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Oximetry methods for evaluating the activity of nanoantioxidants (minireview)

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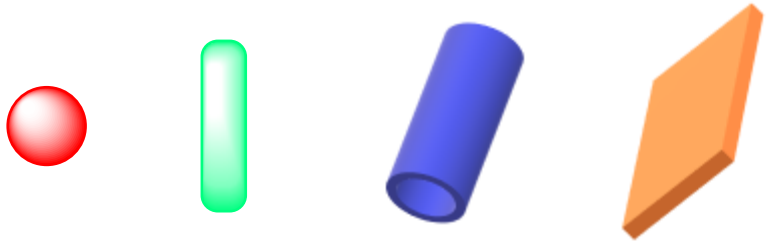
Nanomaterials are probably the most promising and unexplored frontier in the ongoing research for better antioxidants. Testing the efficacy of these materials however requires significant efforts to improve current protocols. In this minireview, we describe the advantages of the methods based on the detection of oxygen consumption during the autoxidation of an organic substrate. The differential oxygen uptake apparatus is a powerful and cost-effective way to measure antioxidant activity from inhibited autoxidation studies, especially in organic solvents. Besides, electrochemical or optical oxygen probes are specifically designed to quantify dissolved O_2 in water, where autoxidations are performed by using micellized linoleate or liposomes as oxidizable substrates. Selected examples of the use of these techniques to rationalize the effect of nanoantioxidants are reported.

Keywords: Antioxidants, nanomaterials, nanoantioxidants, peroxyl radicals, oximetry

INTRODUCTION

Nanomaterials having antioxidant activity are an emerging trend in the field of antioxidant research.[1-3] Nanomaterial are objects having at least one dimension smaller than 1000 nm and, on the basis of their topology, they can be classified into nanoparticles, nanorods, nanotubes and nanosheets Figure 1.[1]



a) Nanoparticles b) Nanorods c) Nanotubes d) Nanosheets

Figure 1. Morphology-based classification of nanomaterials.

At the nanometer size, distinct and peculiar characteristics that differ from those observed in the bulk material may arise, such as bright colors and the ease with which they are absorbed by living organisms.[4] Nanoparticles may contain a magnetic core[5] or may be covered with active surfaces that are recognized by specific cells.[6] Degradable nanoparticles[7] and nanotubes[8] can serve as carrier of drugs, being able to reach specific organs and allowing triggered release of the payload.[9] Nanomaterials have a huge surface area, allowing enhanced reactivity and catalytic activity.[4] Exploiting nanomaterials to develop new antioxidants may lead to the improvement of existing oxidation inhibitors, or even to the discovery of new mechanisms of action. Antioxidants are a well-known class of compounds able to retard the oxidation under air of organic substrates. Discovery of antioxidants and of the radical chemistry associated with their activity dates to the middle of the twentieth century, with the exploitation of natural rubber and the discovery of the first synthetic polymers.[10] The interest toward antioxidants was then boosted by the importance of oxidative stress in aging and in the development of many diseases.[11] Recent innovations in the field of antioxidants include the use of heterocycles (such as 2,4-diazaphenoxazine),[12] of organo-chalcogenides (phenols containing selenium and tellurium),[13-15] and of stable radicals (nitroxides).[16-17] Besides these examples, there is a growing number of antioxidants based on nanomaterials, that are also called "nanoantioxidants" (NAox).[18] Their classification into three main families has been recently proposed: 1) nanomaterials with intrinsic radical-trapping activity; 2) inert scaffolds with covalently linked antioxidants and 3) nanocarriers of antioxidant molecules.[3] Class 1 and 2 NAox can be further divided on the basis of their mechanism of action, which may be that of mimicking antioxidant enzymes such as catalase or superoxide dismutase,

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or "classical" radical trapping, as shown in Figure 2.[3].

