Babes-Bolyai University

Faculty of Chemistry and Chemical Engineering

Extended summary of the Ph.D. Thesis

Biologically-relevant redox reactivity in small molecules

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Cluj-Napoca

2020

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Contents of summary

Keywords: *antioxidants, antioxidant activity, myoglobin, glutaraldehyde, phenothiazine, spectroscopy.*

1. About this thesis

The present thesis aims to examine various facets of biologically relevant redox reactivity in small molecules. Such facets include:

- Application of standard assay methodology for evaluating redox properties of antioxidant-rich plant extracts – with emphasis on newly-developed methods that entail biologically-relevant reagents (and primarily hemoglobin)
- Examination of the interaction of antioxidant molecules with proteins, using spectroscopy
- Development of a new version of a quantitative assay for antioxidant activity, employing a liposome-myoglobin reagent mixture, with increased biological relevance compared to classical antioxidant assays
- Application of computational methods for examining the HOMO-LUMO gap (and henceforth the UV-vis spectra) in a series of compounds with potential biomedical applications
- Examination of redox reactivity in glutaraldehyde stock solutions – further evolved into a more complex study with relevance to their biocide properties and applicability in medical settings

In order to achieve the above-mentioned things, the thesis brings together a diverse set of methods–spectroscopy (UV-vis, fluorescence, NMR, Raman), kinetics (including enzymatic Michaelis-Menten), mass spectrometry, and DFT/TD-DFT calculations. Central to the thesis is the interaction of redox-active small molecules with proteins (especially hemoglobin and myoglobin) and cells.

2. General Considerations

Oxidative stress can appear as a depression of the antioxidant systems, a general increase in ROS generation, or both. If the antioxidant systems do not pacify the reactive oxygen species, they can cause DNA damage, increase lipid peroxidation and/or induce nucleic acid and protein modifications.[1]

Recent and old studies in the antioxidants field have focused on the role of antioxidants, vitamins C, E, caffeic acid, gallic acid, ferulic acid, catechin, rutin, selenium, carotenoids, folic acid have protective role against some disease like cancer, coronary heart disease, arterosclerosis.[11]

Antioxidant activity pathways are various and involve singlet oxygen quenchers, inhibitors of free radical reactions, stopping the propagation of the autooxidation chain reaction, synergism with other antioxidants, and metal chelators that transform metal prooxidants into stable compounds.[9] Representative antioxidant activity assays include the Oxygen Radical Absorbance Capacity (ORAC), DPPH Radical Scavenging Capacity Assay, Total Phenol Assay by using the Folin-Ciocalteu Reagent;[20] more biologically-relevant methods include hemoglobin/ascorbate peroxidase activity inhibition assay (HAPX) and assay based on the inhibition of induced peroxidation of liposomes.[21]

The prooxidant capacity refers to substances capable to induce oxidative stress by repressing antioxidant systems or by generation of ROS. It has been reported that some popular antioxidant flavonoids can also act as prooxidant under certain circumstances and promote oxidation of other substances. Antioxidants are thus considered substances with “two faces” because they can display an prooxidant effect in certain circumstances. Ascorbic acid is an important water soluble antioxidant used in organism defense against ROS, especially for protection of plasma against oxidative stress. Ascorbic acid can behave as prooxidant in the presence of copper or iron with hydrogen peroxide generation.[28,29,30]

Proteins are indispensable macromolecules found in all living organisms. Myoglobin is a monomeric globular protein with important function in transport and storage of oxygen in the muscles. The oxygen is stored for the use in anaerobic stage of respiration. In addition, myoglobin is implicated in NO scavenging and regulation. Hemoglobin is a tetrameric protein, with important role in the transportation of oxygen from lungs to the tissues.[38,39]

Valuable information about protein structures, interactions, exogenous ligand binding, oxidation states and spin states of intermediates, even the function of the enzymes, is collected using the experimental methods such as UV-Vis, Raman, Mössbauer, and circular dichroism spectroscopy, X-ray crystallography, electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR) and computational chemistry.[37,40,41]

3. Applications of the HAPX assay on plant extracts

Salvia species are the most widely used medicinal plant and generally known for their multiple pharmacological effects including antiproliferative, antiinflammatory, anti-nociceptive, antioxidant, antimicrobial, antimutagenic, antidementia, hypoglycemic, and hypolipidemic effects[44]. The evaluation of the polyphenolic content and the antioxidant activity of six *Salvia* L. species from spontaneous Moldavian Flora (*S. aetiopsis, S. austriaca, S. sclarea, S nutans, S. verticilatta,* and *S. nemorosa*), was made using three *in vitro* model systems, DPPH, FRAP and HAPX (Hemoglobin /Ascorbate Peroxidase Activity Inhibition) with data illustrated in Table 3.1.

