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Research review paper

Insights on the emerging biotechnology of histidine-rich peptides

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ABSTRACT

In the late 70's, the discovery of the restriction enzymes made possible the biological production of functional proteins by recombinant DNA technologies, a fact that largely empowered both biotechnological and pharmaceutical industries. Short peptides or small protein domains, with specific molecular affinities, were developed as purification tags in downstream processes to separate the target protein from the culture media or cell debris, upon breaking the producing cells. Among these tags, and by exploiting the interactivity of the imidazole ring of histidine residues, the hexahistidine peptide (H6) became a gold standard. Although initially used almost exclusively in protein production, H6 and related His-rich peptides are progressively proving a broad applicability in novel utilities including enzymatic processes, advanced drug delivery systems and diagnosis, through a so far unsuspected adaptation of their binding capabilities. In this context, the coordination of histidine residues and metals confers intriguing functionalities to His-rich sequences useable in the forward-thinking design of protein-based nano- and micro-materials and devices, through strategies that are comprehensively presented here.

1. Introduction

Protein purification is an essential practice in biotechnology, drug development and proteomics, and a late downstream step in the recombinant protein production task flow (Garg et al., 1991; Gräslund et al., 2008). Considering the diversity of cell factories and target proteins in the protein production scenario, simple, one-step separation protocols are ideally required to fulfill the demands for generality, scalability, and high throughput (Fig. 1A). In this context, affinity tags, namely peptides or proteins with selectivity for specific binders have been incorporated to recombinant proteins by genetic fusion, usually at either the amino or carboxy termini, to selectively retain the whole fusion in affinity chromatography systems based on immobilized ligands (Amarasinghe and Jin, 2015; Kimple and Sondek, 2004; Mishra, 2020). Essentially, any pair of interactors involving at least a peptidic partner should be suitable for the design of entrapment protocols to retain a specific protein among complex molecular mixtures such as cell lysates or culture media.

Since the 70's (Porath et al., 1975) and along decades (Gaberc-Porekar and Menart, 2001; Porath, 1992; Riguero et al., 2020; Spriestersbach et al., 2015; Sulkowski, 1985), immobilized metal affinity chromatography (IMAC) has proved to be a robust choice method for preparative protein purification because of its versatility, cost effectiveness, scalability and simplicity, compliant with high-throughput requirements. The IMAC concept is supported by the relatively strong interactions between transition metal ions (namely Ni^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+}) and the side chains of some particular amino acids. Among them, the amino acid histidine is the tightest binder through the electron donor group on its imidazole ring, that forms coordination bonds with the metal (Watly et al., 2014). If immobilized in a matrix, the metal acts as a molecular hook for histidine-rich protein segments, that can be further eluted from the matrix by adjusting the buffer pH or by adding excess imidazole. Since series of consecutive histidine residues confer a much tighter binding than isolate amino acids (Bornhorst and Falke, 2000; Gaberc-Porekar and Menart, 2005), polyhistidine stretches and specially H6, have become gold standard affinity tags for protein purification

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(Table 1). In this sense, smaller tags with three to five histidine residues can also serve for this purpose (López-Laguna et al., 2019) at the expense of a weaker binding. On the other hand, longer histidine tags (e.g. H8 or H10) are also an alternative when high purity is needed and optimization of H6-tags is not sufficient (Grisshammer and Tucker, 1997). Several downsides, however, include the possibility of inhibiting protein functions, inducing conformational changes, decreasing the protein expression, or requiring higher imidazole concentrations to elute, which ultimately lead to unstable proteins in the solution (Mohanty and Wiener, 2004). Nevertheless, it is historically assumed that H6 is an appropriate choice when designing recombinant proteins unless previous concerns regarding the desired number of histidine residues exist. The binding mediated by histidine residues occurs since at physiological pH, the molecular coordinative foci of imidazole rings are found in the δ1-Nitrogen atom, which is capable to act as a nucleophilic center and share two unpaired electrons with the desired targeting molecule, which will act as an electrophilic Lewis acid. In particular, the d-orbitals of these electrophiles allow the formation of coordinative bonds whose geometrical structures promote the simultaneous gluing of several imidazole rings from adjacent His residues (Valenti et al., 2006). This type of interaction is strongly held into the structure formed by the molecular partners in the complex, supporting mechanical forces that sustain the applicability of polyhistidine tails as molecular ligands (Lopez-Laguna et al., 2019).

