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Evaluation of the Structure-Activity Relationship of Hemoproteins through Physicochemical Studies: Hemoglobins as a Prototype of Biosensor

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Abstract

In the present work, we have studied a group of prerequisites in terms of "structure-function relationship" of hemoproteins, especially hemoglobins, emphasizing the role of the heme and its chemical environment in the biochemical and physicochemical properties of the biomolecule. We have discussed the ferrous center and its properties as coordination center; the macrocyclic ligands, especially the porphyrins; the esterochemical and electronic properties of the iron-porphyrins (heme groups); and the interaction between heme groups and globins, which is related to several redox and oligomeric properties of hemoprotein systems and its potential applications with respect to novel materials. One of the main uses of hemoglobins in new materials is also discussed, which is its employment as a biosensor. Therefore, we have discussed the development of novel biosensors based on hemoglobins and their physico-chemical properties as well as on the main molecules of biological relevance that have been detected by these biosensors, such as hydrogen peroxide (H_2O_2), nitric oxide (NO), and cholesterol, among others. Indeed, several important biomolecules and biological processes can be detected and/or evaluated by devices that present hemoglobins as leading chemical components. Different apparatus are covered with respect to distinct characteristics, such as chemical stability, sensitivity, selectivity, reproducibility, durability, optimum conditions of measurements, etc. and their respective characteristics are analyzed.

Keywords: iron, hemoprotein structure-function relationship, macrocyclic ligands, porphyrin flexibility, biological molecules

1. Introduction

1.1. Hemoproteins

Hemoproteins constitute a very relevant group of proteins which present the heme as a prosthetic group. A prosthetic group is a chemical compound inserted in a polypeptide chain by chemical binding, which is not amino acid residue or peptide binding. The heme group is a coordination compound (metallic complex) that presents a ferric (Fe(III)) or ferrous (Fe(II)) ion as coordination center, which is strongly ligated to a macrocyclic ligand (Lewis base with more than three electron-donor sites), which, in the case of the heme, is a porphyrin. Therefore, the prosthetic group of hemoproteins is an iron-porphyrin compound. It is important to emphasize that the heme group is the main active site of the hemoproteins in which the iron ion plays an important role.

There are several types of hemoprotein in nature. One of the main differences that involve the several kinds of hemoproteins is associated to the number of polypeptide chains that can occur in each hemoprotein. Indeed, we can find hemoproteins with only one polypeptide chain, i.e., hemoproteins that do not have a quaternary protein structure (obviously, these hemoproteins present lower molecular mass), such as, for example, the cytochrome *c* [1]. On the other hand, there are giant hemoproteins with a great number of subunits (therefore, presenting a quaternary protein structure), such as the giant extracellular hemoglobin of *Lumbricus terrestris* (HbLt), which presents around 144 globin chains (subunits with heme group) and approximately 36 *Linker* chains (subunits with structural function that do not have any heme group). This kind of giant hemoprotein presents an extraordinarily high supramolecular mass, which, in the case of *HbLt*, is approximately of 3.6 MDa, presenting a very complex and well-organized tridimensional arrangement [2-4].

A second important difference between the various types of hemoproteins is related to the isolation level of the heme pocket. In fact, in most hemoproteins, there is a hydrophobic isolation around the heme (that is variable in intensity, depending on each specific hemoprotein), which is constituted by the lateral chains of the amino acid residues that are encountered in the neighborhood of the iron-porphyrin system. This isolation is relevant to preclude a significant grade of water molecule penetration in the heme pocket, which can provoke several chemical reactions involving the metallic center of this prosthetic group and, consequently, the loss of its biological function. Furthermore, heme isolation avoids dimerization between heme groups (mainly in the hemoproteins with a great number of globin subunits), which also causes function loss to the several hemoproteins.

Hemoproteins are very interesting chemical systems that can be applied with distinct objectives. In this context, the elaboration of the called "novel materials" have received special emphasis in order to optimize the employment and the durability of the hemoproteins with respect to its application as biosensor, considering the respective chemical and biochemical functions. Alternative employments, such as biosensors, catalysts, blood substitutes, surface modifiers, among others, have been considered and evaluated by several research groups. The results of these initial studies are very promising.