Table 3.1. The antioxidant activity of Salvia species.

Samples	**DPPH IC_{50} (µg/mL)**	**FRAP (mmol Trolox/mg d.w.)**	**HAPX (mg CAE)/g**
S. aetiopsis	158.76 ± 0.82	1399 ± 5.01	6.31 ± 0.21
S. austriaca	123.14 ± 0.70	2066 ± 4.81	8.88 ± 1.56
S. sclarea	97.67 ± 0.56	2791 ± 4.81	146.66 ± 23.69
S. nutans	158.03 ± 0.88	1546± 4.81	62.2 ± 7.31
S. verticillata	42.923 ± 0.23	8044 ± 4.81	1074.3 ± 836.16
S. nemorosa	80.09 ± 0.6	2797 ± 4.81	91.2 ± 10.34
Quercetin	5.62 ± 0.33	-	-
BHT	16.2 ± 0.42	-	-

S. verticilatta presents the highest antioxidant capacity determined by DPPH (42.923±4.81µg/ml), FRAP (8044±4.81 mmol Trolox/mg) and HAPX (1074.3± 836 mg

CAE)/g). *S. nutans*, *S. austriaca* and *S. aetiopsis* have the lowest antioxidant activity (Table 3.1).

The polyphenolic profile and the antioxidant activity for six Salvia species were evaluated in order to complete scientific data related to Salvia genus that may be important sources of natural antioxidants.

4. Antioxidant binding to globins

Under conditions of oxidative stress, the ferrous-oxy hemoglobin in blood suffers not only auto oxidation to Fe(III), but also oxidation to the toxic form, ferryl (Fe(IV)-oxo). Antioxidant compounds can reduce ferryl back to met hemoglobin (Fe(III)), which can be further transformed by met hemoglobin reductase into hemoglobin. Endogenous antioxidants (such as ascorbate or urate) have been shown to be efficient in these reduction processes for completing a pseudo-peroxidase catalytic cycle, with biomedical as well as analytical relevance.

Hemoglobin and myoglobin display fluorescence at ~330 nm due to their Trp and Tyr residues. This fluorescence may be affected by ligands if/when they bind to Hb in the proximity of Trp and/or Tyr residues. Figure 4.1 shows that the fluorescence of Mb is quenched by ascorbate in a concentration-dependent manner.

The interaction with myoglobin at three different temperatures (297 K, 303 K and 310 K) was further studied in order to establish what type of fluorescence quenching mechanism is present, as shown in Table 4.1 illustrates the relationship of the Stern-Volmer dynamic quenching constants and the temperature.

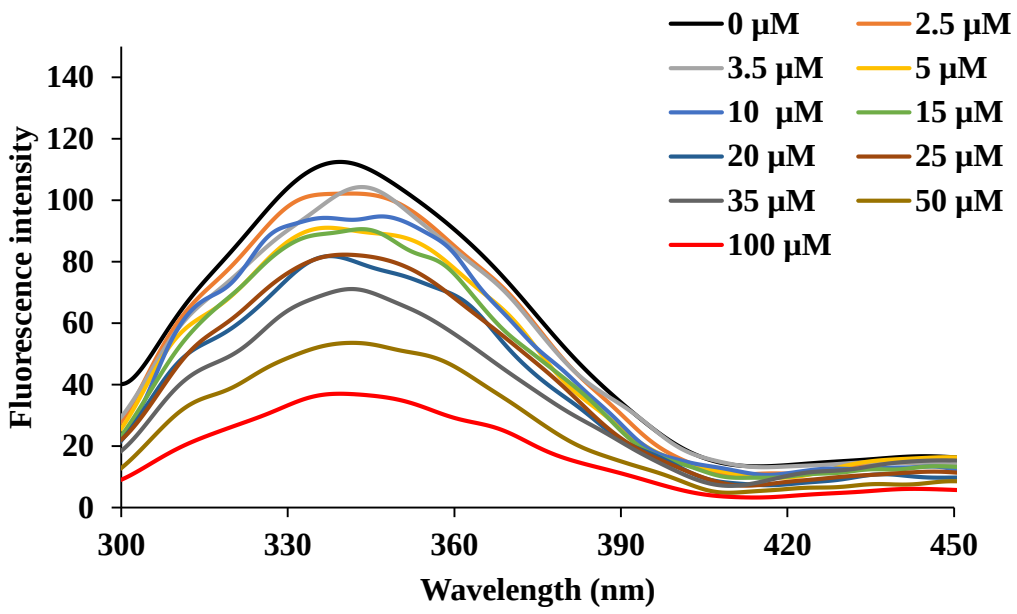


Figure 4.1. Emission spectra of myoglobin in the presence of various concentrations of ascorbate at excitation wavelength at 280 nm, in PBS buffer, at room temperature.