2. Advanced systems for protein purification

The traditional IMAC, with divalent cations immobilized on agarose resin beads, basically Sepharose, remains as a conventional protocol (Loughran et al., 2017). Here, several divalent cations (Co^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}) are useful for purification purposes and each of them shows different binding affinity towards histidine. Its choice therefore, depends on the final application. Co^{2+} for example, forms less stable coordination bonds with histidine residues and is especially suitable when high protein purity is required. On the other hand, Cu^{2+} has the greatest affinity and is useful for enriching low abundant proteins from crude lysates, prior to other purification steps. Ni^{2+} and Zn^{2+} have an intermediate affinity, being nickel the most extended choice (Riguero et al., 2020). In all these cases, divalent cations might suffer from leakage from IMAC columns and elute with the protein of interest. For some particular applications, the presence of metal ions may affect protein activity or promote undesired reactions. Therefore, in these cases, we must ensure that the final product is free of divalent cations. In this sense, some commercial protein purification companies have already developed resins that show tighter bonds with the divalent cation and propose strategies to diminish its presence in the eluted fractions (e.g. using prepacked columns, pre-washing the column with elution buffer prior to protein purification or connecting an uncharged column just after the purification column,in series, to catch leaked ions). An alternative solution is to eliminate eluted ions during the protein dialysis step using a chelator such as EDTA, although this chemical compound can also be a contaminant for the protein of interest and it must be subsequently removed (Mónico et al., 2017).

Advanced strategies based on this principle have been developed to purify His-tagged proteins in a more efficient, sustainable, easier or cheaper manner. In this regard, magnetic nanoparticles have emerged as a simple, fast, and high-throughput system to purify proteins from complex bacterial or animal cell lysates by magnetic separation when functionalized with linker-chelating metal ions (Xu et al., 2004), nickel silicate (Wang et al., 2014a) or simply a nickel coating (Minkner et al., 2020). In 2019, Schwaminger and collaborators went a step further with a proof of principle of a magnetic one-step purification system, using non-functionalized bare iron oxide nanoparticles (BIONs) (Schwaminger et al., 2019). Besides IMAC, liquid-liquid extraction with affinity ionic liquids has been also used for the same purpose, resulting in a suitable method for large-scale purification (Ren et al., 2015). His-tagging has served for the purification of structures more complex than single polypeptides, such as bacterial outer membrane vesicles (OMVs) (Alves et al., 2017), virus-like particles (Gupta et al., 2020) or bacteriophages (Ceglarek et al., 2013).

An additional step in the enhancement of IMAC purification exploits nitrilotriacetic (NTA)-Ni^{2+}-His tag coordination complexes (Fig. 1B). NTA is an aminopolycarboxylic acid with a tripodal tetradentate nature, able to form coordinative interactions with divalent metal ions in a bonding type called chelation. The NTA-metal ion complexes can specifically interact with certain chemically compatible amino acid residues. Among all different options, imidazole rings from histidine residues are extremely good targets, as they offer two non-bonded valences on their surface that are available for further metal ion coordination. In a general manner, more-than-two NTA (bis or tris) structures are needed to ensure a sufficient strong binding between ion and histidine (Fig. 1B). As a result, ternary bis or tris NTA-metal ion-histidine stable complexes are formed. From here, the chemical versatility of NTA allows this technology to be used in a plethora of different protein engineering fields (Wijeratne et al., 2016).

