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Focused review

Towards the functional high-resolution coordination chemistry of blood plasma human serum albumin

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ABSTRACT

Human serum albumin (HSA) is a monomeric, globular, multi-carrier and the most abundant protein in the blood. HSA displays multiple ligand binding sites with extraordinary binding capacity for a wide range of ions and molecules. For decades, HSA's ability to bind to various ligands has led many scientists to study its physiological properties and protein structure; indeed, a better understanding of HSA-ligand interactions in human blood, at the atomic level, will likely foster the development of more potent, and overall more performant, diagnostic and therapeutic tools against serious human disorders such as diabetes, cardiovascular disorders, and cancer. Here, we present a concise overview of the current knowledge of HSA's structural characteristics, and its coordination chemistry with transition metal ions, within the scope and limitations of current techniques and biophysical methods to reach atomic resolution in solution and in blood serum. We also highlight the overwhelming need of a detailed atomistic understanding of HSA dynamic structures and interactions that are transient, weak, multi-site and multi-step, and allosterically affected by each other. Considering the fact that HSA is a current clinical tool for drug delivery systems and a potential contender as molecular cargo and nanovehicle used in biophysical, clinical and industrial fields, we underline the emerging need for novel approaches to target the dynamic functional coordination chemistry of the human blood serum albumin in solution, at the atomic level.

1. Introduction – HSA the multitudinous protein "sponge"

Human serum albumin (HSA) accounts for over 60% (by mass) of human blood plasma proteins. It is a monomeric, globular, largely α-helical protein containing turns and extended loops [1–4] (Figs. 1 and 2). It is present in the circulatory system and is considered to be the most abundant carrier protein in the human serum [5]. HSA is highly soluble in water, and its blood concentrations range from 30 to 50 g/L (~0.53–0.75 mM), within a narrow pH range of 7.35–7.45 [6]. HSA is encoded by ALB (Albumin) gene [7]. The initial translation product, pre-proalbumin, has a molecular mass of 69.3 kDa and consists of 609 amino acids (aa), including an N-terminal ER (endoplasmic reticulum) import signal sequence. Pre-proalbumin is produced in the liver and undergoes a post-translational modification in the Golgi apparatus. The first 24 amino acids from the N-terminus (1–18 signal peptide and 19–24 pro-peptide) are cleaved before the mature HSA protein is released into the bloodstream [5,8,9]. The mature HSA protein has 585 amino acids and a molecular mass of 66.5 kDa.

Multiple ligand binding sites have been identified in HSA, in line with its extraordinary binding capacity with a wide range of ions and molecules [10–12] (Fig. 2). Hence, HSA has become a pivotal contender as a molecular cargo and nanovehicle used in biophysical, clinical and industrial fields (see Fig. 1) [10,13–15].

A main function of HSA is the regulation of blood colloid osmotic pressure [16] and metal ion homeostasis, including the transport and storage of transition metals [11,12]. HSA also plays a crucial role in binding, storing and transporting numerous endogenous molecular ligands such as fatty acids, heme, bilirubin, and prostaglandins, and exogenous ligands such as pharmacological drugs and various transition metal ions [17,18]. HSA can bind to a remarkably wide range of drugs (over 250 registered drugs on the market), [19–23] thus restricting their free, active concentrations in blood; this represents a

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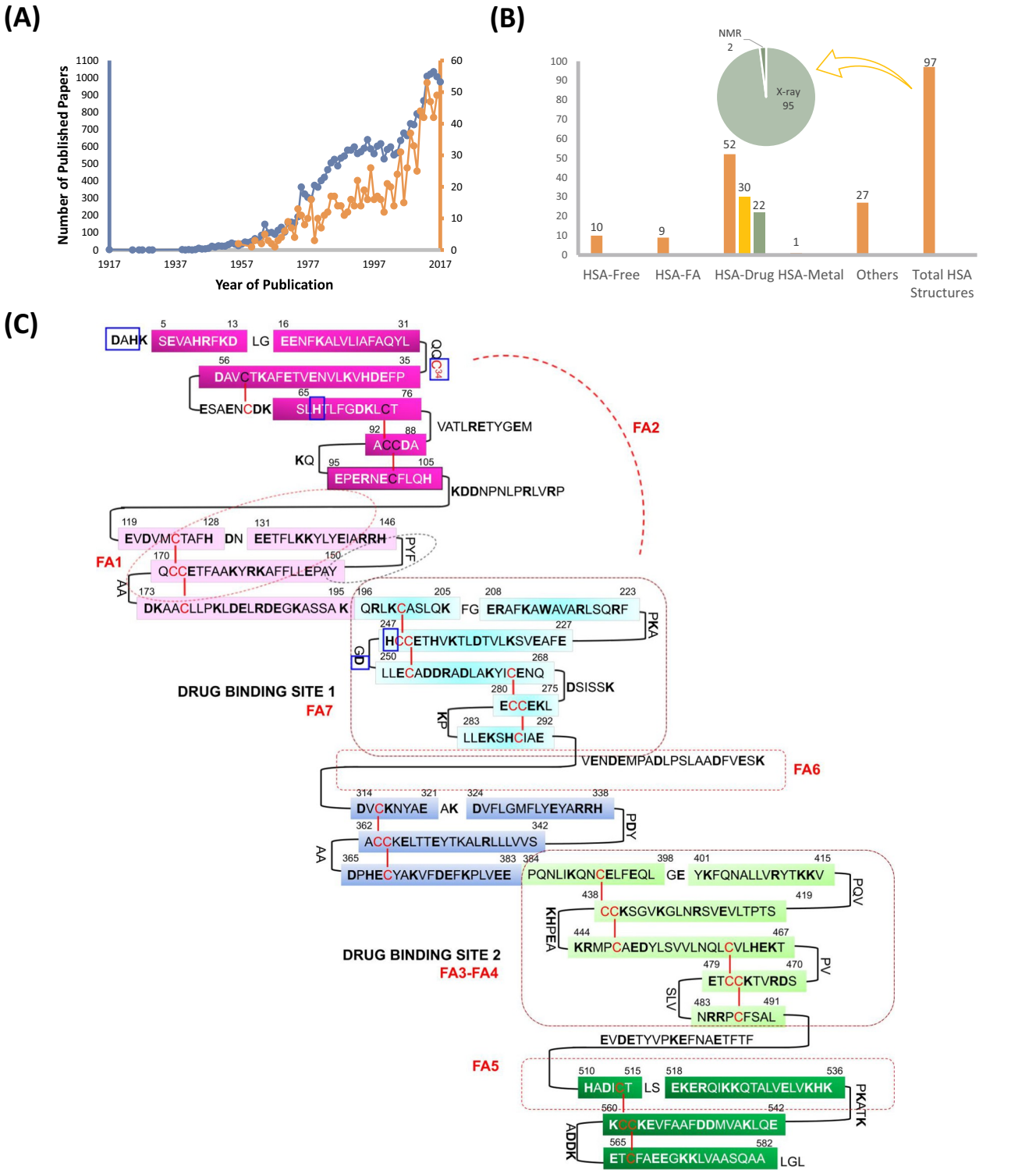

Fig. 1. Human serum albumin – the tripping centenarian protein for research. (A) Number of published articles on human serum albumin (1917–current). The blue line represents the total number of articles on HSA indexed by PUBMED, the orange line represents the total number of articles on HSA-metal ion binding. (B) Number of HSA structures deposited in PDB divided by the type of ligand. HSA-Free: no ligand; HSA-FA (fatty acids): 8 different types with only fatty acid as ligand; HSA-drug: 52 HSA structures in complex with different types of drugs (30 HSA structures with drug ligand only; 22 HSA structures with drug and FA); HSA-metal: Zn^{2+} ions in complex with HSA crystallized from pH 9; Others: HSA structures in complex with inhibitor, receptor, simple sugars, chemical compounds, or antibody. (C) Schematic drawing of HSA topology, the secondary structure elements and disulfide bridges (red vertical lines) of HSA. Each helix is represented by a rectangular bar, each sub-domain by a different color. From top to bottom: IA, IB, IIA, IIB, IIIA and IIIB. Drug binding sites are encircled by black dashed lines, fatty acids binding sides are encircled by red dashed lines, while metal binding residues are marked with blue rectangular boxes. Binding sites for natural ligands are in Bold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