Table 4.1. Stern-Volmer quenching constants and thermodynamic parameters of ascorbate-Mb interaction at pH 7.4.

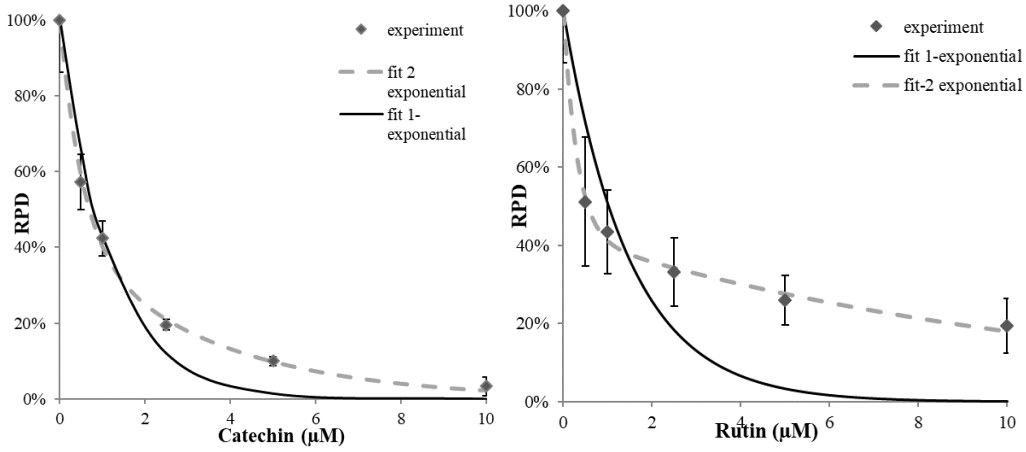
T (K)	KSV (L*mol-1)	R^2	n	Kd (µM)	Ka (x 10^4 L* mol^{-1})	R^2	ΔS J/mol	ΔH KJ/mol	ΔG KJ/mol
297	10191	0.98	1	7.34	3.90	0.99	-100	-55.99	-26.19
303	14180	0.91	1	25.30	2.28	0.92			-25.59
310	15922	0.97	1	1613	1.51	0.93			-24.89

The values of free energy change (ΔG), entropy changes (ΔS) and ΔH are shown in Table 4.1. The negative sign for ΔG implies that the interaction process is spontaneous. The major contribution to ΔG is from ΔH, rather than from ΔS, which reflects that the binding process is enthalpy driven and hydrogen bonding is the major interaction force in the binding of ascorbate to Mb.

5. Kinetic assay for antioxidant reactivity with increased biomedical relevance, based on liposomes and myoglobin

The inhibition of liposome autooxidation by cytochrome *c* can be employed for at least qualitative or semi-quantitative assays of antioxidant reactivity with biological relevance; this type of assay will be hereafter referred to as ILA-cytc (inhibition of liposome autooxidation by cytochrome *c*).[21,98,109,110,117,118] An assay based on liposome oxidation has the advantage of being applicable to water-soluble as well as to lipophilic antioxidants. We report here that myoglobin (Mb) may be used instead of cytochrome *c* in such a liposome assay (hereafter referred to as ILA-Mb) for antioxidant reactivity, with three advantages: (1) employing a more generally accessible protein (Mb vs. cytochrome *c*), (2) reducing the duration of the experiment, and (3) affording a quantitative assay as opposed to the previously described semi-quantitative versions based on cytochrome *c*.

A quantitative measure of antioxidant reactivity is proposed here, the "relative peroxidation degree" (RPD), calculated based on the increase in absorbance at 235 nm over a set time after initiating the reaction. Two options for the value of this set time are explored: 120 and 180 min; these values are chosen so as to explore the upper and lower limits of the ascending part of the absorbance = f(time) curve, noting that in one case (ascorbate vs. catechin at 2.5 µM) the order of reactivity measured at these two time points is different. Here is presented the data for 120 min set time.



