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Biomimetic Models of Radical Stress and Related Biomarkers

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Abstract: The biological consequences of free radical production is the central subject of a very lively scientific debate, focusing on the estimation of the type and extent of damage, as well as the efficiency of the protective and repair systems. When studying free radical based chemical mechanisms, it is very important to establish biomimetic models, which allow the experiments to be performed in a simplified environment, but suitably designed to be in strict connection with cellular conditions. The biomimetic modeling approach has been coupled with physical organic chemistry methodologies and knowledge of free radical reactivity. Molecular basis of important processes have been identified, building up molecular libraries of products concerning unsaturated lipids, sulfur-containing proteins and nucleic acids, to be developed as biomarkers. Ongoing projects in our group deal with lipidomics, genomics and proteomics of free radical stress and some examples will be described.

Keywords: Biomarkers · Cyclonucleosides · Free radicals · Tandem protein–lipid damage · *Trans* lipids

1. Biologically Relevant Small Radicals

Superoxide dismutase (SOD) and nitric oxide synthase (NOS) are two classes of enzymes that control the production of superoxide radical anion ($O_2^{\bullet-}$) and induce the formation of nitric oxide ($^{\bullet}NO$), respectively, as shown in reactions (1) and (2).[1,2]

Under physiological conditions, concentrations of ~0.1 nM $O_2^{\bullet-}$ and ~10 nM $^{\bullet}NO$ play a role in regulating the activation of transcription factors, cell proliferation and apoptosis. During inflammatory response, their concentration can increase up to 100-fold excess. These two radicals are the progenitors of endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS).

The ROS/RNS network includes molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$), as well as radicals such as hydroxyl radical ($HO^{\bullet}$), nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radical anion ($CO_3^{\bullet-}$). The ROS/RNS network also functions as an efficient cellular defense mechanism, by playing an important role for eliminating viral and microbial infections. The overproduction of ROS/RNS has been linked with the etiology of various diseases. The main processes that generate $HO^{\bullet}$ radicals are depicted in reactions (3)–(5) and are the Fenton reaction of H_2O_2, the reduction of HOCl by superoxide radical anion and the spontaneous decomposition of protonated $ONOO^-$, respectively.[1,2]

In our laboratory the effect of radical production has been studied in aqueous systems using a biomimetic modeling approach, coupled with physical organic chemistry methodologies. Molecular basis of biologically relevant radical processes, as well as molecular libraries of products concerning the transformations of unsaturated lipids, sulfur-containing proteins and nucleic acids have been obtained, showing promising applications in biomarker discovery and disease prevention.

2. Biomimetic Model Based on Radiation Chemistry

Radiolysis of neutral water leads to reactive species, including solvated electrons (e_{aq}^-), hydrogen atoms ($H^{\bullet}$) and hydroxyl radicals ($HO^{\bullet}$), together with H^+ and H_2O_2, as shown in reaction (6). The values in parentheses represent the radiation chemical yields (G) in units of μmol J^{-1}.[3]

In a N_2O-saturated solution (~0.02 M of N_2O), e_{aq}^- are transformed into $HO^{\bullet}$ (reaction (7), $k_7 = 9.1\times10^9\ M^{-1}\ s^{-1}$), affording a $G(HO^{\bullet}) = 0.55$ μmol J^{-1}, *i.e.* $HO^{\bullet}$ radicals and $H^{\bullet}$ atoms account for 90% and 10%, respectively, of the reactive species.

In the presence of thiols like the amphiphilic 2-mercaptoethanol (RSH), hy-

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

$$\text{Arginine} \xrightarrow{NOS} \text{Citrulline} + {}^{\bullet}NO \quad (2)$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + HO^- + HO^{\bullet} \quad (3)$$

$$HOCl + O_2^{\bullet-} \longrightarrow O_2 + Cl^- + HO^{\bullet} \quad (4)$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH \longrightarrow {}^{\bullet}NO_2 + HO^{\bullet} \quad (5)$$

$$H_2O \xrightarrow{\gamma} e_{aq}^- (0.27), HO^{\bullet} (0.28), H^{\bullet} (0.06), H^+ (0.27), H_2O_2 (0.07) \quad (6)$$

$$e_{aq}^- + N_2O + H_2O \longrightarrow HO^{\bullet} + N_2 + HO^- \quad (7)$$

$$HO^{\bullet}/H^{\bullet} + RSH \longrightarrow RS^{\bullet} + H_2O/H_2 \quad (8)$$