Although poorly studied in general terms, His-rich tags are suspected to have potential undesired impacts on the structure, function and immunogenicity of some of the target proteins (Khan et al., 2012; Singh et al., 2020). Such side-effects of His-tagging, mild and case-dependent, can be solved by the proteolytic removal of the peptide upon purification, driven by a protease target site introduced during the genetic fusion (Goh et al., 2017) or by regulatable, Ni^{2+}-mediated hydrolysis (Abd Elhameed et al., 2019). More details about the potential bottlenecks generated by His-tagging are discussed below. Because of these issues, alternative affinity tags have been extensively used, such as small peptides (FLAG, c-Myc, Strep-Tag) or longer polypeptidic ligands such

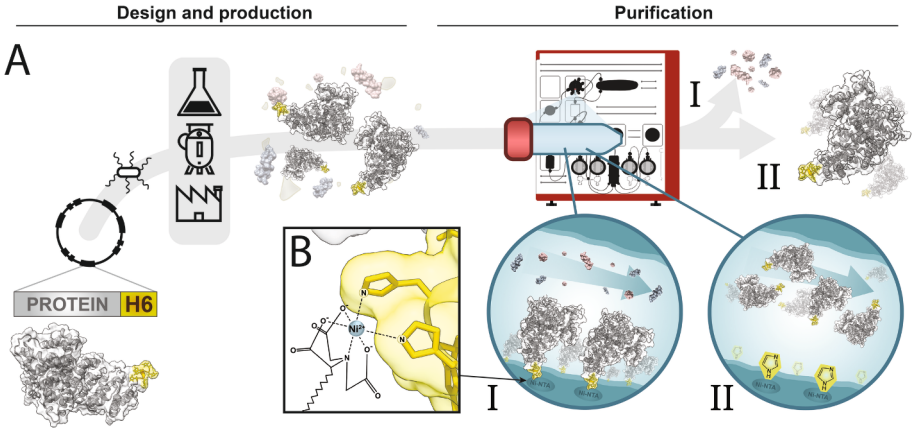

Fig. 1. A. Schematic process to obtain a pure recombinant polypeptide (e.g. His-tagged Human Serum Albumin, PDB: 1AO6) by means of a His-tag genetically fused to the target protein. The sequence of activities involves gene design (in which the desired protein-encoding gene is fused to the selected tag), cloning, transformation in a capable cell factory, production in an adequate scale (lab, pilot or industrial), His-tag based immobilization in an IMAC column (I) and subsequent displacement using imidazole (II). B. Zoomed representation of the coordination bonding between Ni-NTA and the histidine imidazole ring.

Table 1
A summary of applications of His-rich tags in protein sciences.