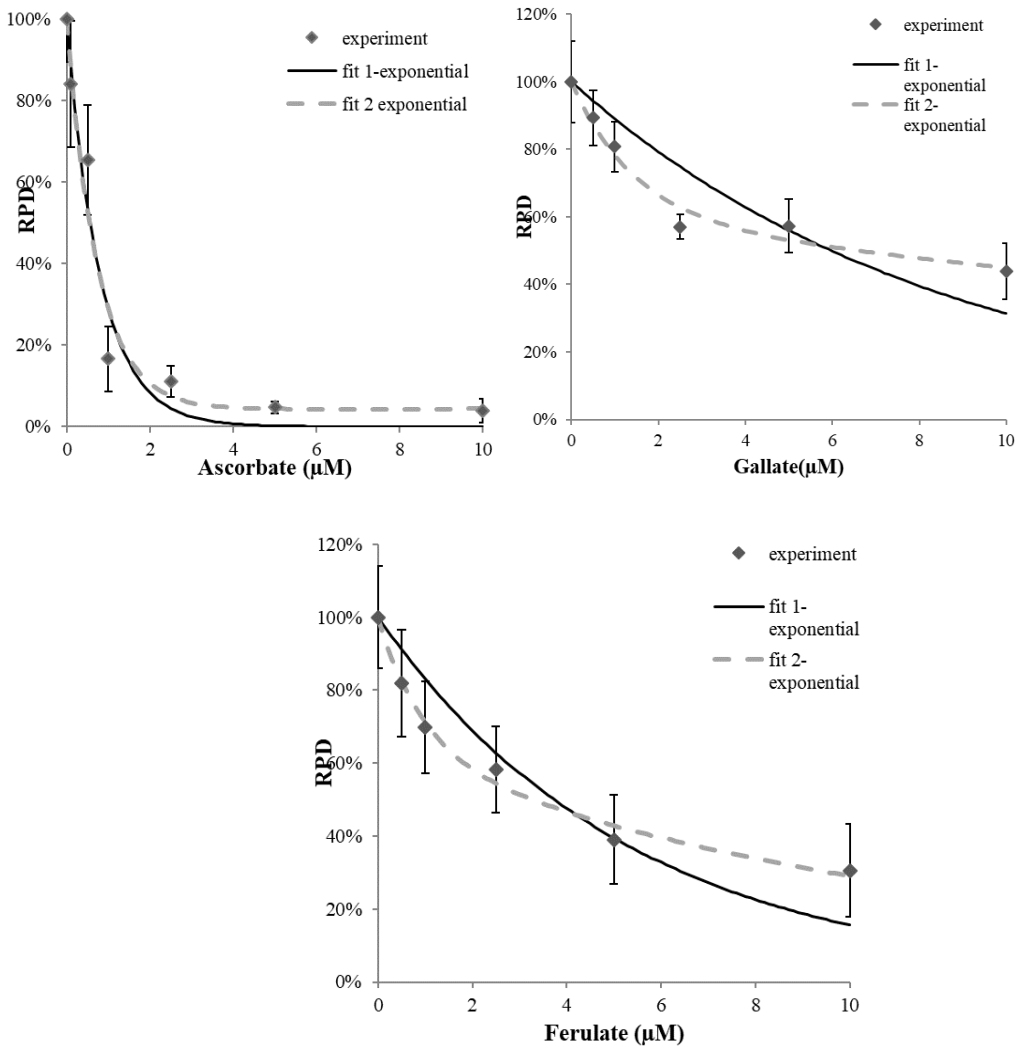


Figure 5.1. Concentration dependence of RPD, based on data at 120 minutes.

Table 5.1. Numerical fits to the data in Figure 5.1.