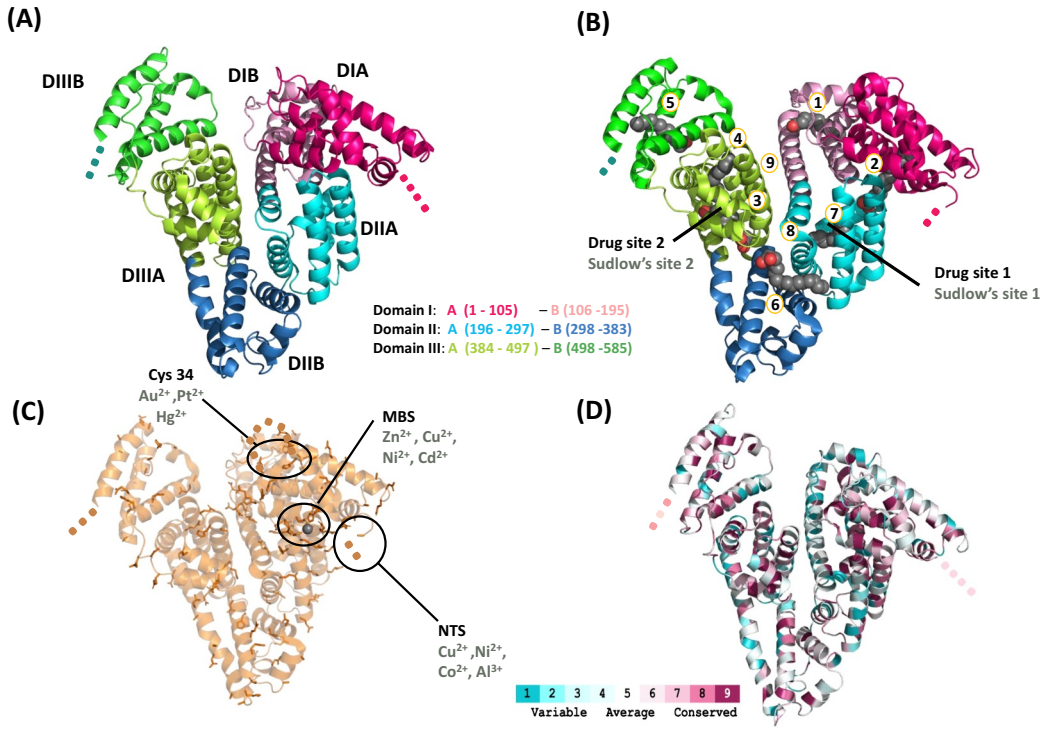

Fig. 2. Human serum albumin dynamic structures and binding sites: (A) Crystal structure of defatted human serum albumin (HSA) PDB: 1AO6. (B) Crystal structure of fatted HSA in the presence of palmitic acid, and location of major binding sites (Binding sites of 2 drug/versatile ligands, 7 fatty acid) PDB:1E7H Subdomains are colored-coded and the fatty acids are represented by stick-models colored in red. (C) Crystal structure of human serum albumin with zinc ion occupy the MBS (PDB: 5IJF) with the location of 3 metal binding sites. (D) HSA structure color coded according to evolutionary conservation of amino acid positions, dark pink being the most conserved amino acids and cyan being the least conserved amino acids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

major challenge in drug development. HSA supports the metabolic modification of some ligands, renders potential toxins harmless [10,14,24–34], accounts for most of the anti-oxidant capacity of human plasma, and displays some enzymatic properties [24,35]. Even HSA degradation products can be beneficial [36]. As an example, peptide fragments originating from the proteolytic degradation of HSA were recently identified as an endogenous antagonist of the CXC chemokine G-protein coupled receptor 4 (CXCR4) [37]. HSA also serves as a biomarker for diagnosis of multiple diseases such as cancer, rheumatoid arthritis, ischemia, post-menopausal obesity, severe acute graft-*versus*-host disease, and diseases related to the glycemic index. HSA is already been used, with success, for the clinical treatment of several human disorders including hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia [38].

Because of its high abundance, its ability to bind to multiple sites, and its multifunctional properties, the investigation of HSA's interactions with drugs and metal-mediated binding interactions can provide critical information about pharmacokinetics, pharmacological activities and drug distribution pathways [5]. HSA's capacity to carry and interact with many essential transition metal ions has already been used in therapeutics and clinical biochemistry for designing metallodrugs based on Au, Pt, and V [12].

Emerging evidence is also now revealing the inhibitory, chaperon-like function of HSA towards the formation and aggregation of amyloid β fibril and the mitigated membrane damage of alpha synuclein (αS) and human islet amyloid polypeptide (IAPP) in the fatty-acid and cholesterol dependent manner [39–42]. This, together with the well-documented impact of transition metal ions on the development and progression of neurodegenerative diseases [43,44] makes it essential to understand the functional dynamic coordination chemistry of HSA. This, in fact, represents a fundamental quest for contemporary inorganic biochemistry.

In this article, we summarize the current knowledge in high-resolution HSA coordination chemistry; we also emphasize the overwhelming need for a detailed atomistic understanding of HSA dynamic structures and interactions, and the impact of the various endogenous and exogenous ligands and transition metal ions on the human serum albumin structure and dynamics, under conditions as close as possible to physiological conditions.

By integrating our knowledge of contemporary molecular biophysics with that acquired in the medical field, we will likely be able, in the future, to directly monitor the atomistic details of HSA in human blood serum and correlate them with various states of health and disease. In many ways, such a dynamic functional high-resolution coordination chemistry of blood plasma HSA would be the realization of "the American dream of contemporary bioinorganic coordination chemistry", providing us with an unparalleled understanding of molecular mechanisms, and, as a long-term goal, much more efficient approaches to therapeutic and diagnostic applications.

2. Dynamic structures of heart-shaped human serum albumin

The first HSA's three-dimensional structure, determined by X-ray crystallography, was published in 1992 (PDB id: 1UOR) [1]. Since then, approximately 100 static structures of HSA and HSA-with bound ligands have been determined, predominantly by single crystal X-ray crystallography, and subsequently deposited in the Protein Data Bank (PDB). Matured and active HSA contains 17 disulfide bridges shaping the protein fold, and one free cysteine at the 34th position (Cys 34) [2]. HSA is a helical, heart-shaped, 66.5 kDa-protein consisting of three topologically identical and structurally similar domains: domain I (DI) (aa 1–195), domain II (DII) (aa 196–383), and domain III (DIII) (aa 384–585). Each domain comprises one antiparallel six-helix (sub-domain A) and four-helix (sub-domain B) motifs [2] (Figs. 1 panel C, 2).

According to published crystal-state X-ray high resolution HSA structures augmented by numerous biophysical studies, there are two main versatile ligand binding sites with high affinity for diverse molecules on HSA that are referred to as 'Sudlow's sites' (I and II). These binding sites are located in subdomains IIA and IIIA (Fig. 2) [29]. In addition, 9 fatty acid (FA) binding sites have been identified, with different affinities for FAs [17], and two of which overlap Sudlow's sites. Moreover, HSA contains four metal-binding sites of different structures, metal ion specificities, and binding affinities [12].