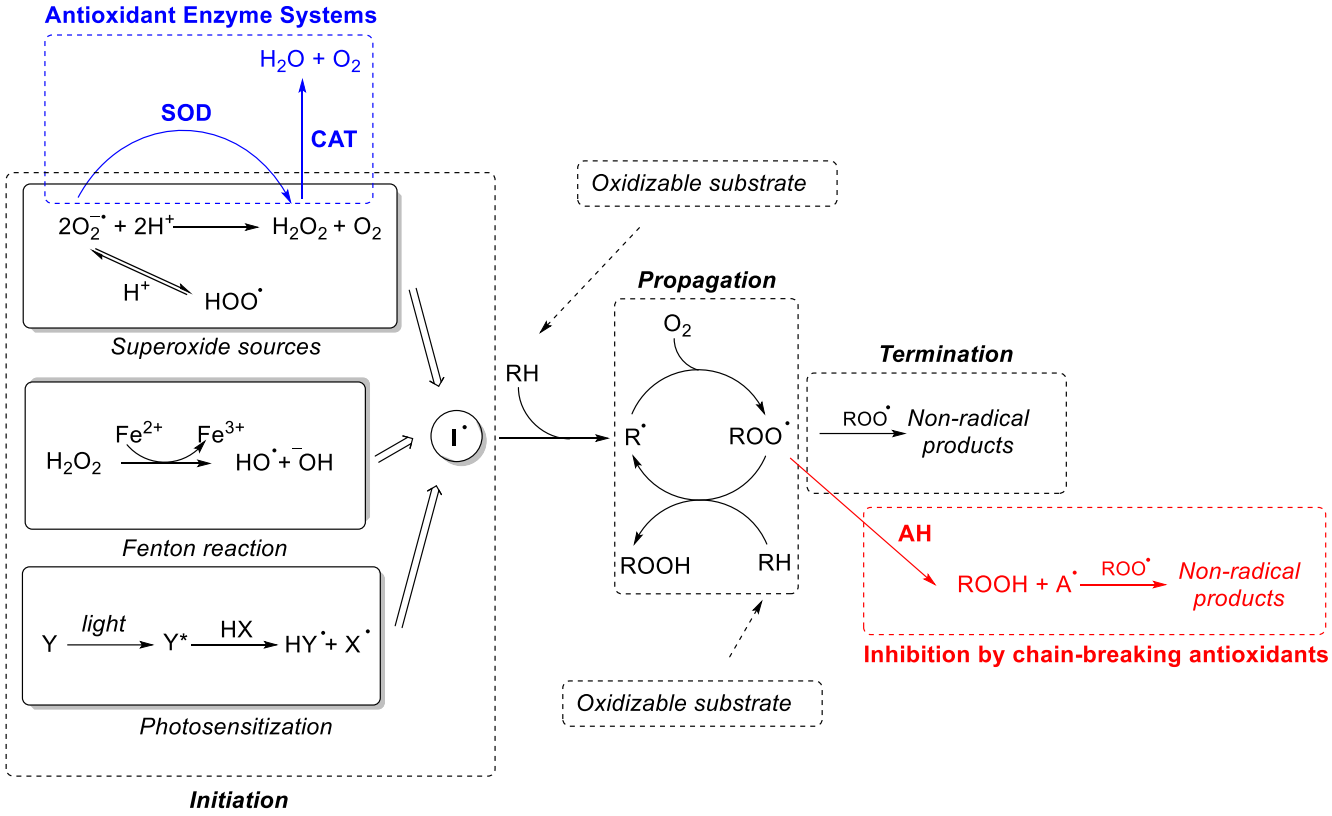

Figure 2. Mechanism of autoxidation of an organic substrate and different kinds of antioxidant action. SOD= Superoxide dismutase; CAT= Catalase.

MECHANISM OF ACTION OF ANTIOXIDANTS

If excluding the indirect activity based on induction of antioxidant enzymes, that is apparent only in biological systems, the direct antioxidant effect can be due to two different mechanisms: 1) reduction of the formation of free radicals and 2) trapping of free radicals.[19] The antioxidants that belong to the former family are called "preventive" and can act in several ways, such as by reducing the concentration of hydroperoxides and by removing transition metals. Antioxidants belonging to the latter family are perhaps the most renowned ones and include polyphenols, vitamins C and E and most synthetic antioxidants. All these compounds possess easily cleavable O-H bond and give rise to stable radicals that do not propagate the oxidative chain. These compounds are called "chain-breaking" because they are able to trap peroxyl radicals that are the chain-carrying species of the autoxidation reaction (Figure 2).[19]

Although various radical species, such as alkyl or alkoxyl, may be formed during the autoxidation of an organic substrate, only peroxyl radicals are usually quenched by antioxidants. The reason is that alkyl radicals react at diffusion-controlled rate with O_2, which is present at millimolar concentrations in most organic samples, so their reaction with antioxidants, usually present at a much lower concentration, is almost negligible.[19] Likewise, alkoxyl and hydroxyl radicals are extremely reactive with all organic substrates, thus this reaction can not be prevented by antioxidants.[19] On the other hand, peroxyl radicals react with relatively low rate constants with organic molecules, with values ranging from 0.1 to 100 $M^{-1}s^{-1}$ depending on the substrate. For this reason, even relatively low concentrations of an antioxidant are able to prevent the propagation of the autoxidation.[20]

MEASURE OF THE CHAIN-BREAKING ANTIOXIDANT ACTIVITY OF NANOANTIOXIDANTS

The measure of antioxidant activity is nowadays a controversial topic, because of the presence in literature of many simplified methods which have little relationship with the mechanism depicted in Figure 2.[21] As a matter of facts, the best methods should be based on the ability to slow down the autoxidation of a relevant organic substrate, under conditions that should be as similar as possible to those occurring in real materials. Spontaneous autoxidation of purified vegetable or animal oils is a slow process that takes several days to proceed, so it is unsuited to the efficient analysis of many samples.