Antioxidants	Fit 1-exponential	R^2	IC_{50}	Fit 2-exponential	R^2	IC_{50}
Catechin	$y = e^{-0.848x}$	0.985	0.8	$y = 0.428*e^{-0.293x}+0.572*e^{-1.935x}$	0.998	0.7
Rutin	$y = e^{-0.679x}$	0.870	1	$y = 0.424*e^{-0.086x}+0.576*e^{-3.223x}$	0.997	0.6
Ascorbate	$y = e^{-1.239x}$	0.965	0.5	$y = 0.042*e^{-0.005x}+0.958*e^{-1.352x}$	0.967	0.5
Gallate	$y = e^{-0.116x}$	0.859	5.9	$y = 0.602*e^{-0.029x}+0.398*e^{-0.699x}$	0.975	6.6

Ferulate	$y = e^{-0.186x}$	0.939	3.7	$y = 0.628*e^{-0.077x}+0.372*e^{-1.051x}$	0.990	3.2

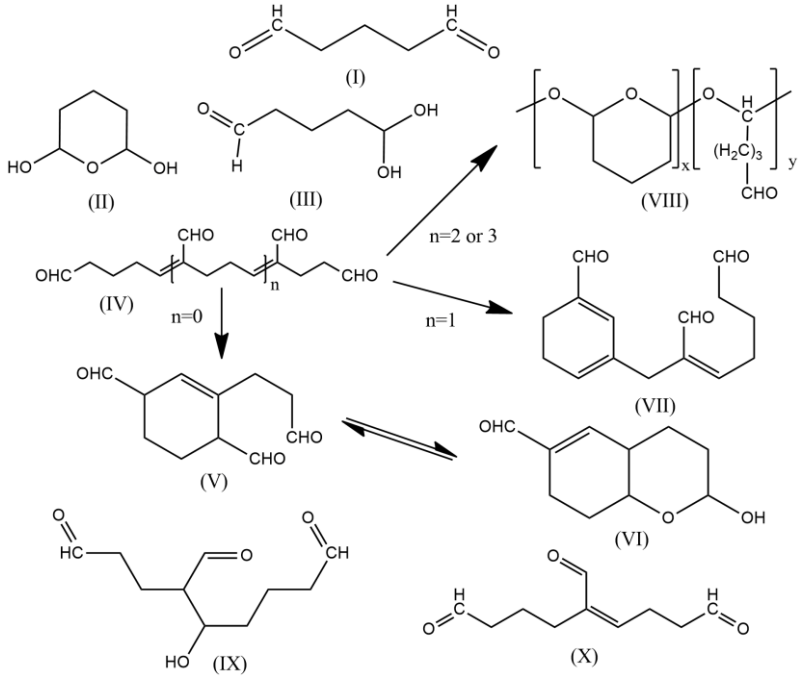
The data in Figures 5.1 allow for calculation of an IC_{50} parameter (concentration at which the antioxidant reaches 50% RPD), which are thus listed in Table 5.1. The order and the magnitude of these values is essentially similar across the five antioxidants regardless of the time of reaction (120 vs. 180 min). Thus, the lowest IC_{50} is shown by ascorbate (~1 μM), followed by catechin and rutin with values essentially identical to each other and only slightly larger than ascorbate, and then by ferulate with a 3-fold increase, with gallate showing the highest value (twice as high as ferulate).

We have described here a quantitative assay for antioxidant reactivity, based on the inhibition of myoglobin-induced liposome autooxidation (ILA-Mb). The low concentrations of antioxidants required for this assay, as well as the presence of lipids, make it more suitable for less water-soluble antioxidants and also allow one to operate at more biologically-relevant concentration ranges of the substances to be tested.

6. On the stability of glutaraldehyde in biocide compositions

Among the wide range of uses of glutaraldehyde (industrial, agricultural, medical), its application as biocide in hospital settings is among the most important.[127] Determination of glutaraldehyde (GA) concentration is essential in contexts such as conformity checks in hospital-used biocides[128] or toxicity assays nature[129] (GA is a volatile substance and the vapors can irritate the skin, eyes, nose and lungs).[130] However, even in "pure" solutions, GA (I in Scheme 6.1) undergoes a wide range of reactions - including at a first stage cyclization (II), hydrate formation (III), dimerization via aldolic (IX) and crotonic condensation (X). Subsequent reactions also occur, as previously described. Scheme 6.1 illustrates the chemical structures of products previously shown to be present in GA solutions.[137] GA's two aldehyde groups allow it to be used as cross-linking reagent especially targeting the amino groups of proteins, producing polymeric as well as nonpolymeric species; at large concentrations, this crosslinking leads to the biocidal effects.[138] Recent public investigations on the conformity of the chemical composition of biocides currently in use in Romanian hospitals[139140] have

reportedly found GA (as a key ingredient) to be up to 10-fold diluted with respect to the nominal concentration listed for the respective products.



Scheme 6.1. Chemical structures of products shown to be present in GA solutions.

Reported here is an exploration of experimental and theoretical data that may allow more informed analyses of GA concentrations in biocides as well as generally in GA-containing solutions.