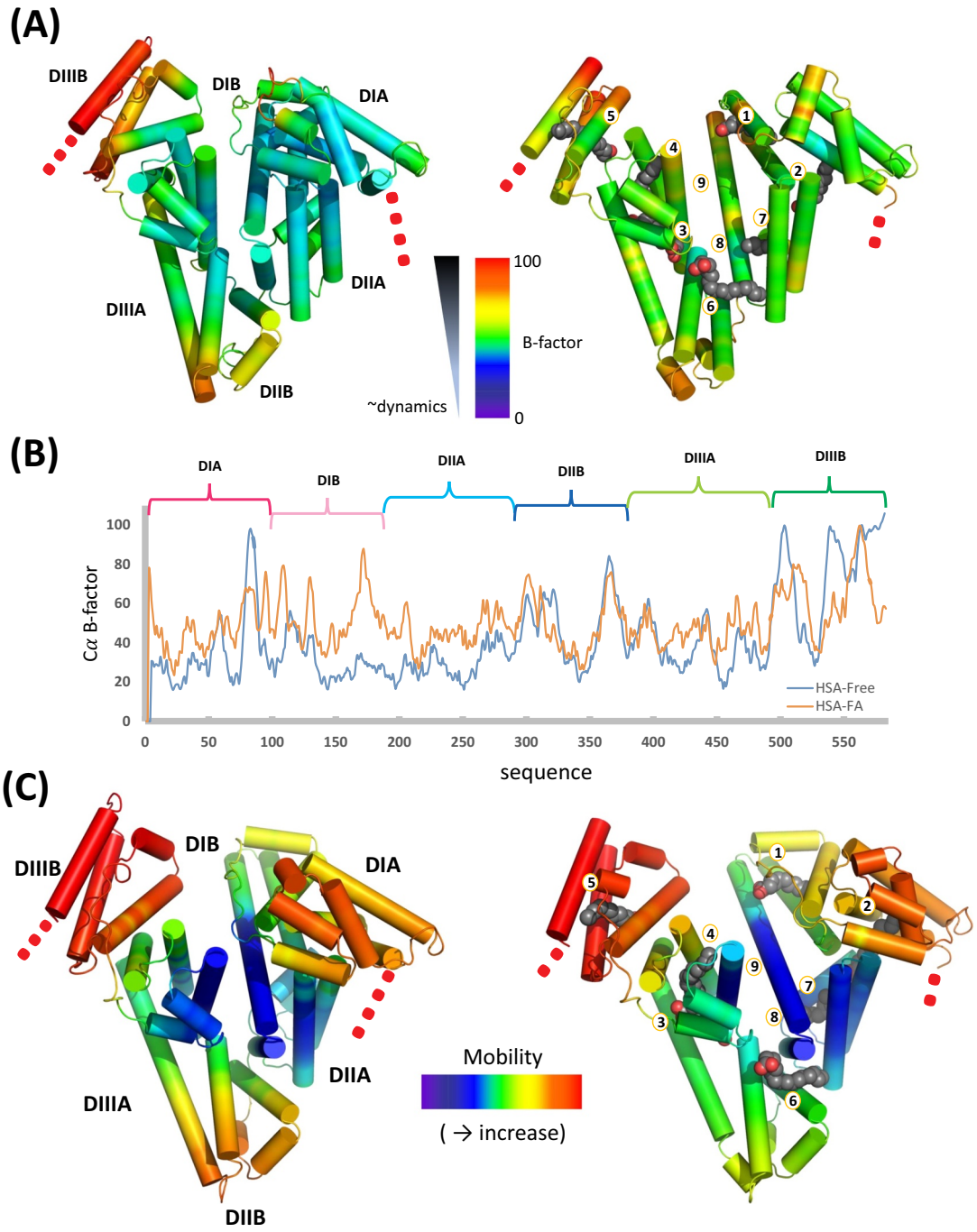

Fig. 3. Ligands affect HSA's dynamics: (A) The 3D crystal structures of HAS with colored Cα B-factors; left panel HSA-free (PDB ID: 1AO6) and right panel HSA-FA (PDB ID: 1E7H). (B) The plot depicting the differences along the HSA sequence for the HSA-free and HSA-FA together with the location of domains. (C) The approximation of the protein conformational dynamics done by the Gaussian Network Model algorithm [188] revealing the differences in molecular motions between the two structurally the most distant Human Serum Albumin crystal structures. The domain names and FA binding sites are marked as in (A).

Despite the substantial diversity of high-, medium- and low-affinity ligands, only two well-defined and distant spatial conformations of HSA have so far been documented: one for the defatted HSA [1,2] and one for fatted HSA [4,45,46]. The 3D structures determined by the X-ray crystallography for those two conformations are represented in Fig. 2. The RMSD (root-mean-square deviation) of Cα atoms, over the entire primary sequence (5–582), between the two main conformations of the defatted (PDB: 1AO6) and fatted (PDB: 1E7H) HSA structures, was found to be approximately 4.6 Å. Subdomain IA and IIIB display large RMSD values suggesting a large conformation change occur [18]. The conformational changes of HSA that were observed can be characterized by a twist motion between DI and DII with an angle rotation of 24°, and a hinge motion between DIII and DII with an angle of 15°. DII was found to be the HSA fragment overlapping the most in both conformations.

Thus, there is a clear imbalance between HSA's multiple binding sites towards numerous ligands, and the fact that only two main conformations of HSA have been identified so far. Differences of dynamics between those two structures in the crystal state are shown in Fig. 3. The existence of a comprehensive protein dynamics, functional mobility, and/or more functional conformations/structures in solution, not found by existing high-resolution biophysical methods, can be expected (*cf.* Fig. 3).

A comparative analysis of the two most distinct HSA conformations indicates that ligand-metal binding affinities can be modulated by the presence of fatty acids, which might be a key factor influencing the distribution and the free concentrations of many HSA ligands. The identified drug-specific binding site I overlaps with FA-binding site 7, drug-specific binding site II with FA-binding sites 3 and 4, respectively [47]. Additionally, a previous study showed that the binding of Zn^{2+} to its primary binding site (site A) was modulated by the binding of FAs at FA-binding site 2. It has been proven that the binding of FAs to HSA serves as an allosteric control that impacts the binding of Zn^{2+} to albumin, and therefore controls the availability of free Zn^{2+} for transport into cells, or use by other proteins [48]. The binding of fatty acids to HSA inhibits the binding of zinc, whereas the presence of zinc does not exclude the binding of fatty acids [48–50]. Given the inherent flexibility of biomacromolecules and the importance of dynamics for the proper functioning of the proteins, we can conclude that the interactions with ligands influence, or even regulate, the activity of HSA (Fig. 3).

One not yet well understood phenomenon is the potential formation and function of HSA quaternary structures. HSA has been shown to aggregate, most likely in dimers, in a concentration-dependent manner (above 1 to 2 mM), *via* weak non-covalent intermolecular interactions. Moreover, the protein is able to form higher-order aggregates (oligomeric) in alkaline solution. Research is still ongoing to examine potential structural changes induced by covalent and non-covalent interactions between HSA molecules, and the resulting effects on the protein's functions and binding sites, under physiological conditions [51,52].

Under oxidative stress conditions, HSA can undergo several structural modifications: the presence of a reduced cysteine side chain (Cys 34) in monomeric HSA facilitates dimerization, by formation of a disulfide bridge between two HSA polypeptides [53]. Approximately 5% of HSA, in the entire amount of blood, is in dimeric form, and is actually considered to be a biomarker for oxidative conditions [54,55].However, we cannot exclude the fact that these HSA dimers are an artifact resulting from HSA's extraction from blood, or its subsequent lyophilization. In addition, HSA dimers have been confirmed to be effective plasma-retaining agents and drug carriers [30,52]. However, despite the potential use of HSA covalent and non-covalent dimers in clinical applications, only a limited amount of information is available on the properties and stability of HSA dimers, under physiological conditions, and on the structure and dynamics of their contact interface(s), at an atomic level.

HSA's molecular shape can be flexible, under different conditions (*e.g.* Figs. 2 and 3). During ischemia, HSA can attain a conformation form called ischemia modified albumin (IMA). The half-life of albumin in serum equals approximately 20 days, so the detection of IMA should be possible a few days following ischemia [56,57]. For this reason, IMA can serve as a blood-borne biomarker for the detection of cardiac ischemia; it has recently been approved by the Food and Drug Administration for clinical use [58].

According to a previously published interpretation, reported by Bar-Or et al. [59], IMA has a structural change at the HSA N-terminal end, which consists of amino acid motive H_2N-Asp-Ala-His, and can bind strongly to cobalt (Co), copper (Cu), and nickel (Ni) ions. Damage in this region, under ischemic conditions, should result in a reduction of the binding affinity towards those transition metal ions. This occurs in the context of elevated endothelial or extracellular hypoxia, acidosis, or presence of free oxygen radicals. Transition metals, such as cobalt, can be used to measure the titer of IMA by an Albumin Cobalt Binding (ACB) test in order to aid the establishment of a diagnosis, particularly for heart disease [60]. The albumin cobalt-binding (ACB) assay is used to indirectly measure IMA. In this assay, the albumin in the patient's serum is incubated with cobalt, and the unbound ions are detected by colorimetric measurement [61]. The ACB test was widely criticized, because of the lack of a well-established correlation between the degree of N-terminus modification in ischemia [62] and the reduced cobalt binding by albumin [63–65].

The bioinformatics tool Consurf Server [66–69] (*cf.* Fig. 2 panel D), allows the analysis of the evolutionary conservation of amino acid positions in homologous proteins. Regions essential for protein structure and function are usually well-preserved among proteins from different species. The analysis of evolutionary conserved regions reveals that the tripeptide at the N-terminal (DAH) with the potency to coordinate numerous metal ions present in HSA is not preserved among all living species included (Fig. 1 panel D). This N-terminal motif was never found in the experimental electron density within the X-ray structures, probably due to its intrinsic flexibility (see Figs. 2 and 3). Usually, the defatted HSA structure misses the first 1–5 amino acid residues, but the structures containing the FA miss only the first two, some three, N-terminal residues that might further indicate an allosteric effect and FA-driven modulation/regulation of the coordination capabilities of the HSA (Fig. 2). This emphasizes the need for high-resolution, comprehensive studies of proteins in solution, and for information on the binding ability to variety of endogenous and synthetic ligands and metals.

These comparative studies of the primary sequence and spatial structures highlight that amino acid sequences, and therefore also parts of 3D structures of serum albumins, which are highly conserved, although substantially differ in several places. Experiments performed on other serum albumins, such as Bovine Serum Albumin (BSA) and Equine Serum Albumin (ESA), thus could potentially reveal useful information that may be applied to HSA. For example, in 2016, a study by Handing et al. reported a structure for ESA crystallized in a complex with an antihistamine drug of interest [70]. The study showed that the binding pockets for the drug in ESA were structurally conserved in HSA, making it likely that the binding of the drug to HSA would be similar. Differences among species, as well as detailed information on binding are therefore important and chemically interesting. This, however, does not negate the need for exact and careful studies done with HSA, as protein dynamics, under physiologically relevant conditions, rather that static studies in the crystal state.

3. HSA interactome - from biophysics to medical diagnostics

HSA is able to interact with a wide range of ligands, increasing the solubility of bound compounds and delivering them to target tissues [71]. Interactions of exogenous (therapeutic and diagnostic) and endogenous (heme and fatty acids) ligands with HSA modulate the overall conformation of the protein (Fig. 2).

