of reduced fluoresceinamine with $ONOO^-$ 10 μM without oxygen at pH 7.4

ANOVA ($R_{Multiple}$=0.976)						
Effect	SS	d.f.	MS	F-ratio (p-value)	b	$(S.E.)_b$
Model	2.306×10^5	9	2.562×10^4	11.181 (0.008)		
Error	1.146×10^4	5	2.292×10^3			
Adjusted total	2.421×10^5	14	1.729×10^4			
Factor						
Intercept	2.103×10^4	1	2.103×10^4	9.178 (0.029)	83.734	27.639
C_{Fl}	3.166×10^4	1	3.166×10^4	13.815 (0.014)	71.896	19.343
C_{NaOH}	2.812×10^4	1	2.812×10^3	12.270 (0.017)	1.186×10^3	338.510
C_{CoCl2}	2.029×10^4	1	2.029×10^4	8.852 (0.031)	-4.453×10^3	1.496×10^3
$C_{Fl}\times C_{NaOH}$	2.030×10^3	1	2.030×10^3	0.886 (0.390)	−12.873	13.678
$C_{Fl}\times C_{CoCl2}$	6.596	1	6.596	2.878×10^{-3} (0.959)	0.734	13.678
$C_{NaOH}\times C_{CoCl2}$	85.185	1	85.185	3.717×10^{-2} (0.855)	2.637	13.678
$C_{Fl}\times C_{Fl}$	1.691×10^3	1	1.691×10^3	0.738 (0.430)	−12.229	14.236
$C_{CoCl2}\times C_{CoCl2}$	1.420×10^5	1	1.420×10^5	61.980 (0.001)	112.078	14.236
$C_{NaOH}\times C_{NaOH}$	3.802×10^3	1	3.802×10^3	1.659 (0.254)	18.336	13.678
Model check						
Main	8.007×10^4	3	2.669×10^4			
Int.	2.122×10^3	3	707.234	0.309 (0.819)		
Int. + Squ.	1.484×10^5	3	4.947×10^4	21.588 (0.003)		
Squ.	1.484×10^5	3	4.947×10^4	21.588 (0.003)		
Error	1.146×10^4	5	2.292×10^3			
Lack of fit						
Lack of fit	1.119×10^4	3	3.731×10^3	28.123 (0.034)		
Pure error	265.339	2	132.669			
Total error	1.146×10^4	5	2.292×10^3			

See footnote of Table 4

From the global analysis of the results it is possible to see that for $ONOO^-$ the greater fluorescence intensity was found with O_2, higher C_{Fl}, C_{NaOH} and pH and lower C_{CoCl2}; for NO the lowest fluorescence intensity was found without O_2, higher pH and lower C_{Fl} and C_{CoCl2}. Also the greater variations of fluorescence intensity were observed for $ONOO^-$ with C_{Fl}, C_{CoCl2} and C_{NaOH} and for NO with C_{Fl} and O_2. These results clearly suggest that the quantification of $ONOO^-$ in the presence of NO it is favoured in the absence of O_2, lower C_{CoCl2} and C_{Fl} and higher C_{NaOH} and pH.

Optimization Box Behnken Experimental Designs

Optimization Box Behnken experimental designs were performed for $ONOO^-$ and NO with and without oxygen. For $ONOO^-$ the pH was also fixed at 7.4. Each optimization experimental design was done with fifteen experiments - three continuous design variables, each one with two levels, without repetitions, three centre samples and one response variable.

$ONOO^-$

The ANOVA results of the $ONOO^-$ optimization Box Behnken experimental designs are presented in Table 4 for the reaction in the presence of O_2 and in Table 5 for the reaction in the absence of O_2.