1.2. Understanding hemoproteins and its potential to application as biosensor

Bioelectronic sensing devices, which are well known as biosensors, represent a growing research area at the interface of chemistry, biochemistry, physics, and nanotechnology, in which the interest focused on applications of redox-active biomolecules (e.g. enzymes and carrier proteins) are often used for the development of this type of analytical devices [5]. In this context, the present study is focused on the structure-activity relationship of hemoproteins through physicochemical evaluations, which includes biochemical behavior and inferences obtained from instrumental analytical methods, with an especial emphasis on spectroscopic and electrochemical analyses. These data sources furnish relevant information regarding several aspects of the chemical reactivity of these extraordinary biological macromolecules. Indeed, the so-called "structure-function relationship" of hemoproteins has different types of implications in terms of biochemical processes, such as heme biological synthesis, physiological disturbances, ligand changes, polypeptide unfolding generated by pH changes and surfactant interaction, thermal denaturation, autoxidation mechanisms, and other processes. These studies constitute relevant contributions to understand the structure of the heme, the globin, and their mutual interactions. This basic research is an important prerequisite to optimize several types of applications of hemproteins. Indeed, these applications require the elaboration of novel materials, which includes, in several cases, an especial role to the hemoproteins, considering its chemical properties. Shumyantseva and coworkers, for example, reported significant advancements in biosensor development based on nanocomposite materials, with hemoproteins as fundamental components [6]. The previous knowledge of the principal chemical agent of the system will favor the interpretation of the first results and the resolution of the eventual initial limitations.

Considering the great potential of employment of hemoproteins in the constitution of new materials, it is relevant to emphasize some important areas in which hemoproteins have been applied, such as biosensors, blood substitutes, and agents of surface modification.

We have studied the great potential, in terms of chemical properties, of hemoglobins regarding their application as biosensors and the main molecules that have been evaluated in the literature through the biosensor based on hemoglobins and/or other hemoproteins.

2. The iron element and its action as metallic coordination center

Iron constitutes 4.7% of the Earth's crust, and it is the second most abundant metal (the first most abundant metal is the aluminum). This remarkable presence is associated to its extraordinary mechanical properties, mainly in the form of steel, making it a very important element with respect to its technological application potential [7]. Due to its electronic configuration, iron (Z=26) presents two more stable oxidation states: iron(III), which is the most stable oxidation state, known as the ferric ion or met-form (this denomination is mainly used by biologists and biochemists when they discuss biological iron), and iron(II), which is known as the ferrous form. Depending on the chemical environment, iron can present other less usual

oxidation states, such as iron(I) and iron(IV), which are much less chemically stable than the ferric and ferrous forms.

Iron as metallic center, mainly when its more stable oxidation states are considered, 2+ and 3+, tends to occur, in terms of coordination chemistry, in hexacoordinated and octahedral complexes, since its electronic configurations are the well-known d^6 and d^5 electronic distributions, respectively. Considering that iron is an element of the first series of transition metals (first period of the transition metals in the periodic table), its electronic configuration can be observed in the high- and low-spin states, since the $3d$ orbital is relatively small, precluding a more intense d orbital splitting, when in contact with weak-field ligand, such as F^- and Cl^-. Therefore, the complexes with Iron(II) and Iron(III) as metallic center can or not to obey Hund's rule.

3. The bioinorganic chemistry of iron

Small quantities of iron are essential for animals and plants. However, similarly to copper and selenium, iron is a very toxic element in higher concentrations in the biological medium [8]. Biologically, iron is the most important transition metal, being associated to several relevant biochemical processes:

a. Oxygen transport in the blood of mammalians, fishes, and other animals: hemoglobin;

b. Storage of Oxygen (O_2) in the muscular tissue: mioglobin;

c. Electron transport in plants, animals, and bacteria (cytochromes and, to plants and bacteria, ferrodoxines);

d. Storage and release of iron in animals (ferritin and transferrin);

e. Nitrogenase components (enzyme of the bacteria that makes fixation of Nitrogen (N_2));

f. Relevant role in several other enzymes, such as aldehyde-oxidase (oxidation of aldehydes), catalases, and peroxidases (decomposition of hydrogen peroxide (H_2O_2)), and succinate dehydrogenase (aerobic oxidation of carbohydrates) [Lee, 1996].