3.1. Endogenous ligands

3.1.1. Fatty acids

FAs are important substrates that play major roles throughout the body. Due to their low solubility in water, FAs in blood plasma bind to HSA as the main fatty acid binding protein and carrier [72]. Fatty acids are considered to be the primary physiological ligands of HSA. A maximum of nine long chain FAs equivalents can be bound at different binding sites (*i.e.*, FA1–FA9), having different affinities [73,74] and arranged asymmetrically throughout HSA. (Figs. 2 and 3). The FA binding sites have been classified into two main classes: high affinity binding sites (FA2, FA4, FA5) and low affinity binding sites (FA1, FA3, FA6, FA7) [62].

After dietary intake, short- to- medium fatty acid chains are absorbed into the blood [75]. However, the free non-esterified long-chain fatty acids (mainly C16 and C18) are stored in the body's adipose tissues in form of triacylglycerol (TAG), which serves as a major metabolic fuel. A controlled process called 'lipolysis' helps to breakdown TAG and release FFAs (free fatty acids) into the blood, which then can be transported by albumin to other tissues to be used [72,76].

Under normal physiological conditions, most FFAs in the blood plasma are bound to HSA (0.1–2.0 M equivalents) and their binding

affinities (K_d) to HSA range from 1.5 to 90 nM [31]. It is thought that only under elevated FFAs conditions, more than 2 M equivalents of FFAs are bound to HSA [77].

3.1.1.1. FA1 (heme pocket). FA1 partially overlaps with the heme binding site, and the D-shaped cavity located in the interior of subdomain IB is surrounded by a four-helix cluster. When either ligands are bound, the binding site is closed by Tyr138 and Tyr161. The binding of all FAs in FA1 is in the same orientation: hydrogen of the carboxylic group is bonded to Arg117 and water molecule, which at the same time is coordinated by both Tyr161 hydroxyl group (in the side chain) and Leu182 carbonyl oxygen atom. In the case of saturated long-chain FAs their tail curls around the interior of the surface cavity, so that the tip of the hydrophobic tail is constantly approaching His146 at the end of the cavity [73].

FAs and heme-Fe(III) binding induce a substantial conformational rearrangement of the subdomain IB [78]. The methyl group at the end of FAs tail is bound to FA1 around the back wall, and therefore leaves plenty of room for drugs or drug-like molecules (indomethacin and triiodobenzoic acid), to co-bind [4,79].

3.1.1.2. FA2. The location of FA2 is found to be between the subdomains IA and IIA, and is considered to be the most enclosed binding site. It is a high affinity binding site, and can be instantly populated at 1 M equivalent [48,80]. Multiple studies reported evidence indicating that the binding of FAs to FA2 site stabilizes the B-conformation of HSA; it also suggests that this site is responsible for the ligand-induced conformational transition. Indeed, the formation of a pocket that can accommodate FA requires the rotation in domain I (relative to domain II), and this rearrangement can be stabilized by the binding of FA to FA2 [15,81,82]. FAs anchor their head groups in the subdomain IIA by forming a hydrogen bond with side chains of Tyr150, Arg257, and Ser287, while the methylene tail extends linearly in the hydrophobic cavity between subdomain IA and IIA [73].

3.1.1.3. FA3–FA4. FAs bind in FA3 *via* hydrogen bond interactions between the hydrogen present in FA head groups, Ser342 and Arg348 (IIB subdomain), and Arg485 (IIIA subdomain). FA4, on the other hand, exhibits similar binding patterns found when the carboxylate head groups of FAs interact *via* hydrogen binding to the amino acid residues found on the exterior of the subdomain IIIA (Arg410, Tyr411, and Ser489), and where the tail extends further through a narrow hydrophobic cavity in the subdomain IIIA [73,79,83]. Studies show that Sudlow's site II consists of two regions: the apolar region, filled with FA a methylene tail that bounds to FA3, and a polar region occupied by a FA carboxylate group bound to FA4 [38]. To this day, no drug has been observed to enter the FA4 narrow, long hydrophobic tunnel that holds FA's methylene tail. In addition, it has been shown that FAs present at FA3 do not show any interactions with the polar region centered on Tyr411 [73,79,83].

3.1.1.4. FA5. FA5 is located in the subdomain IIIB and can form a hydrophobic channel. FAs are bound to FA5 in an extended conformation; the FA carboxylate head group interacts with Tyr401 and Lys525 residues, and the methylene tail extends through the tunnel [73].

FA4 and FA5 are categorized as high-affinity sites for FAs, and they are found to be the most enclosed binding sites of FA on HSA. Such an environment provides proper conditions for FA's methylene tail binding in an almost linear conformation, whereas the carboxyl group can form specific salt-bridge interactions with basic side-chain on amino acids [74].

3.1.1.5. FA6. FA6 is located at the interface between the subdomains IIA and IIB, and can be filled with medium and long fatty acids. Its binding properties are different from those observed for FA1-FA5, mainly due to the absence of amino acid side-chain cluster that helps to stabilize the FA carboxylate *via* electrostatic interactions. However, Arg209, Lys351, and Ser480 side-chains recognize the FA carboxylate. The methylene tail's middle section is strongly anchored by salt-bridges from Arg209 to both Asp324 and Glu354. FA6 is a rather open site to which FAs bind with a low affinity [73,74].

3.1.1.6. FA7 (Sudlow's site I). FA7, or Sudlow's site I, forms a hydrophobic cavity located in the subdomain IIA. This site preferably binds to bulky heterocyclic anions, with warfarin being the prototypical ligand [28,31,45,47,79,83–87]. Compared to its analogous cavity in the subdomain IIIA (*i.e.*, FA3–FA4 or Sudlow's site II), FA7 is considered to be smaller [79,83]. The Arg257 residue provides stabilization to FA carboxylates by polar interaction(s). Lys199, Arg218, Arg222, and His242 show no direct involvement in FA binding. However, such residues appear to involved in providing stabilization to other ligands at the site, *e.g.* metal ions. FAs bind to this site by their tail being co-planar with aromatic drugs that are already bound; therefore, FA7 is considered to have a low-affinity binding site [73,74].

In 2018, a single high-affinity FA binding site in HSA was characterized by a joint fluorescence, dialysis and X-ray analysis. For a fluorescence-labeled NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl)-fatty acid conjugate, binding was observed in the IIA subdomain by interactions with residues of Gln196, Lys199, Ser202, Trp214, and His242. In spite of a NBD-labelling reduced affinity (approximately 20-fold), the observed K_d was still 27 ± 2 μM [88].

FA7 is considered a versatile binding site; HSA can covalently bind to different ligands, due to the presence of the nucleophilic side-chain Lys199. Molecular dynamic (MD) simulation studies of FA7 revealed that the ionization state of the Lys199 and Lys195 residues influenced the structure and dynamics of the FA7 site [89].

3.1.1.7. FA8. The gap between subdomains IA–IB–IIA and subdomains IIB–IIIA–IIIB hosts FA8. Only short-chain FAs, such as capric acid, can bind at this site, due to volume restrictions. On one end, the hydrophobic cavity is formed by capric acid's methylene tail that is bound at FA6, and on the other end, polar amino acids (*i.e.*, Lys195, Lys199, Arg218, Asp451, and Ser454) contribute to the formation of an open ring to provide stability to the FA carboxylate [73].

3.1.1.8. FA9. FA9 is located at the upper of the gap between subdomains IA–IB–IIA and subdomains IIB-IIIA-IIIB, producing an open binding environment. The stability of the ligand depends on the formation of a salt-bridge between Glu187 of domain I and Lys432 of domain III [4]. Additionally, FA9 is formed upon a FA-induced conformational transition, thus providing an additional binding site in FA-saturated HSA [73,79].

FA9 and FA8 are recognized as supplementary binding sites, due to the fact that FA8 shows a ligand occupancy only in the presence of short-chain FAs and FA9, at a saturating FA concentration [73].

As mentioned previously, FAs are HSA's primary ligands under physiological conditions. Additionally, it has been shown that the binding of FA can change the polarity, and increase the volume, of drug-binding site 1. Thus, FAs have the ability to modulate the affinity of HSA to other ligands, *e.g.* Zn(II) and Co(II), at all physiological levels, not just under extreme conditions [48]. It has been proven, in BSA, that there is a complete inhibition of Zn(II)'s coordination to site A by five equivalents of myristate, binding at site B was also severely affected. Even though Zn(II) and Co(II) binding preferences are not clear, the presence of myristate has been shown to reduce the BSA's binding affinity to Co(II) [90] at all physiological levels, not just under extreme conditions [48,49].

In certain disease states, these effects are exacerbated, as the fatty acid:HSA mole ratio may be as high as ~6:1. These pathological conditions are associated with high (micromolar to millimolar) levels of bilirubin, hemin, or renal toxins [*e.g.* 3-carboxy-4-methyl-5-propyl-2-

Table 1
List of amino acids with metal binding sites in HSA or other peptides and proteins.