Application category	Strategy	Used material or technical basis	Approaches	Representative references
Protein purification	Traditional Chromatography	Immobilized Metal Ion Affinity Chromatography (IMAC)	Sepharose beads with immobilized divalent metal ions	Loughran et al. (2017)
	Alternative Material-based strategies	Magnetic NPs	- Linker chelated metal ion NPs. - Nickel (II) Silicate NPs. - Nickel (II) Coated NPs. - Iron oxide NPs (BIONs).	Minkner et al. (2020); Schwaminger et al. (2019); Wang et al. (2014a); Xu et al. (2004)
	Alternative Liquid-based strategies	Affinity ionic liquid (AIL)	Liquid-liquid extraction with affinity ionic liquids	Ren et al. (2015)
Protein labelling and detection	Traditional Ab-protein recognition	Western Blot	Anti His-tag Abs emitting detectable signals	Hirano (2012); Kreisig et al. (2014)
	His-tag radiolabeling	$^{99m}Tc(CO)_3$-His-tag	Technetium-99 attached to His-tags, emitting radioactive signals	Garousi et al. (2020)
	scFv-Bioimaging	Histidine tagged monoclonal scFv	scFv Abs recognizing biological compounds and His-rich tags allowing cell or tissue imaging (e.g. by immunofluorescence, immunoelectron microscopy, ELISA or Western Blot)	Min and Yamabhai (2020)
	Mono or Tris NTA-based complexes	Signaling compound-NTA-Ni^{2+}-His-tag	NTA- Ni^{2+} complexes label His-tagged proteins and conjugated compounds (e.g. $ATTO_X$, $ALEXA_X$, OG488, FEW_{646}, BODIPY, arylazide, QD and gold nanoparticles) emit detectable signals	Banerjee et al. (2016); Chao et al. (2017); Kitai et al. (2011); Kollmannsperger et al. (2016); Soh (2008); Wang et al. (2014b); Zheng et al. (2016)
Protein immobilization	Basic Immobilization strategies for biosensing	Surface-NTA-Ni^{2+}-His tagged proteins	NTA-Ni^{2+} complexes conjugated to chemically adequate surfaces allowing the detection of His-tagged proteins, nucleic acids, biomarkers, toxins, immunoglobulins or pesticides	Dai et al. (2018); Kang et al. (2017); Liu et al. (2010); Mikula et al. (2018); Povedano et al. (2020); Ravikumar et al. (2018); Vallina-Garcia et al. (2007); Zhang et al. (2014)
	Alternative Immobilization strategies for biosensing	Au-coated probes-NTA-Co^{3+}-His-tagged proteins	Au-coated probes conjugated with NTA-Co^{3+} complexes used to create SPR Optic biosensors immobilizing His-tagged antigens	Horta et al. (2020)
		Chitosan-Zn^{2+}-His-tagged proteins in a quartz crystal microbalance (QCM)	Chitosan-Zn^{2+} complex immobilize and allow the detection of His tagged mycotoxin in hapten biosensors using the QCM technology	Soleri et al. (2015)
	Biocatalysis in glass surfaces	Fe^{3+} porosity glass carriers with immobilized His-tagged enzymes	Controlled porosity Fe^{3+} glass carriers used to immobilize His-tagged enzymes to catalyze reactions	Pellis et al. (2017)
	Biocatalysis in self-immobilized insoluble enzymes	Zn^{2+} assisted generation of reusable insoluble protein microgranules	Generation of H6-tagged β-galactosidase microscale granules with enhanced stability	Sanchez et al. (2021)
	Biocatalysis in bioreactors	Semicontinuous or continuous -flow bioreactors with His-tag immobilized enzymes	His-tagged enzymes immobilized in different functioning bioreactors promoting successive and multi-step product transformations	Britton et al. (2017); Plz et al. (2020)
		Microfluidic bioreactors with magnetic beads attaching His-tagged enzymes	Microfluidic packed-bed bioreactors using magnetic beads to attach His-tagged enzymes to perform desired enzymatic reactions	Peschke et al. (2019)
	Bioengineering	Glass-plate Ni^{2+} chelated surface with immobilized His-tagged growth factor	Glass-plate Ni^{2+} surfaces used to enhance *in vitro* proliferation of human mesenchymal cells by immobilizing FGF	Shakya et al. (2020)
Protein site-specific conjugation	Site specific conjugation by metal cation-coordination.	Desired compound-NTA-Ni^{2+}-His-tagged proteins	Biological compounds or materials such as DNA, nanolipoproteins (MPLA-NiNLP), Au-polymer NPs or QD can be chemically attached to NTA-Ni^{2+} complexes and be specifically coordinated to His-rich tags from proteins	Goodman et al. (2009); Rai et al. (2016); Shimada et al. (2008); Wilkins et al. (2019); Zheng et al. (2016)
	Site specific conjugation by alkynyl group addition	Baylis-Hillman ester - ternary NTA-Ni^{2+}	Ternary NTA-Ni^{2+} complexes can be used to bring an alkynyl group-carrying ester moiety near the imidazole ring of a desired histidines for its two stage derivatization with molecules.	Liu and Melman, 2013)
	Site specific conjugation by histidines *bis*-alkylation	Cation free Sulfone – His *bis*-alkylation.	Sulfone-mediated bis-alkylation of PEG-*bis*-sulfone or PEG_{10}-mono-sulfone molecules to His-tag in desired proteins (e.g. interferon α-2a or anti-TNFα domain Ab)	Cong et al. (2012); Peciak et al. (2019)
	Site specific conjugation by cyclic ketone generation	Michael addition of 2-cyclohexanone in His residue.	Orthogonal late-stage installation of aminooxy functionalized NMR tags or dyes in exposed His residues on protein surface (e.g. lysozyme C)	Joshi and Rai (2019)
	Site specific conjugation by histidine acylation.	D-gluconic acid δ-lactone (GDL) and 4-methoxyphenil esters for specific acylation of amino-terminal His-tags ($GHHH_n$).	Site specific acylation allows the direct addition of a biotin or an azide functional group for its subsequent reaction with DBCO-functionalized molecules into His-rich tags of different proteins (e.g. GFP, MBP or SUMO).	Martos-Maldonado et al. (2018)
Protein assembling	Ion-based protein gluing to manufacture soluble nanostructures	His-tag/divalent cation/His-tag	Divalent cations used as coordinative agents of His-tags (interacting with the δ1 nitrogen of the histidine imidazole ring) and promoting	Bai et al. (2013); Brodin et al. (2015); Cespedes et al. (2018); Dexter et al. (2006); Diaz et al. (2018); Haglin et al. (2017);