The models including the variable interactions and the square effects with O_2 (p=0.043; $R_{Multiple}$=0.950) and without O_2 (p=0.008; $R_{Multiple}$=0.976) are globally statistically significant and adequately model the variation of the experimental data. For the two optimization experimental designs only the square effects contributes significantly for the model variation (with O_2, p=0.017; without O_2, p=0.003). The statistically significance of the square effects indicates that the shape of the response surfaces estimated by the model adequately corresponds to the shape of the response. The inclusion of the square effects shows with O_2 (p=0.172) a statistically not significant and without O_2 (p=0.034) a statistically significant lack of fit of the model. Without O_2 and only with the inclusion of the square effects (data don't shown) a global model (p=0.0001; $R_{Multiple}$=0.972),

Table 6 ANOVA of the system response obtained for the fluorescent intensity using a Box Behnken experimental design in the evaluation of the reaction of reduced fluoresceinamine with NO 10 μM with oxygen

ANOVA ($R_{Multiple}$=0.970)						
Effect	SS	d.f.	MS	F-ratio (p-value)	b	$(S.E.)_b$
Model	2.533×10^5	9	2.815×10^4	8.916 (0.013)		
Error	1.579×10^4	5	3.157×10^3			
Adjusted total	2.691×10^5	14	1.922×10^4			
Factor						
Intercept	1.179×10^4	1	1.179×10^4	3.734 (0.111)	62.685	32.440
C_{Fl}	1.867×10^5	1	1.867×10^5	59.147 (6.00×10^{-4})	174.602	22.703
C_{CoCl2}	4.312×10^3	1	4.312×10^3	1.366 (0.295)	-1.772×10^3	1.516×10^3
pH	1.597×10^4	1	1.597×10^4	5.060 (0.074)	44.686	19.865
$C_{Fl}\times C_{CoCl2}$	4.631×10^3	1	4.631×10^3	1.467 (0.280)	−19.444	16.054
$C_{Fl}\times pH$	3.295×10^3	1	3.295×10^3	1.044 (0.354)	16.402	16.054
$C_{CoCl2}\times pH$	2.711×10^3	1	2.711×10^3	0.859 (0.397)	−14.876	16.054
$C_{Fl}\times C_{Fl}$	2.161×10^4	1	2.161×10^4	6.844 (0.473)	43.713	16.709
$C_{CoCl2}\times C_{CoCl2}$	1.499×10^4	1	1.499×10^4	4.748 (0.081)	36.411	16.709
$pH\times pH$	443.489	1	443.489	0.140 (0.723)	−6.263	16.709
Model check						
Main	2.070×10^5	3	6.900×10^4			
Int.	1.064×10^4	3	3.546×10^3	1.123 (0.423)		
Int. + Squ.	3.567×10^4	3	1.189×10^4	3.767 (0.094)		
Squ.	3.567×10^4	3	1.189×10^4	3.767 (0.094)		
Error	1.579×10^4	5	3.157×10^3			
Lack of fit						
Lack of fit	1.519×10^4	3	5.062×10^3	16.879 (0.057)		
Pure error	599.786	2	299.893			
Total error	1.579×10^4	5	3.157×10^3			

See footnote of Table 4

inclusion of square effects (p=0.0001) and lack of fit (p=0.058) with similarly responses surfaces were found.

From the results found by the optimization experimental designs the C_{Fl} (with O_2, p=0.008; without O_2, p=0.014) and C_{NaOH} (with O_2, p=0.061; without O_2, p=0.017) induces a statistically significant increase and the C_{CoCl2} induces with O_2 a statistically non-significant (p=0.156) and without O_2 a statistically significant decrease (p=0.031) of the fluorescent intensity. The interactions are statistically non-significant.

As shown in Fig. 3 a similar saddle shape was found for the reduced fluoresceinamine and cobalt chloride concentration vs. sodium hydroxide concentration response surfaces. In these response surfaces a clear region of minimum is defined and the region of maximum is outside the limits of the experimental region at higher C_{NaOH}, without $CoCl_2$ at a C_{Fl} from 0.5 to 2 mM. For the reduced fluoresceinamine concentration vs. cobalt chloride concentration a different shape was found with and without O_2. For this response surface with O_2 a clear region of maximum was found (C_{Fl} – 0.9 to 2 mM; C_{CoCl2} – 0 to 1.142×10^{-2} M). The maximum for all these response surfaces are limited by the practical constraints as the solubility and precipitation of fluoresceinamine and zero C_{CoCl2}.