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drogen abstraction by HO• and H• directly produces thiyl radicals (reaction (8), k_8 = 6.8×10^9 M^{-1} s^{-1} for HO• and k_8 = 1.7×10^9 M^{-1} s^{-1} for H•).[3]

To investigate the reactivity of reductive species (e_{aq}^- and H•), HO• radicals have to be scavenged. To achieve this, the most efficient procedure is based on the addition of an alcohol, the most commonly used being *t*-BuOH (0.2–0.5 M), which efficiently scavenges HO• radicals but not H• atoms (reaction (9), k_9 = 6.0×10^8 M^{-1} s^{-1} for HO• and k_9 = 1.0×10^5 M^{-1} s^{-1} for H•).[3,4]

Thiyl radicals can be also generated by means of direct photolysis of 2-mercaptoethanol in the presence of *i*-PrOH (>0.2 M). In this condition, the generation of thiyl radicals and H• atoms occurs as shown in reaction (10) and the H• atoms are efficiently quenched by the alcohol (reaction (11), k_{11} = 7.4×10^7 M^{-1} s^{-1}). The $(CH_3)_2C(•)OH$ radical in turn reacts with the thiol to give the thiyl radical (reaction (12), k_{12} = 1.2×10^8 M^{-1} s^{-1}).[3]

$$HO^•/H^• + t\text{-BuOH} \longrightarrow (CH_3)_2C(OH)CH_2^• + H_2O/H_2 \quad (9)$$

$$RSH \xrightarrow{h\nu} RS^• + H^• \quad (10)$$

$$H^• + i\text{-PrOH} \longrightarrow (CH_3)_2\dot{C}OH + H_2 \quad (11)$$

$$(CH_3)_2\dot{C}OH + RSH \longrightarrow i\text{-PrOH} + RS^• \quad (12)$$

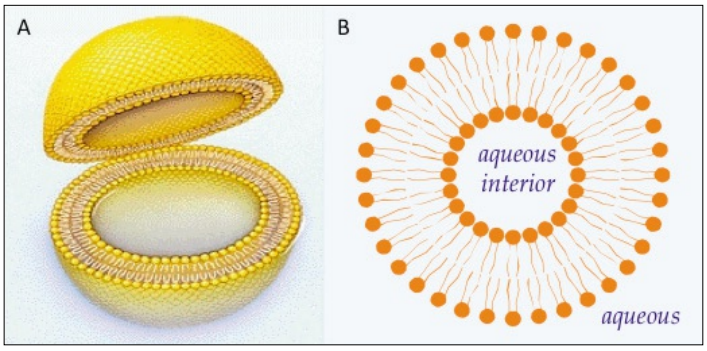


Fig. 1. Large unilamellar vesicles in two different representations.

3. Vesicles as Model Membranes

The natural structure of cell membranes is a double layer of phospholipids, which are amphiphilic molecules capable of self-organization.[5] The hydrophobic part mostly consists of fatty acid residues, that are carboxylic acids with a long hydrocarbon chain (up to 26 carbon atoms), saturated or unsaturated with up to six double bonds. A specific structural feature of naturally occurring mono- and polyunsaturated fatty acid (MUFA and PUFA) residues is the *cis* double bond geometry, whereas PUFA have the characteristic methylene-interrupted motif of the unsaturated chain.

Liposomes are the universally accepted models for cell membranes as they can closely simulate the bilayer structure. Liposomes can be represented as shown in Fig. 1, *i.e.* a double layer formed by spontaneous organization of the phospholipid components in water, delimiting an aqueous cavity. The fatty acid tails can be saturated or unsaturated, and the disposition of the double bonds in the vesicle depends on the supramolecular arrangement of the bilayer. Monolamellar vesicles are the closest models to membranes and they can be formed by different techniques, such as the extrusion[6] and the injection[7] methodologies.

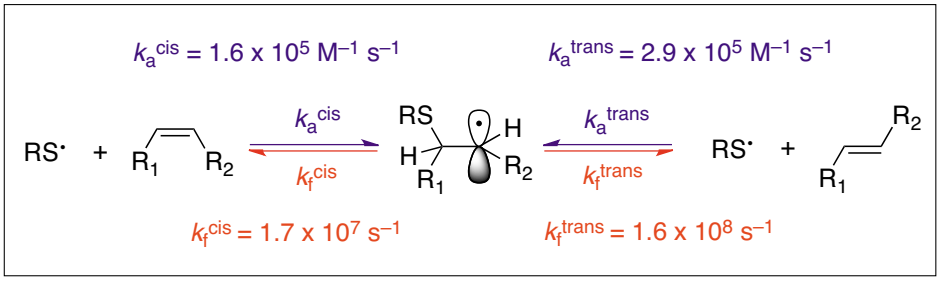
4. *Trans*-Fatty Acid Residues as Biomarkers of Radical Stress