(*continued on next page*)

Table 1 (*continued*)

Application category	Strategy	Used material or technical basis	Approaches	Representative references
			adequate nanostructuring (10-350 nm, e.g. dimers, coiled coils, nanodiscs, nanocapsules, nanorings, helical nanotubes, nanowires, interfacial films or tumor targeted NPs) in a reversible way (upon addition of EDTA)	Maniaci et al. (2019); Serna et al. (2020a); Serna et al. (2020c); Tunn et al. (2018); Unzueta et al. (2020); van Eldijk et al. (2016); Zhang et al. (2012)
	Ion-based protein gluing to manufacture insoluble functional depots	His-tag/divalent cation/His-tag	Divalent cations used as precipitator agents to create functional secretory-like granules at the microscale in a reversible way (upon addition of EDTA)	Chen et al. (2019); Jiang et al. (2011); Sanchez et al. (2020); Serna et al. (2020b)

Ab: Antibody; scFv: Single-chain variable fragments; Ni^{2+}: Nickel (II); NTA: Nitrilotriacetic acid; $ATTO_X$ & $ALEXA_X$: $ATTO_{565}$ or $ATTO_{655}$ and $ALEXA_{647}$ or $ALEXA_{488}$; QD: Quantum Dots; SPR: Surface plasmon resonance; Au: Gold; Co^{3+}: Cobalt (III); FGF: Fibroblast growth factor; DTPC: DNA-template protein conjugation strategy; PEG: Polyethylene Glycol; NMR: Nuclear magnetic resonance; GFP: Green fluorescent protein; MBP: Maltose-binding protein; SUMO: Small ubiquitin-like modifier protein; EDTA: Ethylenediaminetetraacetic acid; DBCO: Dibenzocyclooctyne.

as Maltose Binding Protein (MBP) or Glutathione S-Transferase (GST) (Pina et al., 2014). Also, several novel tags are already under development (Spy Tag, Strach Binding Protein, Fluropatite, Diatomite, Beta-GRP) trying to reduce protein contaminants and/or production costs (Khairil Anuar et al., 2019; Wood, 2014). Related to that, a specific solution to increase His-tagged protein purity is the use of low background cell factory strains, such as *Escherichia coli* LOBSTR, which is engineered to eliminate major His-rich contaminants (Andersen et al., 2013).

3. Protein labelling and detection

3.1. Protein immunodetection

Specific protein recognition appears as a prior method in proteomic profiling, and monoclonal or polyclonal antibody (Ab)-protein immunodetection has raised as one of the most popular methodologies for protein identification (Signore et al., 2017). Upon Western Blot, protein detection ensures adequate polypeptide identification and quantification even in complex samples (Obermaier et al., 2015). Considering the large number of H6-tagged recombinant proteins (Loughran et al., 2017), the importance of using anti-polyhistidine tag antibodies has emerged in parallel (Kreisig et al., 2014). In anti-H6-based detection techniques (Table 1), a primary monoclonal Ab recognizes the H6 tail, followed by a secondary Ab that recognizes the first attached Ab (Fig. 2A). Primary (or secondary, binding to primary) Abs are typically labelled with organic fluorophores (e.g. Alexa Fluor) or enzymes (e.g. alkaline phosphatase and peroxidase) that emit a measurable signal (Hirano, 2012) allowing its quantitative detection.