Ligand	Number of residues in HSA	Essential metal binding	Toxic metal binding	Reference
Cysteine	35	Zn(II)	Hg(II), Pb(II), Cd(II), Au(I), Pt(I)	[12,38,95]
Histidine	16	Zn(II), Cu(II)	Cd(II), Ni(II), Ag(I)	[12,38,95]
Tryptophane	1	Cu(II)		[96]
Glutamic acid	62	Ca(II), Mg(II), Fe(III), Fe(II)	La(III), Yb(III)	[12,38,97]
Aspartic acid	36	Ca(II), Mg(II), Fe(III), Fe(II), Cu(II), Ni(II), Zn(II), Cd(II)	La(III), Yb(III)	[95,97]
Lysine	59	Cu(II)	Ni(II), Co(II), Cd(II), Al(III)	[95,97]
Arginine	26	Cu(II)	Ce(III)	[95,98]

furanpropanoic acid (CMPF), indoxyl sulphate] that bind to the protein, causing significant drug binding defects [89,91,92].

High levels of FAs in blood plasma can be associated with a variety diseases, including cancer, diabetes, and obesity and diabetes [93]. Studies have shown that pregnancy can cause an increase in plasma fatty acid level. In early stages of pregnancy, maternal fat is deposited; later on, it is broken down by lipolytic hormones, leading to an increase of FA level in blood plasma. As a consequence, a peripheral insulin resistance is developed, and the body shifts from a carbohydrate oxidation to a fat oxidation mode, thus maximizing access to glucose for development of the fetus [94].

3.2. Essential metal ions

Seven amino acids are generally considered as naturally mediating binding of metal ligands to proteins. They are listed in Table 1, together with metal ions that are potentially coordinated by them; also indicated is their occurrence in the amino acid sequence of HSA. Of 585 amino acid residues in HSA, 235 have the ability to bind to metal ions (40%). Nevertheless, to this day, only 4 metal binding sites have been characterized.

A comprehensive review of research studies on the coordination of HSA with metal ions, up to 2013, was published by Bal et al. [12]. Here, our focus is on studies published after 2013, particularly those on the high-resolution structure and dynamics of HSA.

3.3. N-terminal binding site (NTS)

The N-terminal binding site (NTS) is composed of Asp1–Ala2–His3 (Fig. 1); it is considered to be the first metal binding site that was identified on HSA. Another acronym of this binding site is 'ATCUN', for the Amino Terminal Cu(II) and Ni(II) binding motif [99,100]. In NTS, two metals are bound in a square-planar arrangement formed by three N-terminal amino acid residues: the N-terminal amine Nα atom of Asp1, the deprotonated amide Nα atoms of Ala2 and His3, and the Nδ atom of His3 side-chain imidazole [99,101]. The ATCUN motif is present in most albumins from different mammalian species, with the exception of porcine and canine albumins, which lack His3 [12]. The crystal structure of HSA shows a high conformational flexibility of the NTS motif in the absence of a bound metal, and a lack of defined structures of the first few N-terminal residues [100]. It is noteworthy that the N-terminal X-X-His motif is present in many other proteins, such as the peptide hormone Hepcidin; it also binds to Ni(II) and Cu(II) ions [102].

Copper is an essential metal ion for humans, as it is involved in many cellular processes such as metabolic oxidations and electron transfers [103]. However, its transport and cell localization must be controlled in order to avoid copper-dependent redox cycling. HSA is one of the copper-trafficking proteins [104] and up to four equivalents of Cu(II) can bind with high affinity mainly to the NTS' side [105]. Under normal conditions, only ≈2% of HSA in the blood serum binds to Cu(II) ions (equating to approximately 15% of total copper in blood plasma [12,106]). Nevertheless, HSA is the second biggest carrier of copper in human blood serum, just after ceruloplasmin [107]. There is no HSA-Cu(II) complex crystal structure, and the solid state coordination chemistry is based on the copper complex of a model peptide that contains HSA's first four N-terminal amino acids [108]. Sadler et al. showed that even Lys4 could be involved in the metal [*i.e.* Co(II), Ni(II), Cu(II), Cd(II) and Al(III)] binding site [101]. HSA has a high affinity to Cu(II), and the value of the dissociation equilibrium constant for the HSA-Cu(II) complex formation is 6.7×10^{-17} M.

Cobalt is another essential metal ion present in the blood as Co(II) ion, and HSA is its principal carrier in plasma [12]. Co(II) binds to NTS, in an octahedral coordination. The β-COO− group of Asp1 and the ε-NH_2 group of Lys4 in axial position contribute to the metal ion chelate. In a recent study by JPC et al., it was shown that cobalt binds not only to NTS motif, but also to sites A and B, with, in fact, a much higher affinity [62]. ITC experiments have shown that cobalt-albumin binding is influenced by the fatty acids (*e.g.* myristate), in a similar manner to Zn(II), and that elevated ACB readings can be elicited simply by high FFA concentrations [90].

NTS is an important metal binding site; surprisingly, about 25% of HSA molecules in blood have Asp^1–Ala^2 cleaved off.

3.4. Metal Binding Site A (MBS-A)

MBS-A is a multi-ligand binding site located at the domain I/II interface and residues from both domains are involved in interactions with the ligands. As a consequence, the binding of the ligand affects the conformation of the protein. MBS-A's location overlaps with the FA2 binding site, and consequently, one ligand binding controls allosterically another ligand coordination. A comparison of the HSA sequence, across multiple mammalian species, shows the presence of MBS-A site for all species (Fig. 2D). MBS-A selectively binds to Zn(II) and Cd(II), while has a low affinity for Ni(II) and Cu(II) [11].

Zinc is an essential metal ion needed for various physiological processes. It plays a catalytic and structural role in different proteins such as zinc finger domains, and it is a component of catalytic centers in some enzymes [12]. Zinc acts as an activator or inhibitor of biochemical processes by affecting the concentration and activities of the following hormones: insulin [109], glucagon [110], and leptin [111,112]. Insulin is required for proper HSA synthesis (diabetics have decreased albumin levels), which, in-turn, may influence the molecular transport of fatty acids [113].

HSA is considered to be the main carrier of Zn(II) in plasma (whole blood concentration ~100 μM [114]), with 75–90% of Zn(II) in blood bound to HSA [31,50,115]. Stewart et al. estimated that ~2% of circulating albumin molecules carried a zinc ion, and that the modulation of their mutual affinity might have significant consequences [48]. Albumin transports the newly-absorbed Zn(II) to the liver [116], and facilitates its uptake by endothelial cells [117] and erythrocytes [118].

Despite the important role of HSA in zinc transport, the structure and location of the zinc binding site in albumin has only recently been identified [113]. The first X-ray crystallography data of Zn(II)-HSA binding at the atomic level was published in 2016 (PDB: 5IJF) [3]. The crystal structure of the defatted HSA-Zn(II) complex, at pH 9, showed only one strong binding site (MBS-A), and up to nine secondary binding sites with a low metal affinity. Unfortunately, the resolution of the structure (refined at 2.65 Å) did not allow to pinpoint their exact

location. Even isothermal titration calorimetry (ITC) showed only two Zn(II) binding sites in the wild type HSA (MBS-A, and one of the secondary sites) [3,11].

According to an X-ray analysis-based model published in 2018, MBS-A is defined as a five-coordinate site having 1.98 Å average Zn–O/N distances and a sixth 2.48 Å weak Zn-O/N bond, made by coordination to residues at domain I (His67 and Asn99) and domain II (His247 and Asp249), as already suggested for Cd(II) binding [11,119] water molecule takes part in the coordination [11,119]. All residues taking part in the coordination are found to be conserved across all mammalian serum albumins, and no functional HSA variants having mutations in these residues have been found to this day. The MBS-A coordinates Zn(II) either in a trigonal bi-pyramid or in a distorted octahedron (when taking into account the backbone carbonyl oxygen of His247 [50]).

Recently, ITC was used to study the allosteric modulation of zinc binding in the presence of FAs. These studies showed that the binding of FAs (FAs with ten or more carbon atoms) to the FA2 side led to zinc release, but not *vice versa* [120]. This mechanism is described by the 'spring-lock' model, in which the coordination from domain I (His67 and Asn99) and domain II (His247 and Asp249) is disengaged, causing the release of Zn(II) from site A [11,50].

Moreover, Asp248 present in domain II of BSA (corresponding to Asp249 in HSA) has been identified as a part of a Ca(II) binding site; an interplay between zinc and calcium ions is also possible [121]. In addition, the binding of Zn(II) to albumin affects Mn(II)'s coordination [113]. The *cis*-Platin anticancer drug forms a crosslink between the two histidine side-chains (His67 and His247) and can prevent the binding of Zn(II) at the MBS-A site [122].