From the global analysis of the response surfaces it was found for $ONOO^-$ at pH 7.4 with and without O_2 the following regions of fluorescence intensity:

a) Maximum: C_{Fl} - 0.50 to 2.00 mM; C_{CoCl2} - 0 to 12.25 mM; C_{NaOH} - 0.095 to 0.100 M.
b) Minimum: C_{Fl} - 0.25 to 0.50 mM, C_{CoCl2} – 13.20 to 22.62 mM, C_{NaOH} - 1.667×10^{-2} to 6.667×10^{-2} M.

NO

The ANOVA results of the NO optimization Box Behnken experimental designs are presented in Table 6 for the reaction in the presence of O_2 and in Table 7 for the reaction in the absence of O_2. The models including the variable interactions and the square effects with O_2 (p=0.013; $R_{Multiple}$=0.970) and without O_2 (p=0.068; $R_{Multiple}$=0.938) are glob-

Table 7 ANOVA of the system response obtained for the fluorescent intensity using a Box Behnken experimental design in the evaluation of the reaction of reduced fluoresceinamine with NO 10 μM without oxygen

ANOVA ($R_{Multiple}$=0.938)						
Effect	SS	d.f.	MS	F-ratio (p-value)	b	$(S.E.)_b$
Model	511.456	9	56.828	4.075 (0.068)		
Error	69.726	5	13.945			
Adjusted total	581.182	14	41.513			
Factor						
Intercept	86.162	1	86.162	6.179 (0.056)	5.359	2.156
C_{Fl}	147.984	1	147.984	10.612 (0.023)	4.915	1.509
C_{CoCl2}	103.262	1	103.262	7.405 (0.042)	274.254	100.785
pH	31.644	1	31.644	2.269 (0.192)	1.989	1.320
$C_{Fl}\times C_{CoCl2}$	16.341	1	16.341	1.172 (0.329)	1.155	1.067
$C_{Fl}\times pH$	129.535	1	129.535	9.289 (0.029)	3.252	1.067
$C_{CoCl2}\times pH$	11.662	1	11.662	0.836 (0.402)	0.976	1.067
$C_{Fl}\times C_{Fl}$	47.012	1	47.012	3.371 (0.126)	2.039	1.111
$C_{CoCl2}\times C_{CoCl2}$	18.251	1	18.251	1.309 (0.304)	−1.270	1.111
$pH\times pH$	1.206	1	1.206	8.651×10^{-2}(0.781)	0.327	1.111
Model check						
Main	282.890	3	94.297			
Int.	157.538	3	52.513	3.766 (0.094)		
Int. + Squ.	71.028	3	23.676	1.698 (0.282)		
Squ.	71.028	3	23.676	1.698 (0.282)		
Error	69.726	5	13.945			
Lack of fit						
Lack of fit	26.251	3	8.750	0.403 (0.769)		
Pure error	43.475	2	21.737			
Total error	69.726	5	13.945			

See footnote of Table 4

ally statistically significant and adequately model the variation of the experimental data. For the optimization experimental designs with O_2 the square effects (p=0.094) and without O_2 are the interactions (p=0.094) that contributes more for the model variation. Even so the inclusion of the square effects shows with O_2 a statistically significant (p= 0.057) and without O_2 a statistically non-significant (p= 0.769) lack of fit of the model.

From the results found by the optimization experimental designs the C_{Fl} (with O_2, $p=6.000\times10^{-4}$; without O_2, p=0.023) induces a statistically significant increase in the two optimization experimental designs. Beside this with O_2 the pH (p=0.074) and without O_2 the C_{CoCl2} (p=0.042) are statistically significant. Also a statistically significant interaction between C_{Fl} and the pH (p=0.029) is found by the NO experimental design without O_2.

As shown in Fig. 4 a different shape was found with and without O_2 for the three response surfaces. For all the response surfaces evaluated, regions of minimum fluorescence intensity are clearly defined. Also the regions of maximum are outside the limits of the experimental region at higher C_{Fl} and pH from 7 to 8 without $CoCl_2$ in the presence of O_2 and at C_{CoCl2} from 11.70 to 22.62 mM in the absence of O_2.