Small amounts of azoinitiators, which generate initiating radicals at a constant rate, are the most practical way to make autoxidations fast and reproducible.[21] The ongoing autoxidation of a given substrate can be followed by several methods: iodometric titration of peroxides, conjugated dienes formation (in the case of polyunsaturated lipids), formation of volatile acids, probe bleaching and oxygen consumption.[19,21] These golden standard methods allow one to follow in real time lipid autoxidation and provide the maximum amount of information about antioxidants. However, as these methods are time consuming and may require specific equipment or reactants, simplified (and less informative) tests based on spectrophotometric measures at a fixed time of product formation or probe bleaching are many times adopted. Two popular examples are the TBARS (thiobarbituric acid reactive substances) method and the β-carotene bleaching test. The former relies on the spectrophotometric detection at a fixed time of malondialdehyde, a secondary oxidation product of fatty acids; the latter on the measure of the decrease of the absorption of β-carotene when it is added to an oxidizing lipid.[19,21]

Besides methods based on the autoxidation of an organic substrate, there are many other ones that have no relationship with autoxidation, but are claimed to provide information about antioxidants, such as ORAC (Oxygen radical absorbance capacity) test, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, FRAP (Ferric Reducing Antioxidant Power) to mention only a few.[19,21,22] These tests should be used with caution and only in preliminary screening because may provide false positive results and incorrect structure-activity relationships.[21]

ADVANTAGES OF OXIMETRY ON COLORIMETRIC OR INDIRECT METHODS

Adapting existing protocols for the assessment of the antioxidant activity of nanoantioxidants is challenging, because of their distinct chemico-physical characteristics. Assays relying on spectrophotometric or spectrofluorimetric determinations suffer from the light scattering displayed by many nanomaterials. Metal nanoparticles may have intense colors deriving from plasmon resonance, while carbon-based nanomaterials are strongly absorbing.[3] In this context, methods based on O_2 consumption have no limitation respect the use of nanoantioxidants. The instrumentation to perform these measures belong to two broad families: i) pressure gauges and ii) O_2 probes. The former method is usually based on differential pressure transducers, that measure the small pressure differences between a sample and a reference reaction flask, and that can be applied to both organic solvents and aqueous solution (Figure 3A).[20,23] The latter method comprises optical or electrochemical O_2 sensing. Optical oxygen detection is based on the fluorescence quenching, caused by O_2, of a fluorescent probe absorbed on the tip of an optical fiber immersed in the sample. This equipment can be used both in air and in water, but is incompatible with organic solvents.[24] Electrochemical detection of water dissolved O_2 is based on a polarographic Clark electrode, that produces a current that is proportional to the amount of O_2 that reaches the electrode tip after crossing a polymeric membrane. This method has been used to measure chain-breaking antioxidant activity in micelles or liposomes,[25,26] and to assess the catalase-like activity of nanoantioxidants, by measuring the O_2 formation. A typical experimental setting for the measurement of the antioxidant activity by using an oxygen uptake apparatus consists in choosing a suitable solvent, that may be water or an inert organic solvent (such as acetonitrile or chlorobenzene), and an oxidizable substrate, such as styrene, cumene, tetrahydrofuran, methyl linoleate, etc. Then, an azoinitiator soluble in the chosen solvent is added to the sample flask, such as azobis(isobutyronitrile) for organic solvents and 2,2'-azobis(2-methylpropionamidine) dihydrochloride or 4,4'-azobis(4-cyanovaleric acid) for water. Alternatively, the azoinitiator can be added to both the sample and the reference flasks, but in order to stop O_2 consumption in the reference flask, an excess of antioxidant is added to the latter. When a constant O_2 consumption is reached, a solution of the antioxidant is injected into the sample flask. Schematic examples of the O_2 uptake traces are reported in Figure 3B. After calibrating the instrument by performing the autoxidation of a reference substrate, the slope and the duration of the inhibited period (see trace b in Figure 3B) provide a quantitative evaluation of the rate constant of reaction with peroxyl radicals, and of the stoichiometry of the radical trapping, respectively.[20]