3.5. Other metal binding sites

Calcium (1600 μM total blood concentration, [114]) and magnesium (1700 μM total blood concentration, [114]) are the most abundant essential metal ions in human blood. Almost half of the total Ca(II) is bound to plasma proteins, mainly to HSA [123], even if it has a low affinity for it (K_d of 0.67 mM) [121,124]. Calcium is one of the most important regulators of cell migration (a process that is still not fully understood) [125]. Different reports describe calcium binding to be non-specific to albumin, with involvement of various carboxylate side-chains on the albumin's surface [99]. On the other hand, studies by Majorek et al. showed three defined Ca(II) binding sites on BSA [121]. X-ray structure showed key site A ligand-Asp248 (corresponding to Asp249 in human albumin) as a Ca(II) binding site. This finding is in agreement with other Nuclear magnetic resonance (NMR) studies, in which Ca(II) ions interfered with the ^{113}Cd signals, for both sites A and B of HSA [126].

Long-chain FAs, particularly oleic and linoleic acids, increase Ca(II)'s binding to HSA. In addition, there is an overlap of residues involved in the binding of Ca(II) and Zn(II) [3]. Two Asp13 and Asp254 residues, corresponding to site II residues in BSA, have also previously been implicated in Ca(II)'s binding [127]. This means that there could be cross-talk between Zn(II) and Ca(II) binding at this site, similarly to what is observed at site A [3].

Mg(II) and Ca(II) have similar affinity to HSA, and the approximately same blood molar concentration of approximately 45%; they are both in a bound state (to proteins). Usually, Mg(II) occupies no more than one site on HSA [31]. Mg(II) binds with a low affinity (K_d of 10 mM) to HSA, at binding sites identical to those with Ca(II) [128].

Next to MBS-A, other zinc binding sites were suggested [129,130] and subsequently validated by X-ray determination of their structure [3]. Secondary binding sites coordinate Zn(II) ions by three protein residues, whereas tertiary sites bind Zn(II) by one or two side chains. There are two secondary (II and III) and six tertiary (IV-IX) zinc binding sites. In site II, Zn(II) is coordinated by the His9 residue and by two carboxylates located at Asp13, and Asp255 sites. Between IB and IIA domains, site III is formed by the coordination of His157 and His288, with carboxylate from Glu153. Each of six tertiary Zn^{2+} binding sites bind metal ion only with two residues (IV–IX), four are formed by a single histidine (*e.g.* His3) and a single carboxylate (Glu or Asp); one is formed by an aspartate residue and a main-chain oxygen (site V) one is located on a crystallographic contact (site VI). The tertiary binding sites have low binding affinities and are not likely to impact the transport of Zn(II), *in vivo*.

There was a lot of uncertainty about site B and whether Zn(II) ions are bound there [11,126]. The X-ray structure showed weak anomalous electron density near His9/Asp13 in the HSA structure at low zinc concentration. This site appears to be an attractive candidate for site B [3]. Also X-ray structure of ESA (PDB: 5IIU) support this data. The coordination sphere of Zn(II) consists of one nitrogen atom from His and two oxygen atoms from Asp. This results are in broad agreement with previous ^{113}Cd(II) NMR data [124]. Secondary site II that consists of His9/Asp13/Asp255 has been shown to be conserved in both HSA and BSA.

In healthy individuals, most of the circulating Fe(III) ions are bound to transferrin, and only approximately 10^{-5} M is bound to small chelating molecules (*i.e.*, citrate) or to proteins. Indeed, Fe(III)'s binding to HSA is considered to be an important determinant of the plasma's antioxidant capacity [131]. HSA has a low-affinity binding to Fe(III), and its dissociation equilibrium constant has yet to be determined; the dissociation constant of Fe(III)–BSA's complex is $K_d = 3.5 \times 10^{-8}$ M [132]. Surprisingly, Fe(III) does not compete with Ca(II) for its HSA binding site [133].

The importance of metal ions – HSA interactions have led to an increasing number of detailed investigations with the goal of understanding the high-resolution dynamic structures. A metal ion HSA structures obtained by X-ray analysis show four distinct metal binding sites that vary in structure and metal ion specificity.

3.6. Thyroxine binding sites

In the FA-free HSA there are four binding sites of the T4 (thyroxine) hormone: Tr-1, Tr-2, Tr-3 and Tr-4. Tr-1 is located in subdomain IIA, Tr-2 in subdomain IIIA, and Tr-3 and Tr-4 are located in subdomain IIIB. Tr-binding sites partially overlap the FA7, FA3–FA4, and FA5 binding sites [134]. T4 molecules bind to Tr-3 and Tr-4 with a configuration that partially overlaps the FA binding to FA5 [45]; T4 binding to Tr-2 is sterically hindered and it binds at the FA4 entry, protruding towards the solvent.

FAs compete with T4 for binding to all four Tr sites, and high mole ratios of FA:HSA inhibit the binding of thyroxine. However, in some cases, the binding of FAs leads to conformational changes that produce the rearrangement of the 3D structure of HSA [45,83,135], and forms a 5th T4 binding site, called 'Tr-5' located between domains I and III (*i.e.*, FA9). This site is built by connecting the two domains (I and III) with a long helix and a couple of helices from subdomain IIIA.

3.7. Bilirubin binding site

HSA binds to bilirubin in subdomain IB, close to the heme site. Due to the steric obstruction of the open tetrapyrrole, bilirubin accommodates in the preformed, L-shaped pocket, located at the entrance of the FA1 cavity [78]. Residues Glu110–Glu119 connect subdomain IA with subdomain IB, and play an important role in strapping the pigment into place, along with the formation of salt-bridges with Arg117 and Arg186 residues. It contacts closely with apolar side chain of amino acids in helices h8-h10 (Ile142, Phe149, Leu154, Phe157, Gly189, and the aliphatic portion of Lys190).

3.8. Exogenous ligands

3.8.1. Drugs

The high blood concentration and multi-binding properties of HSA make it an important carrier of drugs that target various organs and

tissues. According to Drug-Bank version 5.1.1 released on July 3, 2018, approximately 2.5% of total drugs (234 approved, 12 experimental, 3 investigational, 3 withdrawn) are reported to interact with HSA, [19–23]. HSA-drug binding influences the pharmacokinetic behavior of many drugs and affects their efficacy and rate of delivery. Moreover, it restricts the distribution of the drug and free-form, active concentration [10,79]. Therefore, acquiring in-depth knowledge of HSA-ligand binding and its interactions can be very valuable for the design and development of new drugs.

An analysis of the crystallographic structures of HSA, in a drug-bound state, has revealed two specific primary drug binding sites (Figs. 1 and 2) and many secondary binding sites. The two main drug binding sites are called Sudlow's sites I and II, and are located at sub-domains IIA and IIIA, respectively [29]. Drugs generally circulate in two forms: bound (covalently and non-covalently) or in free form (unbound) [136]. Some fatty acids can directly compete with drugs for binding, or lead to allosteric effects that affect the binding of drugs and other ligands to HSA [137].

3.9. Site 1 (Warfarin site) (Sudlow's site I)

Drug site 1 is a pre-formed binding pocket within the core of six helices of the subdomain IIA and a loop-helix feature (residues 148–154) of subdomain IB. The entrance to drug site 1 is enclosed by subdomains IIB and IIIA. The interior of the pocket is mostly apolar, but with two clusters of polar residues: an inner one towards the bottom of the pocket (Tyr150, His242, Arg257) and an outer one at the pocket entrance (Lys195, Lys199, Arg218, Arg222). The large binding cavity is comprised of a central zone with three distinct compartments. The back-end of the pocket is divided by Ile264 into left and right hydrophobic sub-chambers, with a third sub-chamber protruding from the front of the pocket, delineated by Phe211, Trp214, Aala215, Leu238 and the aliphatic portions of Lys199 and Arg218. The wide entrance and presence of flexible side-chains provides enough space for maneuvering at the mouth of the pocket.

Drugs that bind to site-1 form hydrophobic contacts with the pocket and different interactions with specific residues present in the inner and outer polar clusters. Various drugs form hydrogen bond interactions with the hydroxyl group of Tyr150, and this residue has a central role in the drug's bindings. In addition, a number of hydrogen-bond and salt-bridge interactions are formed with His242, Lys199 and Arg222 residues. It is possible that water might form bridge interactions with such residues; however, they are not visible at the current resolution level of crystal structures.

Site-1 is specific, due to the prevalence of basic residues, and the absence of acidic ones. Ligands that are specified to bind to site 1 acquire anionic or electronegative features in the center. [38,78,96]. Molecules with two anionic or electronegative features, on opposite sides, can simultaneously interact with the two polar patches of the site-1. The binding of small drugs leads to only small side-chain movements, whereas bigger drugs display greater movement in proximity of Tyr150 and Trp214.

Drug site 1 coincides with a fatty acid binding site (FA7) [13], which is a low-affinity site. When fatty acids bind, Tyr150 that is located at subdomain IB moves and interacts with the carboxylate group of FA bound to the site that straddles domains I and II (fatty acid site FA [138]). This drives the rotation of domain I, in respect to domain II, and has an effect on site 1. When Tyr150 interacts with the FA, it limits its availability to make the central contribution to drug binding in the site-1 pocket. In this case, different drugs interact with various basic and polar ligands (on both sides of the binding pocket), mostly with the side-chains of Lys199, Arg222 and His242. In particular, the residues Arg218 and Arg257, have the ability to interact with some specific compounds [24].