4. The macrocyclic ligands

Macrocyclic ligands contain at least three donor atoms and the macrocyclic ring should consist of a minimum of nine atoms [9]. Therefore, all macrocyclic ligands are chelants; however, the chelant ligands are not necessarily macrocyclic ligands, since the chelant compounds include ligands that make only two ligations to the center of coordination of the metallic complex. Among the more studied macrocyclic compounds nowadays are the cryptates of lanthanides due to their ability to coordinate with antigens and antibodies [10]. Indeed, Jean-Marie Lehn, who was awarded the Nobel Prize in Chemistry in 1987, obtained a macromolecular system

with the presence of donor groups in a tridimensional arrangement of flexible form, which allowed a cation to be complexed and, moreover, encapsulated. The first species that presented this capability was denominated "criptantings", and its metallic complexes, "cryptates" [10].

It is important to note that there are excellent reasons for nature choosing macrocyclic derivatives for the relevant bioinorganic complexes, such as enhanced kinetic, thermodynamic, and thermal stabilities [9].

5. Porphyrinoid macrocylic ligands

Considering the structural complexity presented by macrocyclic systems, such as natural photosynthetic ones, significant scientific effort has been devoted toward the preparation and study of structurally simpler systems. These works are focused on the reproduction of some of the fundamental steps that occur in nature with macrocyclic ligands. For instance, we can cite natural photosynthesis (one of the most important being photoinduced charge separation (CS)) [Bottari, 2010]. Among the chromophores that have been used as molecular components in artificial photosynthetic systems, the large family of porphyrinoid systems, which constitute the ubiquitous molecular building blocks employed by nature in photosynthesis, have been the preferred and obvious choice, due to their intense optical absorption and rich redox chemistry [11].

Tetrapyrroles, which includes porphyrins, are conjugated heteromacrocyclic molecules consisting of four pyrrole rings joined by bridging carbon atoms. They are excellent ligands for many metal ions and, as such, are widely employed in biological systems as prosthetic groups involved in central biochemical processes such as oxygen transport, photosynthesis, respiration, etc. The insertion of metals into various tetrapyrroles is catalyzed by a group of enzymes named chelatases, e.g., the nickel, cobalt, and magnesium chelatases and ferrochelatases, with ferrochelatases being the best-studied enzyme from this group [12].

6. The porphyrin as biological molecule: a versatile macrocycle

Natural porphyrin derivatives, including hemes, chlorophylls, and bacteriochlorophylls, are integrated into protein scaffolds that are essential for their biological activities. For example, the protein matrices of hemoproteins provide active centers with a special hydrophobic pocket having a specific steric hindrance. In the case of hemoglobin and myoglobin, the embedded active iron centers are protected sterically and hydrophobically against irreversible oxidation, thereby enabling reversible O_2-binding [13]. In the light harvesting complexes of purple bacteria, wheel-like supramolecular assemblies of many bacteriochlorophyll units are also performed with the assistance of proteins; they play an important role in the efficient capture of energy from sunlight and transference of this energy to the photosynthetic reaction center. Because of their morphological similarities to proteins, three-dimensional dendrimer archi-

tectures can act as attractive scaffolds for the site-specific positioning of porphyrin function-alities in nanoscale-size regimes [13].

The order of stability of porphyrin complexes with metallic ions in 2+ oxidation state is $Ni^{2+}>Cu^{+2}>Co^{+2}>Fe^{2+}>Zn^{+2}$. The kinetics of formation of these metalloporphyrins has also been measured. It has been found to be in the order $Cu^{2+}>Co^{2+}>Fe^{2+}>Ni^{2+}$ [14]. Considering the higher abundance of iron porphyrins, interesting questions are inferred regarding the origin and evolution of biological systems if the natural abundance of iron was not over a thousandfold greater than those of cobalt and copper [15].