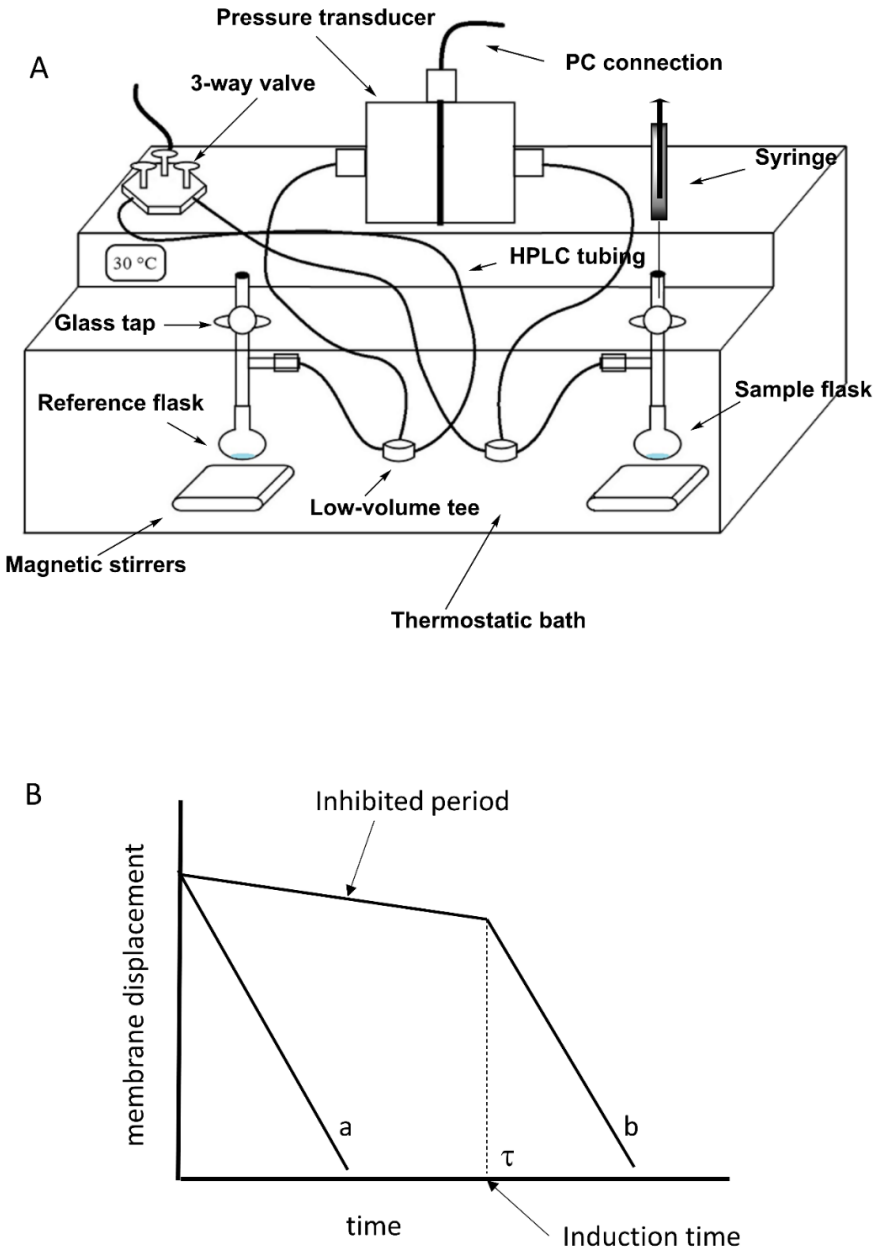

Figure 3. A) Schematic diagram of an oxygen uptake apparatus, based on a pressure transducer. B) oxygen consumption in the absence (a) and in the presence (b) of a chain-breaking antioxidant.

The first use of a differential pressure transducer to measure the activity of nanoantioxidants was reported by our research group in 2014 for magnetic nanoparticles bearing pendant Trolox (a synthetic analogue of α-tocopherol). This material is based on magnetic cobalt nanoparticles, coated by some layers of graphitic carbon, that confers stability and easy surface functionalization (Figure 4).[27] The same technique was also adopted by us to evaluate the activity of nanoantioxidants based on halloysite nanotubes (HNT), that is a natural aluminosilicate clay, having a hollow tubular structure consisting of (acid) siloxane groups on the outer surface and (basic) aluminol at the inner surface. The first material that was prepared was curcumin linked to the surface of HNT through a disulfide bond that could be cleaved in the presence of thiols (Figure 4).[28] Then, we studied a synergic co-antioxidant based on Trolox units covalently linked on the surface, and quercetin absorbed in the inner lumen. By using autoxidation studies, we demonstrated that the antioxidant activity of Trolox was enhanced by quercetin that was slowly released (Figure 4).[29] The last example regards the use of HNT loaded with ascorbic acid (vitamin C) as a strategy to reduce its oxidative degradation while preserving its antioxidant activity (Figure 4).[30].

