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Quantitative structure-chemiluminescence intensity relationships of 4-substituted phenols acting as luminol signal enhancers

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ABSTRACT

Luminol-peroxidase-H_2O_2 is among the most popular systems in chemiluminescence-based analytical applications. The addition of a forth compound into this system has been thoroughly explored over the years, namely an enhancer promoting potency in the signal attained. Improvements in this regard serve to increase sensitivity and applicability of various biochemical methods. Studies exploring the ability of compounds to act as enhancers have proposed several chemical groups as possible candidates, amongst which 4-substituted phenols have been widely employed. Although some studies have explored the effect of the substituent on enhancer potency, no quantitative structure – property relationships (QSPR) employing molecular descriptors for this group of compounds have been presented. Current study provides two such cross- and externally validated models, constructed by the projection to latent structures by means of partial least squares (PLS) multivariate linear regression method: i) a PLS-DA model contributing to the classification of such compounds into classes (relative to the signal), and ii) a PLS model for predicting the signal acquired. Validation was based on statistical metrics $Q^2_{ext(F3)}$ for the test set and Roy's metrics $r^2_{m(Av)}$ & $r^2_{m(\delta)}$, assessing both predictive stability and internal validity. Applied in tandem, those models can assist in the recognition of available compounds as potential enhancers or inspire the synthesis of novel analogues. The significance of some characteristics governing the compounds' behaviour are also discussed based on the molecular descriptors chosen.

1. Introduction

Chemiluminescence (CL) has gained great significance in many biochemical applications, due to its important analytical advantages [1]. Among various CL systems the ones that include horseradish peroxidase (HRP) as the signal generating enzyme have prevailed for the determination of various compounds through coupled enzymatic reactions [2,3]. HRP catalyses the reaction between hydrogen peroxide and a substrate, such as luminol. The latter when oxidized emits light, following the decay of the excited-state oxidation product. CL intensity can be significantly increased by adding in the substrate solution a molecule that acts as an enhancer, with 4-iodophenol (4-IOP) [4,5] being the most popular enhancer of the HRP-catalysed CL oxidation of luminol. Such enhanced CL reactions provide more intense, prolonged and stable light emission [6]. Regarding enhancers, a variety of substituted phenols has been applied as luminol signal enhancers, such as firefly luciferin, 6-hydroxybenzothiazole derivatives [7], arylboronic acid derivatives [8] and even more complicated analogues [9].

In a previous study [10], a comparison of the enhancement effect of four 4-substituted phenols on the HRP-catalysed CL oxidation of luminol was described. The obtained results revealed that the utilization of different enhancers can lead to dramatic changes in CL intensity and consequently to the characteristics (limit of detection and concentration range) of a CL-based enzyme immunoassay. This impact is attributed to the specific properties of the substituent at the 4-position of the phenol. The differences are so remarkable that the selection of the proper enhancer could serve various needs in bioanalysis. To this purpose, the construction of a model that could correlate chemical structure with signal intensity would help in recognizing the key characteristics serving this purpose, as well as identify other related molecules as possible enhancers.

This is the concept behind quantitative structure-property relationships (QSPR) [11]. Information in molecular structure is expressed through molecular descriptors [12] that are in turn used to construct mathematical models through various methods, among which, methods based in regression are quite popular [13]. Those models serve in recognizing and classifying compounds into groups, as well as getting estimates of the response (property) value for novel molecules. Regarding CL applications, the first attempts to predict CL behaviour of various compounds, including pharmaceuticals and pesticides, using QSPR studies were presented some years ago [14,15].

Projection to latent structures by means of partial least squares (PLS) is a multivariate linear regression method that can be thus employed [16]. Its prime characteristic is the uncovering of linear latent variables out of the provided original descriptors. The compounds are then

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projected as points in the chemical space formed of those, which is significantly simpler than the original [17]. More specifically, discriminant analysis by PLS (PLS-DA) can discern among compounds belonging into groups relevant to their difference in response, by focusing on their apparent structural differences. For PLS-DA, group membership becomes the categorical discrete response variable, and in turn the output of such a model is the probability that a compound belongs to each group, while in classical PLS applications the response is a continuous numerical variable. Both, being statistical in nature, the constructed models require validation of their performance. For this purpose, there are a number of internal and external validation methods and metrics, making evaluations of different aspects of the model's quality [18–20]. Subsequently, the models can be applied for predicting the 'behaviour' of novel compounds, given that they belong in the same chemical space, by calculating their *p*-values through Hotelling's generalized Student's test for application in multivariate analysis [21].

In the current study it was aimed, besides a PLS-DA model contributing to the classification of such compounds into classes, to create a PLS–QSPR model devoid of redundant descriptors, while retaining the ability to recreate the information found in the response, a quality, usually accounted by the correlation coefficient R^2Y metric and its cross-validated forms. Further analysis of the model can provide foresight into important chemical characteristics of compounds for use in chemiluminescence signal amplification or even aspects of the mechanism.