Cobalt, for example, is the 30th most abundant element in the Earth's crust. However, the only known cobalt-containing biological system is the coenzyme B_{12}, which contains cobalt bonded to five nitrogen atoms and a carbon atom of an adenosine ligand. The carbon-cobalt bond in this molecule qualifies coenzyme B_{12} as the first example of a biological organometallic compound, as the closely related vitamin B_{12} contains a cyano ligand in place of the adenosine ligand and other cobalt-bound organic substituents are encountered (the denomination "cobalamin" is used for this class of B_{12} derivatives) [16].

It is important to note that porphyrins and related molecules are of importance not only in biological systems but also in several applications, such as organic semiconductors and non-linear optical materials, due to extended π-electron systems. Recently, many studies have been focused on the use of artificial photosynthesis to develop light-energy conversion systems [17].

7. The heme group (iron porphyrins)

Many, though not all, naturally occurring porphyrins contain iron, and a significant portion of porphyrin-containing proteins possess heme as a prosthetic group. The most abundant heme, heme *b*, is found in the hemoproteins myoglobin and hemoglobin and contains two propionate, two vinyl, and four methyl side chains [18]. Oxidation of a methyl side chain to a formyl group and substitution of a vinyl side chain with a 17-carbon isoprenoid side chain converts heme *b* to heme *a*, the prosthetic group of the mitochondrial enzyme cytochrome *c* oxidase. C-type hemoproteins, such as cytochrome *c* and the *bc1* complex, contain heme *c*, in which the two vinyl side chains of heme *b* are covalently attached to the protein [18].

The great number of studies that employ porphyrin-like compounds in different chemical contexts denotes the extraordinary interdisciplinary and multidisciplinary characters of these macrocyclic compounds. The application of porphyrin-like compounds, metallated or not, in PDT [19], catalysis, electrochemical studies, biomimetic studies, and others is a definitive fingerprint of the great biochemical and physicochemical relevance of this area.

8. Porphyrins and metalloporphyrins: very mechanically flexible chemical systems

Studies focused on the non-planar distortions affecting the structures of sterically crowded porphyrins have shown that these macrocycles are considerably more flexible than originally

suspected. This conformational flexibility may play an important role in controlling a wide range of physicochemical properties of the heme-cofactors present in heme proteins [20]. In fact, porphyrins and other tetrapyrroles exhibit a considerable flexibility of the macrocycle conformation. Examples of non-planar tetrapyrrole conformations have been observed in a variety of protein complexes, such as chlorophyll-reaction center and antenna systems, heme proteins, and cytochromes. Different macrocycle conformations are believed to be responsible for diverse functions of the same chromophore in different protein environments. This allows an explanation of the spectral properties of antenna complexes and the unidirectionality of electron transfer in reaction centers [21].

The types of distortion are often conserved for proteins with the same function but isolated from different species [22]. It suggests that the kind of interaction between a prosthetic group and a polypeptide chain is, at least partially, related to the spatial conformation presented by the prosthetic group. In this way, since non-planar distortion is energetically unfavorable for iron-porphyrins, for example, the conservation of the heme conformation strongly suggests that the biological function of hemoproteins might be modulated by protein control over the conformation of the heme prosthetic group [22]. Indeed, in the absence of external forces and steric crowding of the porphyrin substituents the macrocycle exhibits $D4h$ symmetry. This is because the energy of the π electronic system is lowest for a planar conformation [23].