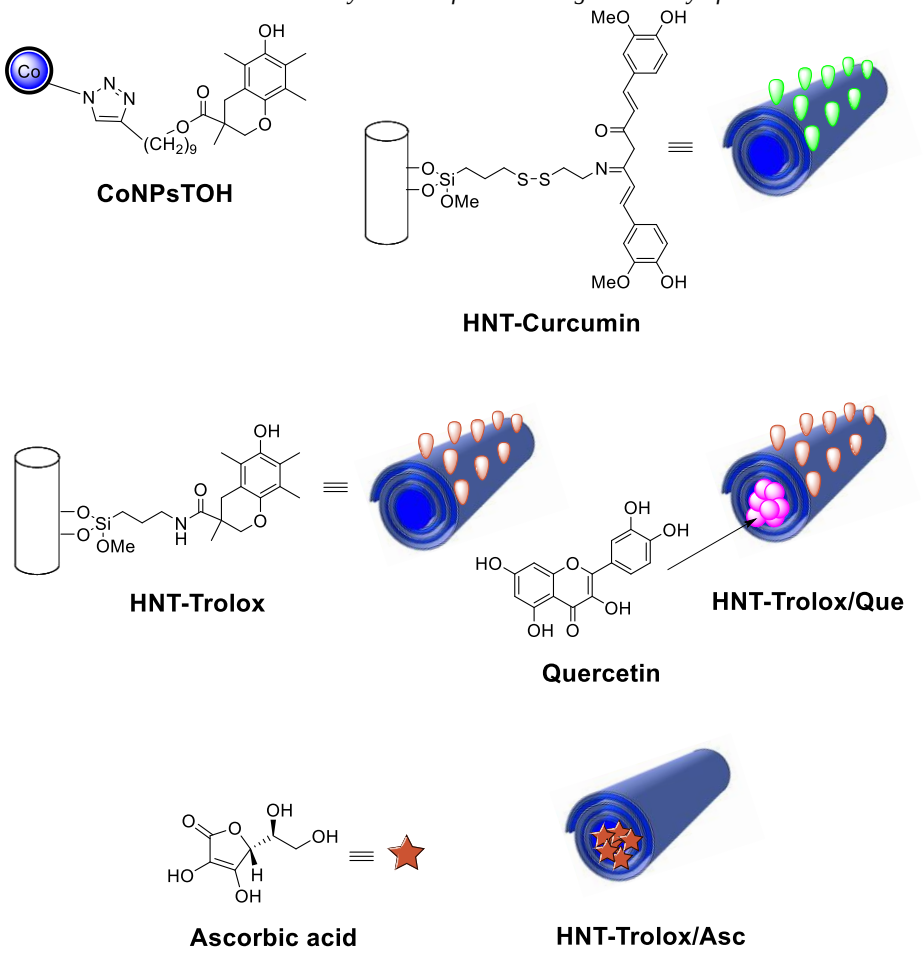

Figure 4. Nanoantioxidants whose antioxidant activity has been studied by measuring O_2 consumption by using an oxygen uptake apparatus based on a pressure transducer.

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Electrochemical oxygen probes are also extensively used to measure the catalase-like activity of nanomaterials, as they determine O_2 produced by H_2O_2 decomposition, at least until O_2 saturation is reached. Examples reported in literature include cerium oxide, cobalt oxide, gold, platinum, palladium, iron oxide nanoparticles.[3]

CONCLUSIONS

The use of nanomaterials as antioxidants is a trend that has been increasing in the past few years,[31-33] and that requires significant efforts to adapt current protocols to properly evaluate the antioxidant efficacy. In this minireview, the advantages of methods based on the detection of oxygen consumption during the autoxidation of a suitable substrate have been presented. The differential oxygen uptake apparatus is a powerful and cost-effective way to measure antioxidant activity from inhibited autoxidation studies, especially in organic solvents. Besides, dissolved oxygen probes allow similar measures in aqueous solution, by using micellized linoleate or liposomes as oxidizable substrates.[25,26] Oximetry techniques, that have been exploited in the past to rationalize the activity of small-molecule antioxidants,[34-35] are expected to be useful also to explore the behavior of nanoantioxidants.

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