Glutaraldehyde generally exerts its biocide activity by crosslinking the amine groups on proteins via imine group formation. Titration with amines has indeed been previously proposed as a tool for quantitating aldehydes in solution, following the distinct absorbance of the newly-formed imine group.[149] Indeed, the imine reaction product resulted from glycine and GA can be followed at 270 nm, without interference from the much weaker absorption peaks of GA at 235 nm and 280 nm.[150] Figure 6.1 shows such data; after only two weeks of incubation at 40°C the concentration of glycine-titratable aldehyde groups (GTAP) appears reduced to half. Considering that a simple aldol condensation reduces the number of titratable groups by one third, then Figure 6.1 suggests that already after two weeks at 40°C all of the GA has condensed.

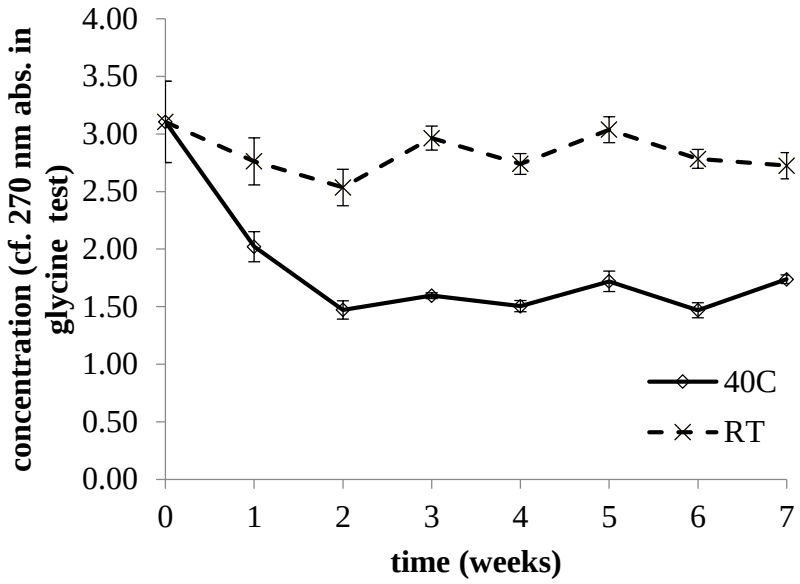


Figure 6.1. Time evolution for 8 weeks of GA concentration at 40°C and at room temperature, as measured by titration with glycine.

Glutaraldehyde is extensively employed for the polycondensation of hemoglobin (Hb) and other proteins.[115143151] Figure 6.2 shows such data, where the efficiency of the condensation is estimated using SDS-PAGE, with Table 6.1 showing the changes in relative proportions of fractions of various molecular weights.

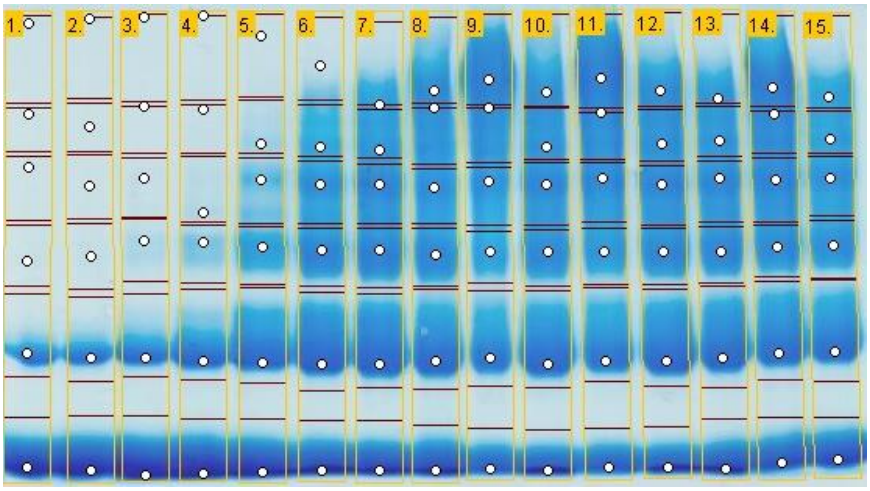


Figure 6.2. 12% SDS-PAGE of 1.5 mM hemoglobin polymerization with varying concentrations of GA: 1, 2: 0 mM, 3: 0.5 mM, 4: 1 mM, 5: 3 mM, 6: 5 mM, 7: 7 mM, 8: 3 mM, RT, week 8, 9: 5 mM, RT, week 8, 10: 3 mM, 40°C, week 8, 11: 5 mM, 40°C, week 8, 12: 3 mM, 1,2% isopropanol, 13: 3 mM, isopropanol, 60°C, 5h, 14: 5 mM, isopropanol, 60°C, 5h, 15: 3 mM isopropanol, 40°C, week 8.