Therefore, porphyrins and metalloporphyrins are conformationally flexible chemical structures, and a great number of examples of distorted macrocycle conformations have been observed crystallographically. In biological systems, distortions form planarity have been observed in photosynthetic reaction centers, light-harvesting complexes, hemoproteins, and methyl coenzyme M reductase [24]. The conformation of the heme group of each of the more than 25 peroxidases and their mutants is predominantly saddled, while the heme groups of cytochrome c tend to be ruffled [25]. Metalloporphyrins undergo remarkable non-planar distortions of the macrocycle that disturb the chemical and photochemical properties of these important protein cofactors. In fact, mechanical (different substituents or polypeptide interactions) and/or electronic factors can significantly alter the chemical reactivity of the ferrous center, affecting the ligand affinity and several consequent aspects. Furthermore, the tertiary structure of the surrounding protein can manipulate these distortions as a way of regulating biological functions. Cytochromes c, for example, presents an energetically unfavorable conserved non-planar distortion of the heme, which probably is associated to its role in its electron transfer function [26].

Indeed, redox potentials, axial ligand binding affinities and dynamics, catalytic selectivity, and basicity, as well as both the biological and non-biological insertion of metal ions are among the many functional properties shown to be sensitive to deformations of the macrocycle, mainly with respect to out-of-plane distortion [27].

9. Hemoproteins as biosensors

The great ability to bind relevant biological molecules, which is an important property of the heme ferrous center, makes that the hemoglobins can be considered prototypes of biosensors.

Biologically relevant molecules, such as NO, CN⁻, CO, F⁻, Cl⁻, and others, are typical potential ligands to bind ferrous ion. It is important to mention, for example, the work of Michael K. Chang [28], that employed heme to sense molecular oxygen (O_2); carbon monoxide (CO); and nitric oxide (NO), which identified heme-driven conformational changes can happen of a very peculiar form. Considering the group formed by NO, CN⁻, CO, F⁻, and Cl⁻, we have to mention that some of these ligands can also bind ferric ion, which can be generated depending of the medium conditions. Indeed, the met-hemoglobin (ferric hemoglobin) presents a very stable ferric oxidation state (Fe(III)), which represents an oxidized state to the hemoglobin (which presents biological function when its coordination center is the ferrous center), therefore, without native biological function (other hemoproteins present functional character in its ferric state). Thus, considering the mechanical stability of the hemoglobin, together with its significant redox stability (two possible oxidation states to the coordination center of the heme (prosthetic group), the hemoglobins, as well as other hemoproteins, could be applied as biosensors.

Considering the relevant biomolecules mentioned above, we can emphasize, for example, the work of Palaniappan and co-workers [Palaniappan, 2008], which attempted to develop a sensor to detect nitric oxide (NO) in gaseous state, employing the NO-specific hemoprotein known as guanylyl cyclase (sGC) entrapped in a mesoporous silica network [29].

In this context, it is important to note that the level of spatial arrangement and redox stabilities is decisive to the efficiency and durability of the hemoproteins in its application as a biosensor. Normally, hemoproteins with higher supramolecular mass and/or higher number of polypeptide chains (protein subunits) are more stable considering the spatial configuration (quaternary structure) and redox state. In this way, a priori, hemoproteins with high mass, number of polypeptide chains, and redox stability are potential prototypes of biosensors. This is the case of several groups of hemoproteins as, for example, the giant extracellular hemoglobins [30-32]. The giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp), for example, has a mass of approximately 3.6 MDa, 180 subunits (144 globins with heme, and 36 *Linker* chains, without prosthetic group) and great stability against autoxidation and other chemical processes [33-36]. Hemoproteins with these properties can be prepared to act as different types of biosensors, depending on previous knowledge regarding its structure-activity relationship.

On the other hand, there is an intrinsic difficulty to elaborate the novel materials to form the biosensor based on hemoglobins, mainly the ones with higher supramolecular mass. Indeed, the electron transfer reactivity of hemoglobin on conventional electrode surfaces is physiologically limited as the function of the normal electro-active center, which is the iron atom in the heme, is deeply buried in its electrochemically "insulated" peptide backbone [37]. In this way, the hydrophobic isolation of the ferrous ion in the heme pocket, which is favorable to avoid the oxidation of the heme and the dimerization between heme groups through μ-oxo bridges (chemical bounds between the metallic centers (iron center) of two heme groups (iron porphyrins), is also associated to a significant difficulty, in terms of construction of the biosensor, which is the significant distance between the ferrous metallic center and the chemical neighborhood, limiting the electron transfer associated to the metallic center as well as the chemical contact among relevant biological molecules and the metallic center. In fact, several studies based on hemoproteins adsorbed or immobilized on different electrodes

demonstrated that high supramolecular mass is not amenable to direct electrical communication with the electrode [38].