Scheme 1 shows the reaction mechanism of *cis–trans* isomerization that consists of a reversible addition of radical RS• to the double bond. Indeed, the reconstitution of the double bond is obtained by β-elimination of RS• and the result is in favor of *trans* geometry, the most thermodynamically favorable disposition.[8] It is worth noting that i) the radical RS• acts as a catalyst for *cis–trans* isomerization, and ii) positional isomers cannot be formed as reaction products because the mechanism does not allow a double bond shift.[9,10] Considering polyunsaturated substrates, the isomerization mechanism occurs as a step-by-step process, *i.e.* each isolated double bond behaves independently as discussed above.[9,10]

Vesicles formed by unsaturated lipid were found to be a good biomimetic model for the double-bond isomerization. From the early studies,[9,10] arachidonic acid residues in membrane phospholipids emerged as relevant markers to be investigated, in order to distinguish endogenous *trans* isomers, formed by radical processes, from the exogenous *trans* isomers, known to derive from partially hydrogenated fats in the diet. Considering the biosynthetic paths of omega-6 fatty acids, the double bonds 5 and 8 of arachidonic acid can evidently be formed by desaturase enzymes and therefore have only a *cis* configuration, unless these positions are involved in a radical-based isomerization process in the membranes.

We extended the biomimetic investigation to biological systems, in order to prove the 'endogenous' *trans* lipid formation under strictly physiological conditions. It was important to deal with '*trans*-free' conditions, which means that the presence of any external source of *trans* fatty acid isomers is carefully checked. Cell membrane lipid composition was monitored during incubation in the absence and presence of thiol compounds, ensuring that no contribution of *trans* compounds could come from the medium. The *trans*-arachidonate content determined in membrane phospholipids of human leukemia cell lines (THP-1) provided the first indication of the occurrence of an endogenous isomerization process.[11] This opened new perspectives for the role of *trans* lipids in the lipidome of eukaryotic cells[12] and was followed by several other investigations in living systems.[13]

More recently peroxidation and isomerization processes, both supported by the free radical reactivity of thiol compounds, were evaluated in a biomimetic model of linoleic acid micelles. Indeed, the catalytic



Scheme 1. Reaction mechanism for the *cis–trans* isomerization catalyzed by thiyl radicals; Rate constants of addition (k_a) and fragmentation (k_f) at 25 °C.

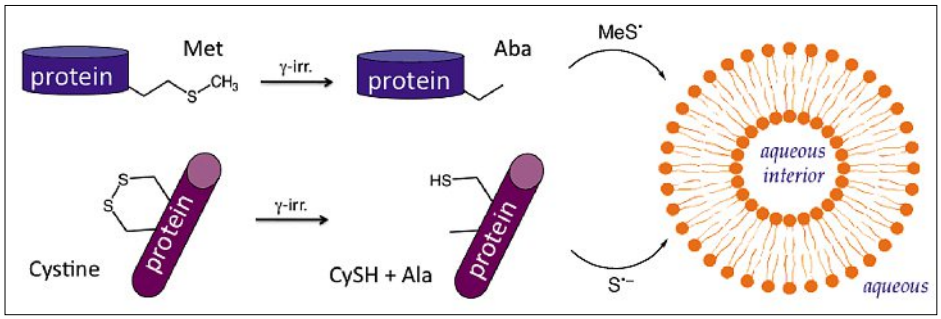


Fig. 2. Tandem protein–lipid damage under reductive radical stress.

process of *cis–trans* isomerization can occur parallel with radical chain peroxidation, producing in the same experiment *trans* fatty acids and hydroperoxides.[14]

Mono-*trans* isomers of cholesteryl linoleate and arachidonate have been synthesized by thiyl radical-catalyzed *cis–trans* isomerization and fully characterized. This small *trans* library allowed the identification to be carried out in human plasma by GC, Raman, and IR analyses, demonstrating for the first time the presence of specific regioisomers connected to free radical stress.[15]