From the global analysis of the response surfaces it was found for NO with and without O_2 the following regions of fluorescence intensity:

a) Maximum: C_{Fl} - 1.6 to 2 mM; C_{CoCl2} – 11.70 to 22.62 mM; pH - 7 to 8.
b) Minimum: C_{Fl} - 0.25 to 1.56 mM; C_{CoCl2} - 0 to 22.62 mM; pH - 6 to 8.

Peroxynitrite Quantification

Attending to the regions of maximum fluorescence intensity for the $ONOO^-$ and of minimum fluorescence intensity for the NO the quantification of $ONOO^-$ was performed with a C_{Fl} 0.50 mM and 1.45 mM, pH 7.4, without $CoCl_2$ and C_{NaOH} 0.1 M with and without O_2. In Fig. 5 are presented typical response profiles of $ONOO^-$ and NO. Tables 8 and 9 present, respectively, the quantification results obtained with different standard solutions of $ONOO^-$ and NO, from 0 to 5 μM found by the reaction with C_{Fl} of 0.50 and 1.45 mM in the presence and in the absence of O_2, and those obtained in serum, in the absence of O_2, and macrophages, in the presence of O_2, fortified samples by the reaction with C_{Fl} of 0.50 mM.

From the evaluation of the typical response profiles (Fig. 5) it is possible to see that for $ONOO^-$ a similar and for NO a different response profile was obtained for the two C_{Fl} with and without O_2. As expected an always lower fluorescence intensity was obtained for $ONOO^-$ and NO with a C_{Fl} 0.50 mM. A minor difference was obtained for $ONOO^-$ and a greater difference for NO. For each C_{Fl} it was obtained for $ONOO^-$ with and without O_2 a similar fluorescence intensity and for NO without O_2 a lower fluorescence intensity next to zero. It is also clear from this evaluation that for a quantification of $ONOO^-$ in the presence of NO better results could be found without O_2, a C_{Fl} 0.5 and 1.45 mM and with O_2 at a C_{Fl} 0.5 M. A greater NO interference is found with the time and with higher C_{Fl}.