Table 6.1. Profile of relative intensities of protein bands.

Sample / ~MW (kDA)	250	130	100	70	55	35
0 mM GA	0	0	0	0.01	0.49	1
0.5 mM GA	0.01	0	0.01	0.06	0.61	1
1 mM GA	0	0	0.03	0.18	0.81	1
3 mM GA	0	0.05	0.34	0.49	1	0.82
5 mM GA	0.08	0.43	0.65	0.56	1	0.67
7 mM GA	0.28	0.51	0.68	0.58	1	0.67
3 mM GA, RT, week 8	0.65	0.64	0.58	0.57	1	0.77
5 mM GA, RT, week 8	1	0.6	0.52	0.36	0.75	0.52

These data do confirm that over time GA has gradually been consumed from the solution. However, they also suggest that some of the GA derivatives formed during storage (dimers, oligomers, etc. cf. Scheme 6.1) may be more efficient crosslinkers than the monomer. This then puts a limit on the degree to which an SDS-PAGE assay may be employed for assessing the concentration of GA solutions.

Titration with glycine, followed at 270 nm, that the sample exposed two weeks at 40°C appear to reduce the number of titratable carbonyl groups to a degree equating 100% bi-molecular condensation of all GA in solution (i.e., where no free/intact/monomeric GA has remained in solution). SDS-PAGE experiments reveal an unpredictable character of GA over time in the polymerization process. Protein crosslinking reactions suggest some of the degradation products of GA (dimer, oligomers) may be more efficient than the monomer in protein crosslinking. Of the methods explored here for estimating the integrity of GA solutions, the most convenient appears to be titration with glycine using the absorbance at 270 nm.

7. HOMO-LUMO gaps and UV-Vis spectra in phenothiazine candidates for tumor-labeling agents

Ovarian cancer is the second most common cause of death in Europe. For now, the best treatment for ovarian cancer surgery and chemotherapy.[153] The aim of this study is to discover a new, better and efficient solution, to find the suitable substance to use in near-infrared (NIR)

fluorescence imaging to improve the accuracy of imaging in ovarian cancer surgery. In order to achieve this, we started to look for the best potential substance to use as specific fluorescent probe for the folate receptor alpha for the visualization in real time of ovarian tumors. Alpha folate receptor was discovered in 1991 as a tumor marker for ovarian cancer.[155]

The targeted substance must have absorption maxima in the NIR field, and have zero toxicity.[156] A number of cationic dyes, with a phenothiazinium chromophore system and various auxochromic substituents attached were the main subject to this study. In order to achieve the object of the study, advanced molecular modelling methods were employed to calculated the UV-Vis spectrum of a series with symmetrical and asymmetrical substituents attached to the basic phenothiazine structure.[157]

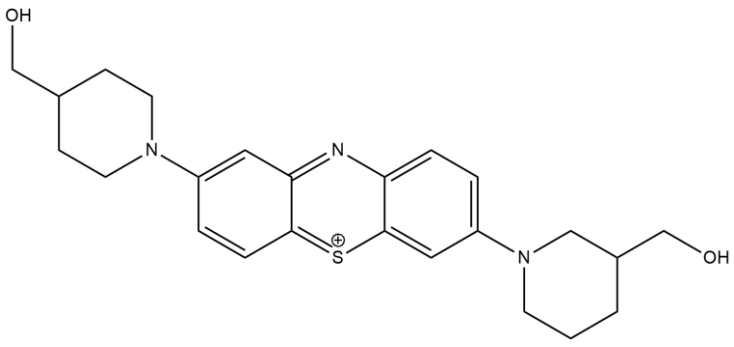
Table 7.1. The relevant maxima positions and intensities with the atomic charges of the central atoms in the models examined.