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid present in fish oils used for omega-3 enriched foods and diets. The natural *cis* double bond geometry can be transformed to the *trans* configuration during the deodorization process utilized in the food industry. The analytical discrimination of the five possible mono-*trans* regioisomers represents a limiting step for the recognition and structure–activity relationship in connection with the harmful effects of *trans*-fatty acids in health. We carried out a dual synthetic strategy, providing new access to mono-*trans* EPA isomers and valuable information on GC and NMR characteristics for further applications in metabolomics and lipidomics. This library was used as an analytical reference for isomer determination in deodorized fish oils and the follow-up of rats fed fish oil diets, evidencing for the first time that mono-*trans* EPA isomers are incorporated in liver mitochondria membranes after dietary intake.[16]

5. Reductive Radical Stress and Tandem Lipid-Protein Damage

There are strong experimental supports that the origin of endogenous formation of *trans* lipids is correlated with radical stress. However, up to now it has not been demonstrated what the real culprits are for the *cis–trans* isomerization. Thiyl radicals are in the pole position on the basis of i) the efficiency of the thiyl radical-catalyzed *cis–trans* isomerization *in vitro*, ii) the presence of many sulfur-containing compounds in the cell, and iii) the evidence of *trans*-lipid increase in cultured cells in the presence of thiols ensuring that no contribution of *trans* compounds could come from the medium.[17]

Possible scenarios for the production of diffusible thiyl radicals were expanded by the discovery of free-radical damage to sulfur-containing proteins resulting in consequent isomerization of membrane lipids. The complex scenario of radical stress reactions affecting peptides/proteins can be better elucidated through the design of biomimetic studies simulating the consequences of the different free radicals attacking amino acids. In this context, ionizing radiations allowed the examination of the specific damage caused by H-atoms and electrons coupled with protons, thus establishing the molecular basis of reductive radical stress. This is an innovative concept that complements the well-known oxidative stress, also in view of a complete understanding of the global consequences of radical species reactivities on living systems. A recent review summarizes the knowledge of the chemical changes present in sulfur-containing amino acids occurring in polypeptides under reductive radical conditions, in particular the transformation of Met and Cys residues into α-amino butyric acid and alanine, respectively (Fig. 2).[18] Concomitant formation of diffusible sulfur-centered radicals (MeS• or S•−), able to migrate into the lipid bilayer, can induce *cis–trans* isomerization of unsaturated fatty acid residues.[19] Reductive radical stress, causing a desulfurization process, is therefore coupled with the formation of S-centered radicals that in turn can diffuse apart and become responsible for the damage transfer from proteins to lipids.

These reductive modifications assayed in different peptide/protein sequences (*e.g.* Met-enkephalin,[20] β-amyloid,[21] RNase A,[22] Human serum albumin[23]) constitute an integration of the molecular inventories that up to now take into account only oxidative transformations. They can be useful to achieve an integrated vision of the free radical reactivity in a multifunctional system and, overall, for wider applications in the redox proteomics field.

6. Purine 5',8-Cyclonucleoside Lesions

5',8-Cyclo-2'-deoxyadenosine and 5',8-cyclo-2'-deoxyguanosine in their 5'R and 5'S diastereomeric forms are tandem-type lesions observed among the DNA modifications and identified in mammalian cellular DNA *in vivo* (Fig. 3).[24] These lesions result from the chemistry of the C(5)' radicals generated by the attack of HO• radicals to 2-deoxyribose units.

Synthetic procedures of compounds **1**–**4** were developed starting from 8-bromopurine derivatives under continuous radiolysis or photolysis.[25,26] These procedures involve a radical cascade reaction that mimics the DNA damage causing the formation of 5',8-cdAdo and 5',8-cdGuo lesions. The reaction of HO• radicals with 2'-deoxyadenosine and 2'-deoxyguano-