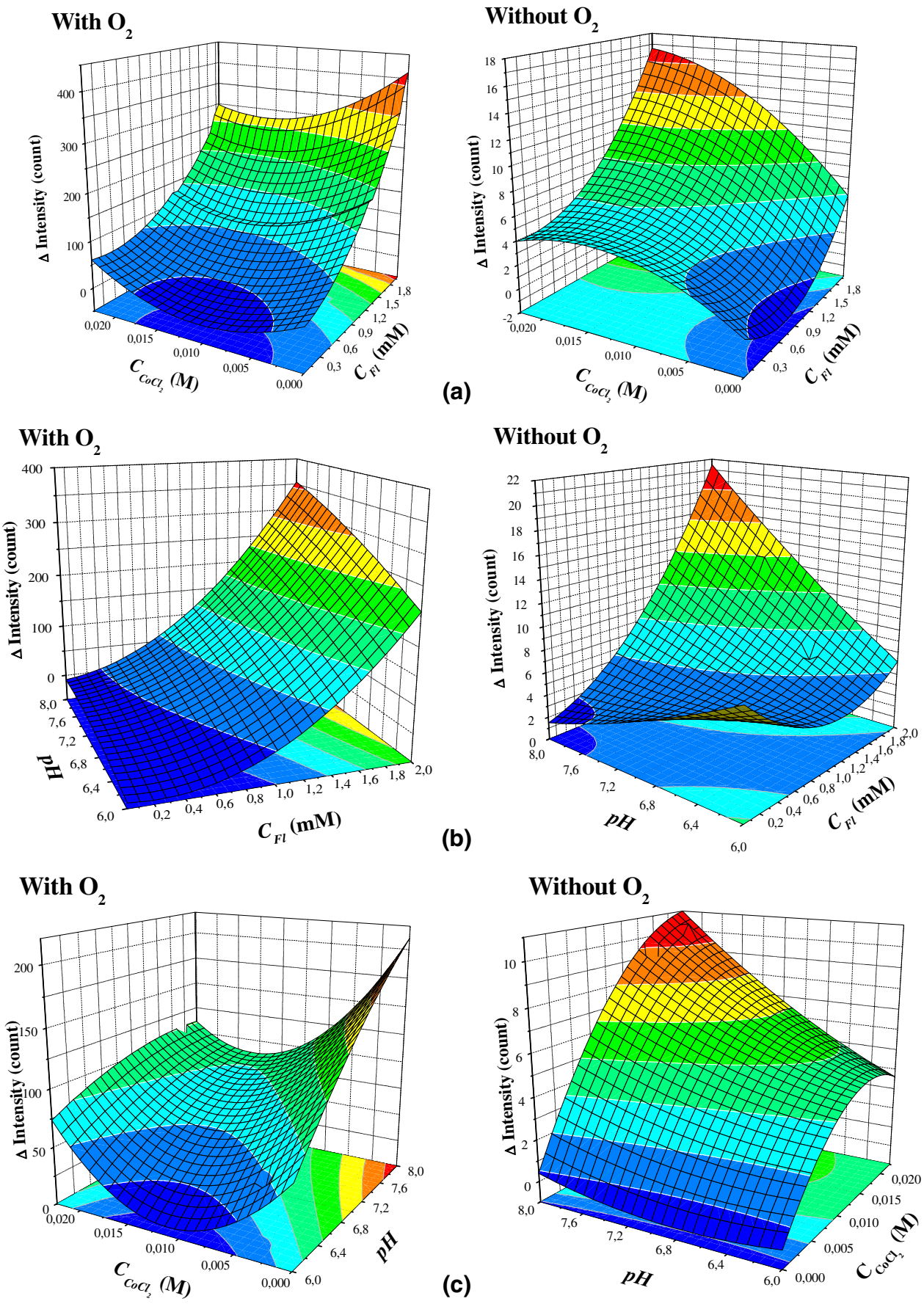
Fig. 4 Response surfaces of fluorescence intensity obtained with the optimization Box Behnken experimental design, through the reaction of reduced fluoresceinamine with NO 10 μM with and without O_2, of (**a**) reduced fluoresceinamine concentration vs. cobalt chloride concentration (**b**) reduced fluoresceinamine concentration vs. pH and (**c**) cobalt chloride concentration vs. pH

In Table 8 it is possible to see that generally better quantification results were found with C_{Fl} 0.5 M in the presence and in the absence of O_2. Slightly higher results were found with C_{Fl} 1.45 M without O_2, namely when the NO concentration is higher than the $ONOO^-$ concentration, and as expected worse results were found with C_{Fl} 1.45 M with O_2. Generally worse quantification results were obtained with the two C_{Fl} with and without O_2 for $ONOO^-$ 1 μM with a higher concentration of NO (2.5 and 5 μM). It is important to stress that some slightly lower quantification results could be explained by the difficult management of the preparation of standard solutions namely of lowest concentrations.

By the analysis of all the linear fit obtained in the range from 1 to 10 μM with four calibration points it was found for C_{Fl} 0.5 mM without O_2 the greater correlation coefficient (R=0.9991), the lowest sensitivity (a=24.03) with a low detection limit (DL_{blank}=0.15 μM) and for C_{Fl} 0.5 mM with O_2 the lowest correlation coefficient (R=0.9811), a higher sensitivity (a=34.36) with also lower detection limit (DL_{blank}=0.10 μM). With C_{Fl} 0.5 mM without O_2 and detection with the QE65000 a linear range from 0.25 to 40 μM and a DL of 0.08 μM were found with the following linear fit parameters:

$$y = 2.63x + 5.36, m = 9, s_a = 1.75, s_b = 0.10, s_{y/x} = 3.94, R = 0.9954.$$

In Table 9 it is possible to see that good quantification results were found for the serum and for the macrophages samples with C_{Fl} 0.5 M. The quantification results for the lower and higher serum sample dilution were obtained respectively with and without subtraction of the serum fluorescence background. For the serum samples good quantification results were found with a higher dilution. Also better quantification results for the two dilutions were