3.2. Tracking oriented protein labelling

Interestingly, the popularity of using NTA-Ni^{2+}-His tag coordination complexes in protein purification has uplifted in novel protein engineering fields (Johnson and Martin, 2005) and single molecule imaging, along with recent H6 radiolabeling (e.g. $^{99m}Tc(CO)_3$) (Garousi et al., 2020) and single-chain variable fragments (scFv) of monoclonal Abs bioimaging (Min and Yamabhai, 2020). Novel formulations of NTA-based complexes are being designed to ensure precise protein detection at sub-nanomolar concentrations and to create clear pictures of protein spatiotemporal behavior under physiological conditions (Kollmannsperger et al., 2016). The specific conjugation of chromophores (e.g. $ATTO_{565}$, $ATTO_{655}$, $ALEXA_{647}$, $ALEXA_{488}$, OG_{488}, and FEW_{646} (Kollmannsperger et al., 2016; Soh, 2008)), small molecules (e.g. distylryl BODIPY or arylazide crosslinkers (Chao et al., 2017)) and other nanoscale materials (e.g. quantum dots, QD (Banerjee et al., 2016) or gold nanoparticles (Kitai et al., 2011)) to these complexes allows exploring biological fundamental processes such as viral infections, protein dynamics and mobility (e.g. QD-NTA-Ni^{2+} labelling respiratory syncytial virus (Zheng et al., 2016), or Au NPs NTA-Ni^{2+} labelling dynactin subunit p62 (Kitai et al., 2011)), protein function (e.g. labelling the DNA repair protein XPA122 (Chao et al., 2017)), subcellular location (e.g. tracing extracellular and intracellular histidine-tagged proteins (Wang et al., 2014b), protein targeting, biodistribution and general protein pharmacokinetics (Garousi et al., 2020). The tripodal tetradentate nature of NTA allows the eventual interaction with exposed H6 tails (Raghunath and Dyer, 2019). Besides the binding affinity of mono-NTA is sufficient for IMAC needs (K_D: 10×10^{-6} M), the molecular labelling of polyhistidine-tagged proteins requires tighter associations to form stable complexes useful in biological conditions. Because of that, three NTAs are usually associated, named Tris-NTA, to provide sufficient binding affinity (K_D: 20×10^{-9} M (Huang et al., 2006)) and to increase the complex stability up to 4 orders of magnitude, thanks to the multivalency of the system. Tris-NTA-Ni^{2+} coordinated complexes are being used by the scientific community to interact with inner-cell histidine tagged proteins without affecting endogenous processes, presenting high specificity and low cell toxicity (Kollmannsperger et al., 2016). These complexes can be bound to H6-tagged cell penetrating peptides (CPPs) to promote cell entrance, and afterwards, spontaneously change its preference to bind H10-tagged proteins of interest inside the cell, due to the 10-fold higher binding affinity of the longer tag (Schneider and Hackenberger, 2017). Also, NTA-Ni^{2+}-red fluorescent probes or similar (e.g. AB or BODIPY) complexes can enter into bacteria cells to selectively label histidine-tagged proteins by using appropriate amounts of detergents (e.g. Tween 80 (Chao et al., 2017)). The traditional proteomic profiling techniques, together with protein radio-labelling methods and NTA-Ni^{2+}-based complexes (Fig. 2B), outpoint the huge versatility and applicability of using H6 (or related tags) as labelling target for protein characterization and *in situ* functional imaging.

4. Protein immobilization in biosensing, biocatalysis and bioengineering

Protein immobilization onto solid surfaces has several applications in protein-based biocatalysis, biosensing and bioengineering (Table 1). In this sense, among the growing variety of developed protein immobilization methods, including entrapment, adsorption, cross-linking or covalent binding, affinity techniques provide a highly specific, controllable, and reversible way to anchor proteins to surfaces without losing their functional structure (Ley et al., 2011; Zhang et al., 2014). Additional benefits of this approach are protein stabilization, protection against environmental stresses and reusability in successive processes (Rodrigues et al., 2013). Taking advantage of their affinity towards divalent metal cations, surfaces or nanomaterials can be decorated with His-tagged proteins, rendering uniformly oriented protein layers with controlled spatial distribution and without multipoint attachments that could impair their functionality (Ley et al., 2011). This chemistry works well under mild conditions, at physiological pH and in presence of salts,