Substituent	**central N charge**	**S charge**	**LWHIM**	**OSLWHIM**	**LWM**	**OSLWM**
methyl/methyl	0.125	-0.531	444	1.240	450	0.008
ethyl/ethyl	0.141	-0.555	449	1.365	900	0.0000165
propyl/propyl	0.142	-0.59	455	1.402	455	1.402
butyl/butyl			457	1.494	926	7.26E-26
t-butyl/t-butyl			632	0.786	1302	2.55E-22
phenyl/phenyl	0.169	-0.527	508	1.380	508	1.380
phenyl-OH/phenyl-OH			583	0.822	583	0.822
phenyl-Oethyl/phenylOethyl			629	1.009	629	1.009
phenyl-$N(CH_3)_2$/phenyl-$N(CH_3)_2$			809	1.149	809	1.149

phenyl-NH_2/phenyl-NH_2			706	0.997	706	0.997
naphthyl/naphthyl			704	0.581	704	0.581
naphthyl-$N(CH_3)_2$/naphthyl-$N(CH_3)_2$			913	0.987	1399	0.003

The aim of these calculation was to identify systems with intense maxima in the NIR field. As shown in Table 7.1, form the aliphatic systems series the closest to the objective is symmetrical t-butyl system, at 632 nm – which is still insufficient. Some aliphatic systems also have maximum in the NIR region, but with very low intensity. Also, the widest delocalization in aromatic systems (naphtyl vs phenyl, or substituents grafting on phenyl/naphthyl) brings an advantage. The most useful synthetic target appears to be the one with (diamino) – naphthyl substituents.

Also, examined was phenothiazinium chromophore with pyrrolidinyl and piperidinyl substitutents in symmetrical and asymmetrical positions. The best substituent with the maximum wavelength is N-(3-hydroxymethyl) piperidinyl in the asymmetric position 3.8 (931 nm).



LWHIM (nm)	**OSLWHIM**	**LWM (nm)**	**OSLWM**
465	0,267	931	0,076

Figure 7.2. 3,8-disubstituted N-(3-hydroxymethyl) piperidinyl cation with the relevant maxima positions and intensities.

The ground state geometry of N-(3-hydroxymethyl) pyrrolidinyl and (3,7-bis-N-(3-hydroxymethyl) piperidinyl) phenazathionium were optimized in vacuum and in solvents using DFT method and compared with the experimental values. Data is shown here only for N-(3-hydroxymethyl) pyrrolidinyl phenazathionium.

For the date referred to N-(3-hydroxymethyl) pyrrolidinyl phenazathionium, the maximum calculated absorption in solvent is for dimethyl sulfoxide, 540 nm and 1.16 oscillator strength (corresponding to ~ 46000 M^{-1} cm^{-1}), and 675 nm determined experimentally. Also, the solvent with the same value of maximum absorption determined experimentally is water.

Table 7.1. Experimental and computational absorption data of N-(3-hydroxymethyl) pyrrolidinyl phenazathionium (1.13 oscillator strength corresponds to ~ 45810 M^{-1} cm^{-1} extinction coefficient).

Solvent	**λabs^{exp}**	**λabs^{DFT}**	**f**	**Major contribution (%)**
Vacuum		506	0.97	H ⟶ L 76%
Acetone	664	536	1.14	H ⟶ L 87%
Acetonitrile	661	536	1.13	H ⟶ L 87%
Dichloromethane	664	539	1.16	H ⟶ L 70%
Dimethyl sulfoxide	675	540	1.16	H ⟶ L 88%
Tetrahydrofuran	-	538	1.15	H ⟶ L 88%
Ethanol	668	536	1.14	H ⟶ L 87%
Water	675	535	1.13	H ⟶ L 88%

λabs^{exp} –experimental absorption maxima value

λabs^{DFT}- computational absorption maxima value

f - oscillator strength

8. General conclusions

Various methods of investigation – mostly based on UV-vis spectroscopy, fluorescence spectroscopy and computational chemistry, but also including NMR, Raman, GC-MS - were employed in order to assessed the multiple facets of biologically relevant redox reactivity in small molecules.

Redox properties of plant extracts with high content in antioxidants were evaluated using standard assays of antioxidant capacity but also newly-developed methods that uses hemoglobin as biologically relevant reagent.

A new version of an antioxidant activity assay was developed using liposome-myoglobin mixture reagent for a better understanding of antioxidant-protein interaction while also having better biological relevance in comparison with classical antioxidant assays.

The redox reactivity of glutaraldehyde solutions was examined in order to establish the exact concentration and the link between the concentration and its biocidal activity through the multitude of the spectroscopic methods used.

Computational methods were employed for investigation of HOMO-LUMO gap and predict UV-Vis spectra for a series of redox-active substances with potential biomedical applications.

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