With O2
Without O2
Δ Intensity (count)
C CoCl2 (M)
C Fl (mM)
(a)
With O2
Without O2
Δ Intensity (count)
pH
C Fl (mM)
(b)
With O2
Without O2
Δ Intensity (count)
C CoCl2 (M)
pH
(c)

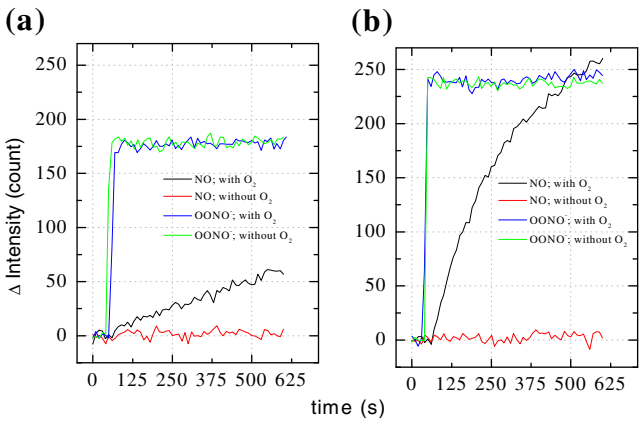


Fig. 5 Typical response profiles of $ONOO^-$ and NO 5 μM by the reaction with reduced fluoresceinamine (**a**) 0.50 and (**b**) 1.45 mM with or without oxygen, at pH 7.4, without cobalt chloride and sodium hydroxide concentration 0.1 M

obtained with the lowest $ONOO^-$ concentrations. The scavenge capacity of some serum components explains the generally lower recovery found with a smaller serum sample dilution. For the macrophage sample and, as expected due to scattering, only with a suspension of 2.5×10^5 cells was possible to obtain good quantification results.

Conclusions

The optimization experimental design methodologies based in a response surface methodology allows to clearly defining optimum experimental regions of the different experimental variables involved in order to a correct quantification of the $ONOO^-$ in the presence of NO. Different regions of maximum and minimum fluorescence intensity were evaluated for the $ONOO^-$ and NO.

For $ONOO^-$ at pH 7.4 maximum region: C_{Fl} - 0.50 to 2.00 mM, C_{CoCl2} - 0 to 12.25 mM, C_{NaOH} - 0.095 to 0.100 M; minimum region: C_{Fl} - 0.25 to 0.50 mM, C_{CoCl2} – 13.20 to 22.62 mM, C_{NaOH} - 1.667×10^{-2} to 6.667×10^{-2} M. For NO maximum region: C_{Fl} - 1.6 to 2 mM, C_{CoCl2} – 11.70 to 22.62 mM, *pH* - 7 to 8; minimum region: C_{Fl} - 0.25 to 1.56 mM, C_{CoCl2} - 0 to 22.62 mM, *pH* - 6 to 8.

The most significant experimental variable that increases the fluorescent intensity is C_{Fl}. Without O_2 for $ONOO^-$ a significant decrease and for NO a significant increase of the fluorescent intensity was found with C_{CoCl2}.

The optimum region in order to the quantification of $ONOO^-$ in the presence of NO is defined by a C_{Fl} from 0.50 to 1.56 mM, C_{CoCl2} from 0 to 12.52 mM, *pH* from 6 to 8 and C_{NaOH} 0.10 M. The best quantification results were found with pH 7.4, C_{Fl} 0.5 mM, without oxygen, without

Table 8 – $ONOO^-$ quantification results obtained in $ONOO^-$ and NO standard solutions found by the reaction of reduced fluoresceinamine 0.50 and 1.45 mM in the presence and in the absence of O_2