3.10. Site 2 (Ibuprofene site/Sudlow's site II)

The second drug binding site (Drug site 2) consists of six helices located in subdomain IIIA; it has been shown that this site is topologically similar to drug site 1, but smaller. It is composed of a large hydrophobic cavity, which consists of a polar structure, and has only one sub-compartment. Drug site 2 displays one major polar patch located at the beginning of the binding pocket on a single side. It is centered on Tyr411, but also includes Arg410, Lys414 and Ser489. Site-2 has the pocket entrance more exposed to the solvent, in comparison with site-1. The formation of salt-bridges between Arg348-Glu450 and Arg485-Glu383 marks the closing end of the pocket. As mentioned previously, water molecules may help to make the binding pocket more adaptable, but there is no evidence of this due to low resolution level of crystal structures.

At the center of the binding pocket of site 2, located in subdomain IIIA, Diflunisal, diazepam, ibuprofen and indoxyl sulphate bind. It has been shown the presence of drug interactions with the hydroxyl group, on the Tyr410 residue, in contrast to Lys414 that shows no interactions. The residues of Arg410 and Ser489 can form salt-bridge and hydrogen bond interactions, and contribute to drug binding except with the drug diazepam. Site 2 generally is an apolar pocket composed of a basic polar patch found at one end; and for this reason, it is generally selective for drugs with a peripherally located electronegative group [38]. Ligand binding to site-2 leads to relatively small side-chain movements, and Val433 and Arg410 residues are mostly influenced by ligand-induced alterations.

Drug site 2 has the ability to bind also to two long-chain FA molecules (sites FA3 and FA4) [29] or to one of thyroxines [134]. FA's methylene tail that is bound to FA3 fills the apolar region of the pocket, whereas the FA carboxylate group bound to FA4 may interact with the polar patch present at the site. To this day, none of drug that have been investigated are able to enter the FA4 hydrophobic tunnel that is occupied by methylene tails of FA bound at this site. In addition, FAs that are bound at FA3 don't show any interactions with the polar patch centered on Tyr411. Noteworthy, fatty acid binding to site-2 leads to a significant conformational change in HSA's structure, one that involves rotations of domains I and III relative to domain II [10,25,133]. In contrast, drug binding at sites-1 and 2 leads only to local conformational changes. Nevertheless, subtle structural effects and the presence of additional binding sites lead to allosteric interactions between drug sites 1 and 2 [96,98].

The site-2 binding pocket is specific, and only for drugs with a peripherally located electronegative group [79,83]. Drugs (*e.g.* ibuprofen) bind in the middle of site II by the formation of several interactions with Tyr411's hydroxyl group, whereas Arg410 and Ser489 residues form salt-bridge and hydrogen-bond interactions with the drug.

Several pharmaceutical companies have developed high-throughput methods to assay the albumin-binding properties of their compound libraries [82,88,89,91,92,138]. However, the interpretation of competition or binding data is complicated, considering the partially overlapping binding compartments in site 1 [85–87], the undefined number of secondary drug binding sites [87,96], and the possibility of allosteric interactions between drugs [97,98] and endogenous ligands [11,99,139].

3.11. Metallodrugs

Medicinal inorganic chemistry, an emerging field in natural sciences, proposes the use of metal ions and drugs based on them in order to treat diseases. Some metal-based drugs (metallodrug) that have been introduced in human biological systems have been successful for diagnostic and therapeutic purposes, particularly in cancer treatment. Organic compounds in complexes with metal ions appear to be more biologically active than the compound alone, for anticancer activity [140]. A large number of metal complexes derived from organic ligands

with anticancer activity have been designed, synthesized, and applied (in the pharmacological field), by using metals such as Pt(II), Pt(IV), Ru (II), Pd(II), Cu(II), Co(III), Au(II), all previously used as potential anticancer, anti-inflammatory, antimicrobial, and antirheumatic drugs [5,141–145]. Such compounds have been tested both *in vitro* and *in vivo* and many of them have reached clinical trials. An extensive review has been published recently by Medici et al. on the most used noble metals in modern clinical medicine, summarizing their use, activities, interactions and importance in the medicinal field [146]. Here, we focus on recent studies involving metal-based complexes, which used HSA as delivery device.

The choice of a suitable drug delivery system can enhance the performance of metallodrugs by improving the drug's effectiveness and decreasing its side effects. HSA has been used as a multi-carrier for therapeutic agents against many diseases, including, but not limiting to, diabetes, cancer, and infectious diseases. Due to the effects of HSA on drug's efficiency and pharmacokinetics, HSA-drug interactions are the main factor to be considered to establish a foundation during the design and development process of therapeutic agents. HSA's unique properties make it a promising drug delivery system, considering that HSA is a non-toxic, non-antigenic, biocompatible and biodegradable protein that lacks immunogenicity [147]. HSA consists of multiple binding sites and active residues such as His, Cys, and Lys, where metal groups on the designed prodrug can react with and subsequently form a complex.

In cancer chemotherapy, platinum in Pt(II) and Pt(IV) complexes represents the first, widely used, metal complex in medicine. *cis*-Platin is considered to be the most successful drug in the field of medicinal inorganic chemistry, along with its analogs (carboplatin, oxaliplatin, satraplatin, and picoplatin). Many studies have demonstrated its effectiveness against a variety of tumors [148–150]. A newly developed approach has been investigated by a group of researchers; this approach involves the generation of novel Pt(IV) prodrugs that mimic the amphiphilic structure of fatty acids [149]. By using HSA as a delivery device, the compound was capable to bind non-covalently to HSA and take advantage of these interactions for transport in the blood. Besides platinum, other copper compounds are considered to be the next generation metal-based anticancer drugs and lately have been immensely investigated. Recently, a group of researchers proposed the development of anticancer copper based pro-drug targeting specific residue on HSA, (His242 and Lys199 in subdomain IIA and His146 in subdomain IB) in order to increase the delivery efficiency and drug's selectivity and decrease its side effects [143,151,152]. First, they designed a prodrug with antitumor activity that has leaving group(s), which can bind to hydrophobic cavity in IIA or IB subdomains. Once it binds, Lys199 or/and His242 in subdomain IA and His146 in subdomain IB coordinate with Cu(II) by displacing the Cu(II) compound prodrug leaving group (s), allowing the Cu(II) compound to strongly bind to the hydrophobic cavity of the HSA IIA or IB subdomain therefore, form a stable HSA complex.

Major limitations, when considering such drugs, are the side-effects, including inherited and acquired resistance and toxicity. Therefore, considerable efforts are being made to overcome such limitations, and to replace those drugs with metal-based prodrugs that are more effective, less toxic, and target specific along with developing efficient drug delivery systems.

Due to their low toxicity to humans, and their role in the treatment of diseases, the potential of metal ions as pharmaceuticals (metallodrugs) is a promising approach, particularly for cancer therapy. It has led to new research efforts aiming to develop new pharmacological drugs for various diseases based on the nature of the HSA carrier.

3.12. Toxic metal ions

3.12.1. The Cys34 binding site

Fully reduced and native HSA contains only one cysteine with a reduced thiol group (Cys-34, domain I), whereas other 34 residues form disulfide bridges that shape the fold of the protein [12,153]. The only one reduced thiol (Cys34) group in HSA accounts for the largest free thiol pool in whole blood serum and acts as physiological antioxidant, while playing a role in the redox regulation of protein structure and function [154,155]. Moreover, the thiolate form of Cys34 is a potential binding site for metal ions that are essential for therapeutic and diagnostic use, such as gold (I) - antiarthritic drugs [156,157]. In addition, the free cysteine residue allows HSA to form covalent dimers.

About 40% of the circulating HSA has Cys34 in reduced –SH form, and up to 60% coupled to low molecular weight thiols, such as cysteine and glutathione. The peculiar topology of the Cys34 site accounts for its clear involvement in Au(I) (from anti-arthritic drugs) and Pt(II) (from cisplatin) complexes [158–160]. Hg(II) added to plasma (both inorganic salts and organomercurials) is bound to HSA at 90%, mainly by the thiol group of Cys34. However, multiple stoichiometries and multiple binding sites have been proposed. NMR and site-directed mutagenesis excluded Cd(II) and Zn(II) ions binding to Cys34 [11].

3.12.2. The primary multi-metal binding site or Cadmium site A (MBS-A)

MBS-A is located at the I/II interdomain contact region, and is surrounded by FA1, FA2, and FA7 binding sites. It is called a 'cadmium binding site', due to its high affinity for Cd(II) ions; its dissociation equilibrium constant (5.0×10^{-6} M [161]) is higher, by an order of magnitude, to those in Cu(II), Ni(II), and Zn(II) ions [126,130]. MBS-A acts as a secondary Cu(II) (and possibly Ni(II)) binding site (the primary one being the NTS), where Cu(II) is coordinated in a tetragonal geometry, as inferred from circular dichroism spectroscopy (CD) and electron paramagnetic resonance (EPR) spectroscopy [99,130].