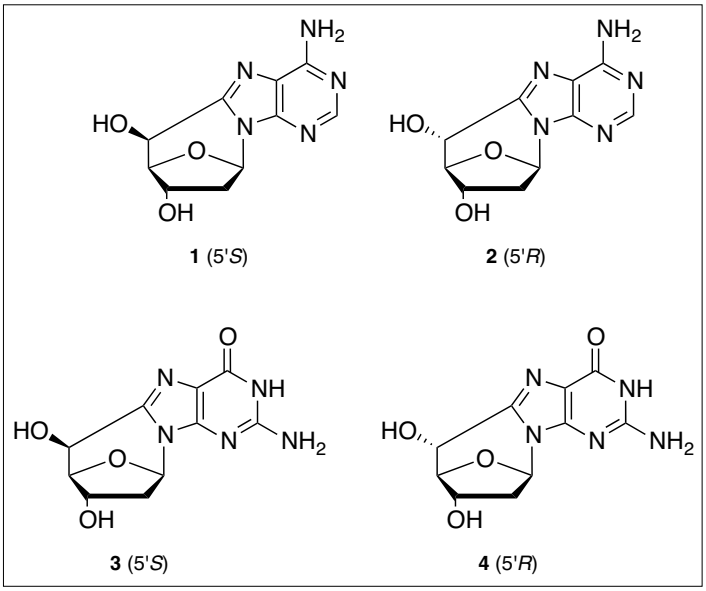
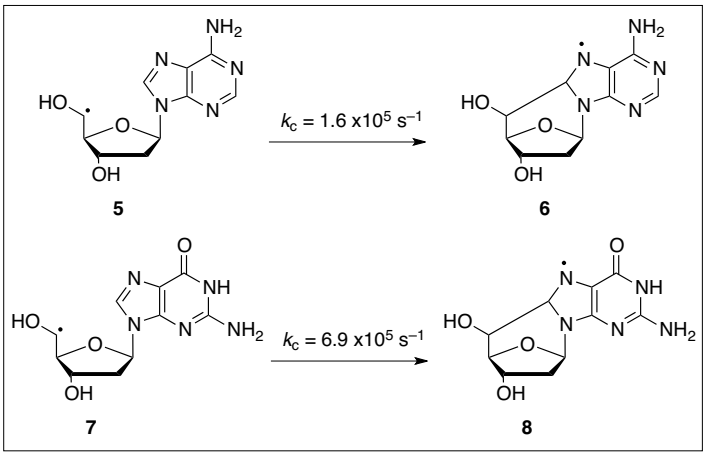


Fig. 3. The four purine 5',8-cyclo-2'-deoxynucleosides.



Scheme 2. Radical cyclization of C(5)' radicals in purine 2-deoxyribo derivatives.

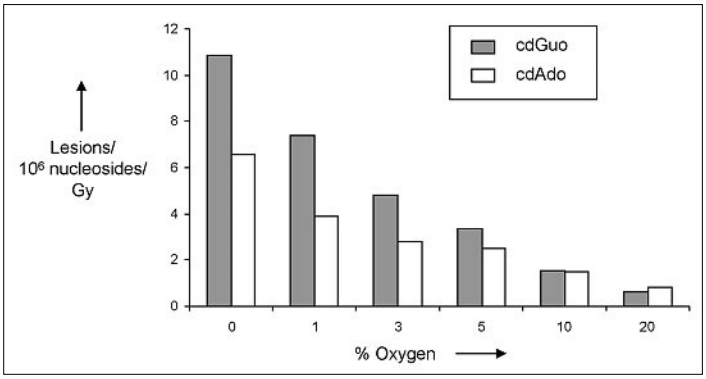


Fig. 4. Radiation-induced formation of 5',8-cdAdo and 5',8-cdGuo in N_2/O_2 saturated aqueous solutions of DNA.

sine under normoxic conditions was also investigated.[27] The minor path of C(5)' radical formation was found to be *ca*. 10% by quantifying the hydrated 5'-aldehyde in both experiments. Pulse radiolysis studies focused on the rate constants of cyclization at nucleoside level. Scheme 2 shows these cyclizations as well as the corresponding rate constants at room temperature. Both cyclizations are in the range 10^5–10^6 s^{-1}, radical **7** being four times faster that radical **5**.[25,28] Quantitative determination of these lesions in biological samples as biomarkers of free radical damage is a challenge. Results reported for irradiated samples of calf thymus DNA have been critically reviewed,[24] underlining the need for further research for the potential involvement of these lesions in human health. Fig. 4 illustrates the purine 5',8-cyclonucleosides measured as the sum of diastereomers by HPLC-MS/MS in enzymatically digested DNA samples: the diastereomeric ratio 5'R/5'S being ~4 and ~3 for 5',8-cdAdo and 5',8-cdGuo, respectively.[29]

7. Conclusions and Future Directions

This mini-review offers an overview of the main processes studied in our laboratory. Ongoing projects also deal with the application of the molecular libraries as biomarkers of free radical stress in lipidomics, genomics and proteomics. Our biomimetic approach is a convenient tool for the examination of free radical reactivity, particularly for a chemical biology approach where the behavior of free radical species in the liposomal or oligonucleotide models can satisfactorily be transferred to the transformations occurring in the biological context. In this view an improvement of interdisciplinarity is needed, among the fields of chemistry, biochemistry, biology and medicine, in order to create a common territory where the achievements of free radical mechanisms can be straightforwardly transferred to a better comprehension of the biological pathways in health and diseases.

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