	C_{Fl} 0.50 mM				C_{Fl} 1.45 mM			
	Without O_2		With O_2		Without O_2		With O_2	
C_{NO}:C_{ONOO^-} (μM)	C_{ONOO^-}±1.27	Recovery (%)	C_{ONOO^-}±2.86	Recovery (%)	C_{ONOO^-}±5.12	Recovery (%)	C_{ONOO^-}±3.92	Recovery (%)
0:1	0.98	98.17	0.72	71.95	0.58	56.10	0.93	93.45
2.5:1	0.60	60.13	0.60	75.59	0.73	73.01	0.78	78.75
5:1	0.63	63.16	1.15	115.49	n.p.	n.p.	1.01	101.01
0:2.5	2.42	96.90	2.30	92.04	2.35	94.26	2.67	107.03
1:2.5	2.20	88.06	2.44	97.59	2.49	99.81	4.21	168.57
5:2.5	2.50	99.97	2.58	101.86	4.98	199.57	6.91	276.20
0:5	4.99	99.79	5.32	106.46	5.02	100.45	5.07	101.42
1:5	4.84	96.87	5.12	102.46	4.85	96.94	5.33	106.58
2.5:5	4.30	86.02	4.92	98.38	6.02	120.30	8.57	171.38
y=bx+a, 1–10 μM (*m*=4)								
	a=36.87; s_a=4.16		a=45.27; s_a=2.29		a=43.42; s_a=4.24		a=29.30; s_a=10.26	
	b=24.02; s_b=0.72		b=34.36; s_b=6.51		b=34.40; s_b=24.38		b=37.20; s_b=1.41	
	$s_{y/x}$=4.95; R=0.9991		$s_{y/x}$=8.63; R=0.9811		$s_{y/x}$=28.97; R=0.9851		$s_{y/x}$=24.77; R=0.9906	
	DL_{blank}=0.15 μM		DL_{blank}=0.10 μM		DL_{blank}=0.41 μM		DL_{blank}=0.18 μM	

n.p. quantification not possible, *m* number of calibration points, s_a slope standard deviation, s_b intercept standard deviation, $s_{y/x}$ standard deviation of residuals, *R* linear correlation coefficient, DL_{blank} detection limit estimated by the standard deviation of five determinations of a blank

Table 9 $ONOO^-$ quantification results in serum and macrophage fortified solutions respectively in the presence and in the absence of NO found by the reaction of reduced fluoresceinamine 0.50 mM

	Serum (without O_2)				Macrophage cells (with O_2)		
	Lower dilution (19×)		Higher dilution (94×)		C_{ONOO^-} (5 μM)		
C_{NO}:C_{ONOO^-} (μM)	C_{ONOO^-}±0.59	Recovery (%)	C_{ONOO^-}±3.60	Recovery (%)	Number of cells	C_{ONOO^-}±3.43	Recovery (%)
5:1	1.03	103.00	1.06	106.08	2.5×10^5	4.73	94.61
1:2.5	2.26	90.54	2.40	96.00	5.0×10^5	3.75	75.04
1:5	4.06	81.20	4.68	93.60	1.0×10^6	2.54	50.73
y=bx+a							
	a=4.44; s_a=0.28		a=5.07; s_a=1.29		a=5.10; s_a=1.76		
	b=2.58; s_b=0.05		b=2.20; s_b=0.14		b=2.73; s_b=0.19		
	$s_{y/x}$=0.42; R=0.9994		$s_{y/x}$=2.29; R=0.9901		$s_{y/x}$=3.12; R=0.9908		
	m=5; 0.5–10 μM		m=6; 0.5–20 μM		m=6; 0.5–20 μM		

m number of calibration points, s_a slope standard deviation, s_b intercept standard deviation, $s_{y/x}$ standard deviation of residuals, R linear correlation coefficient

cobalt chloride and with a previous dilution of peroxynitrite solution with C_{NaOH} 0.1 M. In these conditions a linear range from 0.25 to 40 μM with a limit of detection of the method of 0.08 μM was obtained.

The $ONOO^-$ quantification was achieved with a lower diluted serum samples and lowest $ONOO^-$ concentrations and with macrophage samples with a number of 2.5×10^5 cells.

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