3.12.3. The secondary multi-metal binding site or Cadmium site B (MBS-B)

The fourth metal-binding site on HSA is called 'site B' and it shows a similar binding affinity to Cd(II) as the MBS/Site A. Site B is considered to be the primary binding site for Mn(II) and Co(II). ^{111}Cd and ^{113}Cd NMR experiments on HSA demonstrated the existence of two Cd(II) binding sites, with only one of them showing competition with Zn(II) [11,119]. The first one corresponds to MBS-A; the other one still has to be identified, and is generically labeled as the 'secondary multi-metal binding site' or 'Cadmium site B' (MBS-B). Despite its similar affinity for Cd(II), MBS-B is expected to have a different coordination topology, with one His donor and several carboxylates [11,126]. Even if the exact position of MBS-B is unknown, recent ^{111}Cd NMR on BSA [90] and the recent X-ray studies on HSA [3] suggest that site B may also be an interdomain site. Less substantial findings have been reported for Al(III) binding to HSA. Two Al(III) binding sites have been identified on HSA, but their exact location has not been identified. The binding properties of these two distinct sites appear to undergo reciprocal influences, suggesting a possible interaction between the corresponding protein moieties [161].

3.12.4. Other metal binding sites

X-Ray structural data unambiguously proved, in 2015, that *cis*-Platin mainly binds to His105 and Met329 side-chains; the other binding sites are His288, Met298, Met548, His535, His67 and His247 [162].

The binding of Gd-AAZTAC17 magnetic resonance imaging (MRI) contrast agent to fatted and defatted HSA was studied by relaxometric and modelling studies. These studies revealed a binding of the drug to site I and its removal by warfarin. In the case of fatted HSA (2 to 3 equivalents of fatty acid are normally bound to HSA), the complex affinity was higher than that observed with defatted HSA [163].

Nanoparticles of Fe_3O_4 (IONPs) are bound to HSA by seventeen amino acid residues, includingTyr150, Gln196, Lys199, Lys195, His242, Arg257, Val241, Leu238, Ile290, Ala291, Trp214, Arg218, Leu219, Arg222, Phe223, Leu260, and Ile264. Aromatic amino-acid residues, including Tyr150, Trp214 and Phe223, were found near the binding site of IONPs with HSA [164].

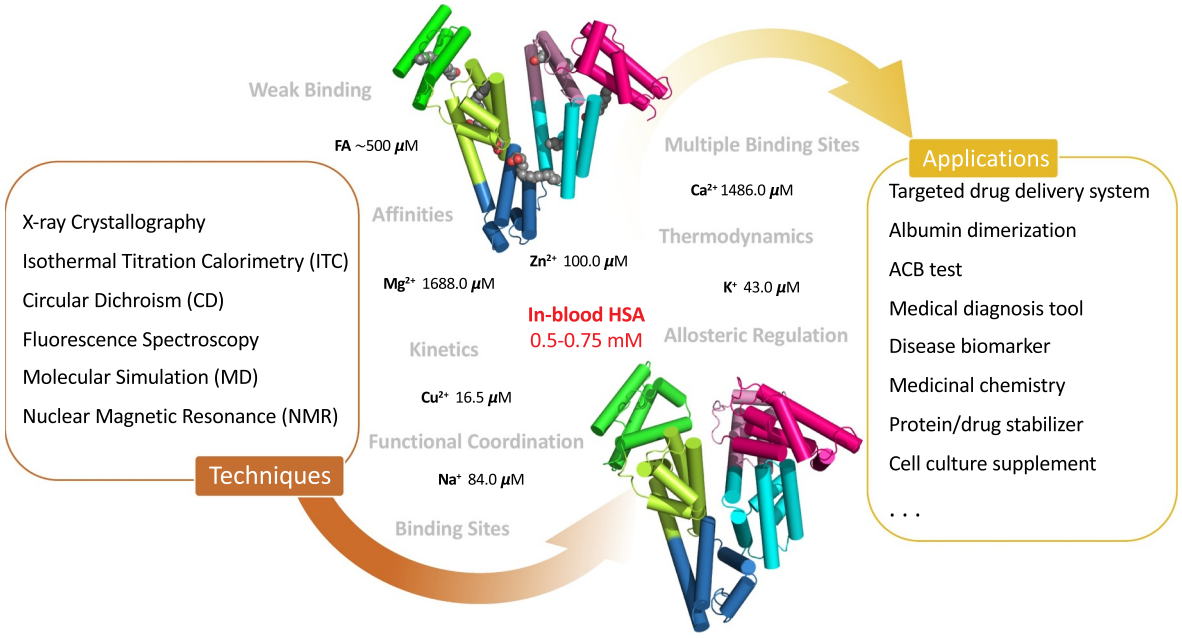

Fig. 4. The dynamic functional high-resolution coordination chemistry of blood plasma HSA – current methodologies and applications: The flowchart of the biophysical studies of HSA and subsequent medicinal and diagnostics applications. The average FA [189] and metal ion concentrations [114] are presented.

Vanadium is not an essential element for humans, but low molecular weight complexes of V(IV) are used as insulin-mimicking drugs [165]. Accordingly, recent calculations of V(IV)O's speciation showed that albumin gained some relevance, at very high exposures to this metal ion [166]. It was also suggested that albumin participates in the uptake of V(IV)O by cells [167]. One EPR spectrum distinguished one "strong" (recently designed withVBS1 [168]) and at least five "weak" binding sites (named VBS2 [168]) with different EPR parameters [169]. The CD spectrum of BSA (with His in the third position) ruled out the coordination of the metal by NTS [170]; EPR competition studies between Zn(II) and V(IV)O suggest that $V(IV)O^{2+}$ has two types of binding sites, one of them corresponding to MSA (VBS1) [168]. When the metal:ligand ratio is increased to 2:1 or higher, the EPR resonances suggest at least five different metal ions bound to albumin, with similar binding modes [168]. The exact number of such non-specific V(IV)O binding sites is not known exactly; it has been shown to be between five and twenty [169,171,172].

4. Summary

In conclusion, high resolution studies on the spatial structure and coordination chemistry of HSA with transition metal ions, mainly Zn^{2+}, have been almost exclusively based on single crystal X-ray crystallography, sometimes with the addition of isothermal titration calorimetry, and occasionally, other spectroscopic methods (Fig. 4). In 2016, the combination of the above techniques delivered first long-awaited structural details on the primary Zn(II) ion binding site in HSA. Although this constituted a substantial achievement, one needs take into account that the HSA:Zn(II) complex was crystallized under non-physiological conditions, at pH 9. In contrast, horse ESA yielded several high and medium resolution X-ray structures with variously complexed Zn ions, at many sites, and under a wide range of pHs, including pH 7.4.

With the recent advancements in the crystallization of HSA, one can expect further studies and results of this type, in the near future, possibly with other transition metal ions. Although rich in high-resolution atomic information, such studies still lack a detailed description of the functional dynamics of HSA protein, and of the site-resolved kinetics of ligand binding. For the time being, such data can only come from global methods like ITC. The striking difficulties of crystallization of HSA, in comparison with ESA, might point out to substantial differences in protein dynamics, thus in the solubility and coordination potency of different binding sites. Such details on the dynamics and structures of proteins remain largely elusive by current X-ray crystallography. The only biophysical method available that could tackle this challenge would be cutting-edge multidimensional NMR spectroscopy optimized for high-molecular weight systems [173–179]. So far, NMR is largely limited by the size of HSA and the problems connected with the production of native and active HSA, at a large scale, from bacterial expression systems [180,181]. Thus, selective deuteration-based labeling techniques cannot be exploited to their maximal potency. An interesting alternative would be a combination of state-of-the-art direct X-detected techniques (X = ^{13}C, ^{15}N), like ^{13}C [182] and more notably ^{15}N nuclei [174,183], as they would not require the deuteration of the protein. Moreover, various fast methods, might be coupled with NMR-DNP (Dynamic Nuclear Polarization) in order to enhance the strength of the NMR signal [184–187]. Still, one needs to bear in mind that HSA is a highly soluble protein reaching blood concentrations of ~0.63 mM, and that can be further concentrated, up to 2 mM, *i.e.* more than one order of magnitude above of what can be reached, on average, for the proteins in the range of 25 to 45 kDa that have been investigated by NMR spectroscopy.

Recently, rapid developments of the state-of-the-art of NMR data acquisition and data analysis might lead soon to major breakthroughs in solution studies of HSA. NMR will be the technique of choice for investigating dynamic protein structures and their modulation by various endogenous and exogenous ligands and metal ions. This new insight will hopefully lead to novel diagnostic and therapeutic tools for biomedicinal research and its applications.

Declaration of Competing of Interest

Authors declare no conflict of interest.

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