Thus, the redox and structural stabilities are significant advantages in order to apply the hemoprotein as biosensor, but the hydrophobic isolation of the heme precludes a high intense level of contact with the electrode surface (difficulting the "electric contact", i.e., the electric current transfer between the hemoglobin the the electrode) in the elaboration of modified electrodes. However, the redox and structural stabilities as well as the hydrophobic isolation of heme can be altered, depending on the chemical medium conditions. Indeed, these modifications can favor or disfavor the biosensor performance, which makes it necessary for several chemical conditions to be tested, aiming at the optimization of the best possible conditions of each hemoprotein in each biosensor prototype. In fact, depending on the chemical properties of the hemoprotein employed as biosensor and its respective matrix in which the protein is immobilized (chemically or physically adsorbed), the electrochemical and/or electroanalytical performance can be drastically changed. Chemical factors as pH, ionic force, contaminant presence, and level of chemical interaction with the matrix, among others, provoke polypeptide unfolding of the hemoprotein, increasing, frequently, the permeability of water and other relevant molecules into heme pockets, which, in its native state, presents high hydrophobic character. In this way, the loss of wild polypeptide arrangement changes the total spatial arrangement of the protein, changing the state of the polypeptide arrangement and the spatial conformations of all globins. Nevertheless, each hemoprotein has chemical and structural peculiarities that can sense the modifications in different ways, depending on all characteristics. Consequently, the evaluation of chemical environment conditions that are suitable to each hemoprotein and each type of biosensor can vary significantly depending on the molecular or supramolecular mass, number of polypeptide chains (protein subunits), type of amino acid residues around the heme pocket, matrix chemical constitution etc. in addition to the characteristics of the own electrode, which are also important factors to the hemoglobins as principal component of the electrodes [39]. In fact, in order to present high sensitivity, reproducibility, and long stability, a simple biomolecule/solid (electrode) interface is required with features of good biocompatibility, depending mainly of the type of applications and sensitive electrochemical activity, being that the electrodes used, i.e. the matrix constitution of the electrode, in these employments play key roles [39]. All these above-mentioned factors can affect the hemoprotein sensibility of the biochemical molecules that must be determined as final task of these type of biosensor. Therefore, each biosensor should be submitted to several tests, in a preliminary way, in various different chemical (or biochemical) conditions, in order to obtain the best standard reference that is possible in a specific context, i.e., the optimum conditions in terms of sensibility, selectivity or specificity, durability, and reproducibility, among other relevant parameters that determine the quality of the biosensor.

Paola Ringhieri, for instance, in her PhD thesis, developed an interesting strategy to overcome this limitation, which is the exploitation of artificial low-molecular mass proteins, to accentuate the electric contact [38]. Ringhieri evaluated a new class of heme-peptide conjugate that are known as "mimochromes", aiming to understand the effects of the peptide chain composition and conformation in modulating the redox properties of the heme and, consequently, the novel biosensor [38].

This above-mentioned limitation can also be solved through adjustments in the medium conditions of the chemical environment around the hemoprotein. In fact, variations of pH, ionic force, polypeptide arrangement, and humidity, among others can decrease the compaction of the polypeptide chains and the consequent heme hydrophobic isolation, favoring the electron transfer as well as the chemical interaction with several relevant molecules.

In this context, many efforts have been made to enhance the electron transfer rate of hemoglobins through mediators, promoters, and several immobilization materials, such as polymer films, surfactants, and nanomaterials [37].