2. Materials and methods

2.1. Reagents

Sulfo-NHS-LC-Biotin was obtained from Molecular Probes (Eugene, OR, USA) and BSA (RIA and ELISA grade) was brought from Calbiochem (Germany). Tween 20 was purchased from ICN Biomedicals (Germany). Hydrogen peroxide (3%, w/v), streptavidin, HRP type VI, luminol, tris(hydroxymethyl)aminomethane (Tris), all enhancers and other reagents were obtained from Sigma-Aldrich (Athens, Greece). All aqueous solutions and buffers were prepared using water de-ionised and doubly distilled (resistivity >18 MΩ cm).

The washing solution used in this protocol was a phosphate-buffered saline (PBS) solution (pH = 7.4) containing 0.05% (v/v) Tween 20. The coating buffer consisted of a 0.1 M carbonate/bicarbonate buffer (pH = 9.6) and the blocking solution was a PBS buffer containing 1% BSA (w/v).

Luminol substrate solution was consisted of luminol (0.10 mmol L^{-1}), hydrogen peroxide (1.0 mmol L^{-1}) and the enhancer in Tris buffer (0.10 M) with a pH value of 8.5. The optimum concentration for each enhancer was determined from preliminary CL measurements.

2.2. Instrumentation and software

All measurements were performed with a Fluostar Galaxy (BMG LabTechnologies GmbH, Germany) multifunctional microplate reader. Luminescence optics was installed for the experiments and all emitting light was recorded without any emission filter. The 96-well microtiter plates (opaque white polystyrene plates with a Maxisorp surface, which exhibited low cross-talk between adjacent wells) were obtained from Nunc (Nalge Nunc, UK). All plates were washed with a fully automated Tecan Columbus (Tecan, Austria) 96-well microplate washer.

Molecular structures were designed in Marvin Beans v.15.3.16.0 64bit (ChemAxon, Cambridge, MA, USA). They were copied in simplified molecular-input line-entry system (SMILES) using the same program suite. PLS, PLS-DA and PCA (as an auxiliary method) were performed using SIMCA-P+ v.7 Umetrics (MKS Corp., Sweden).

2.3. Protocol for CL intensity measurements

The protocol for chemiluminescence measurements was previously described [10]. In brief, the wells of the 96-well plates were coated with 100 μl of streptavidin solution (1 μg ml^{-1}) in PBS and incubated overnight at 4 °C. Next, the wells were post-coated with 200 μl of blocking solution for 1 h at room temperature. After being washed four times with 300 μl of washing solution, the microwells were filled with 100 μl of a diluted (1:500) biotinylated HRP solution for 30 min at room temperature. Then the wells were rewashed six times with the same washing solution and CL measurement was carried out by adding 150 μl of a freshly prepared luminol substrate solution (one for each enhancer) with a multichannel pipette. Compounds tested, as well as their intensity measurements in a Y% normalized form, are presented in Table 1. Transformation into Y% of the intensity response values

Table 1
Compounds included in the dataset, classified by PLS-DA. Experimental response after normalization to a 0–100% scale.

Compound (IUPAC name)	Abbrev.	PLS-DA Class	PLS Set	Response (Y%)
4-hydroxybenzaldehyde	4-HBA	1	Test	6.74
4-[(Z)-2-phenyldiazen-1-yl]phenol	4-AZO	3	Working	83.94
4-hydroxybenzoic acid	4-HAC	1	Working	13.78
4-benzoylphenol	4-BNZ	3	Test	63.46
4-(4-hydroxyphenyl)benzoic acid	4-BCA	3	Excluded	69.50
4-phenylphenol	4-BIP	3	Working	64.45
4-chlorophenol	4-CLO	1	Working	3.635
(2Z)-3-(4-hydroxyphenyl)prop-2-enoic acid	4-CIN	3	Working	18.53
7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one	4-DAI	2	Excluded	30.41
4-(4-hydroxyphenyl)phenol	4-HBI	3	Test	66.34
4-(2,4-dimethoxybenzoyl)phenol	4-DIM	2	Excluded	23.27
methyl 4-hydroxybenzoate	4-EPA	1	Test	10.06
4-hydroxybenzohydrazide	4-HDR	1	Working	20.54
4-(1H-imidazol-1-yl)phenol	4-IMP	3	Working	95.72
4-iodophenol	4-IOP	1	Working	38.52
4-methoxyphenol	4-MEP	1	Working	6.582
4-nitrophenol	4-NIP	1	Working	7.018
4-phenoxyphenol	4-PHO	3	Working	69.06
2-(4-hydroxyphenyl)pyrimidin-5-ol	4-PYR	3	Working	67.49
4-(1H-pyrrol-1-yl)phenol	4-PYP	3	Working	72.69
4-hydroxybenzene-1-sulfonic acid	4-SUL	1	Test	15.57
4-(4-hydroxybenzenesulfonyl)phenol	4-SUE	2	Working	23.94
4-(5-sulfanyl-1H-1,2,3,4-tetrazol-1-yl)phenol	4-THL	4	Excluded	31.64
4-[(1,2,3,4-thiatriazol-5-yl)amino]phenol	4-THZ	4	Excluded	33.82