Moreover, it is necessary to optimize an apparatus that can maintain the hemoglobin supported on this surface. The stability of this surface modification is a prerequisite to the precision grade of the methodology. It is important to note that the acid or basic conditions of the surface are decisive in determining the adsorption of the globin, mainly if the fixation is caused by ionic contacts. In this way, it must be considered the isoelectric point (pI) of the hemoglobin, since the predominance of positive or negative charges on the protein surface depends on the acid/basic conditions of the medium in relation to the pI of the hemoglobin.

The detection of hydrogen peroxide (H_2O_2) and nitric oxide (NO) has received special attention from several groups that apply hemoglobins as biosensors, as hemoglobins have been considered a new class of sensitive, stable, and auspicious electrochemical biosensors [40]. Indeed, electrodes elaborated with hemoglobins as relevant components have generated direct electron transfer of hemoglobins [Dang, 2013]. Dang and co-workers, for example, evaluating immobilized hemoglobin, encountered good response in terms of biocatalytic action in nitric oxide (NO) and hydrogen peroxide (H_2O_2) [41]. Gu and co-workers, for example, aiming to determine amperometrically nitric oxide with immobilized hemoglobin, found electrocatalytic responses that were proportional to the nitric oxide concentration, without any interference of several relevant biomolecules [42].

In this context, the detection of hydrogen peroxide has generated a great number of excellent works due to the long-term stability, good reproducibility, and high selectivity of biosensors produced to this kind of determination [43]. Furthermore, the determination of hydrogen peroxide is highly relevant as a function of its applications as mediator in food, pharmaceutical, clinical, industrial, and environmental analyses [44]. For example, a recent study describes the immobilization of hemoglobin on a Clark electrode surface to develop a novel electrochemical biosensor for the detection of hydrogen peroxide. The principle of the measurements was based on the electrocatalytic activity of the immobilized hemoglobin to the reduction of hydrogen peroxide [45]. A highly sensitive and selective amperometric hydrogen peroxide (H_2O_2) biosensor based on immobilization of hemoglobin (Hb) at multiwalled carbon nanotube–zinc oxide (MWCNT/ZnO) composite modified glassy carbon electrode (GCE) is also reported [46]. Zeolites are also employed as matrix to immobilization of hemoglobin, so this biomolecule can act reducing H_2O_2 [47]. An interesting study demonstrates that titanium dioxide (TiO_2) nanoparticles, through the photovoltaic effect generated by ultraviolet radiation, can significantly improve the catalytic action of hemoglobin as a peroxidase, denoting that the elaboration of photocontrolled protein-based biosensors is very promising [48].

It is important to point out that a recent important work presented a cholesterol amperometric biosensor containing hemoglobin, being that this hemoprotein acts as an efficient electron

mediator, without any interference from phospholipids [Souza, 2013]. The authors claim that the cholesterol could also be detected in real samples from chicken egg yolk, without any influences from phospholipids [49].

It is important to register that the immobilization of hemoglobins has generated highly stable new materials. Batra and co-workers [50], for instance, developed a methodology to detect acrylamide, which was based on covalent immobilization of hemoglobin on carboxylated multi-walled carbon nanotube, with the respective electrode being employed 120 times over a period of 100 days (stored at 4°C) [50]. On the other hand, Saleh Ahammad [51] claims that little attention has been paid to the basic research related to the elucidation of the mechanisms associated to the electrochemical performance of electrodes based on hemoproteins, such as horseradish peroxidase and hemoglobin and that, considering the cost of production of these novel biosensors, higher efforts are required to control the influences of temperature, pH, humidity, and toxic chemicals [51].

10. Conclusions

The perspectives for the applications of hemoproteins, mainly hemoglobins, as biosensors are very promising. However, a more consistent previous study, involving basic research, is a fundamental prerequisite in order to optimize the efficiency of these respective biosensors. The very complex chemistry associated to hemoproteins requires a continuous study focused on its structure-activity relationship to propitiate a higher understanding of the substrate-matrix interaction, surface modification (chemical and/or physical adsorption), and instrumental results. In any case, there has been great progress in this area in the last few years, which has generated very positive perspectives to the future applications of hemoglobins as biosensors.

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