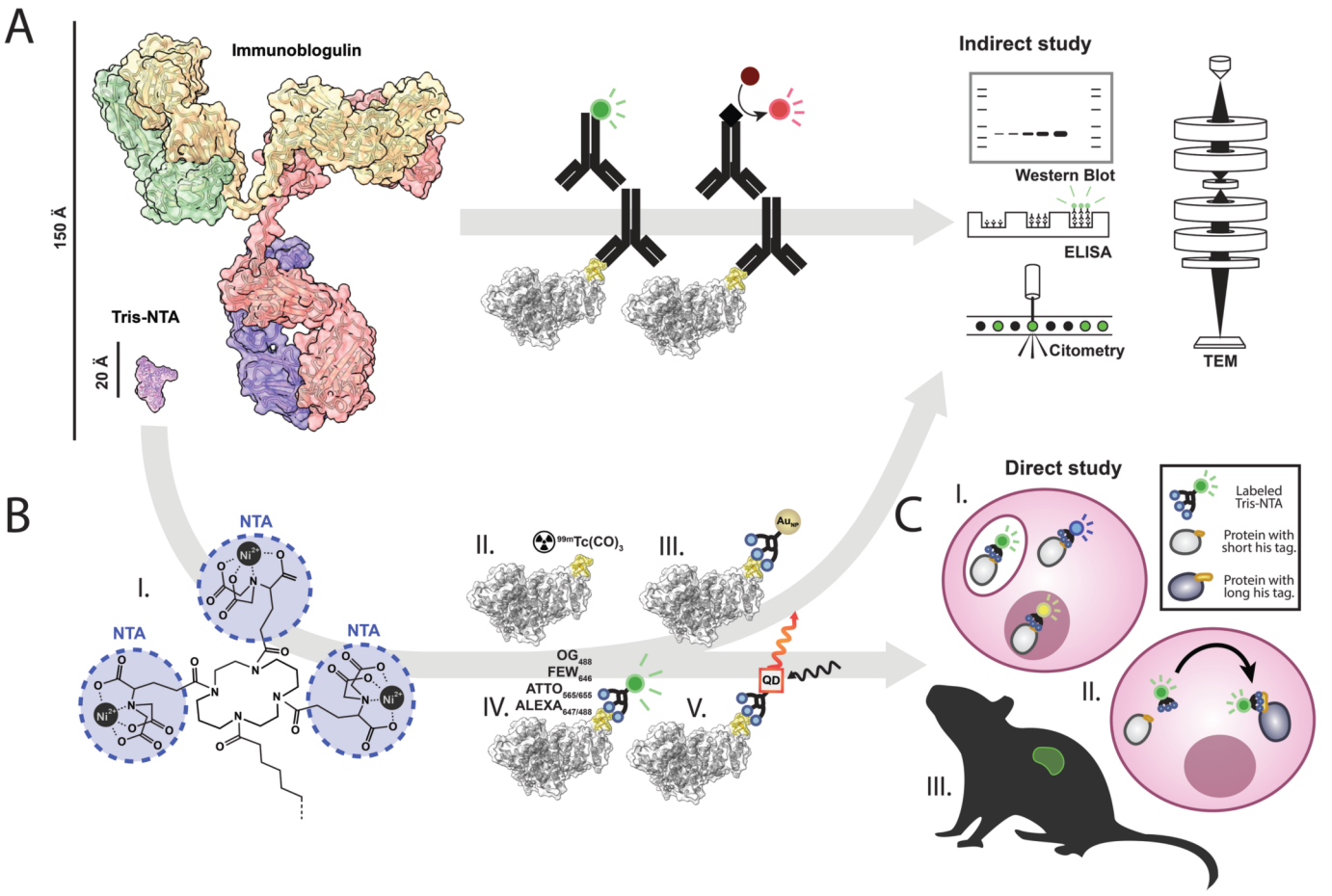

Fig. 2. A. Overview of size, common labeling strategies, and popular detection techniques available for the study of His-tagged proteins using antibodies. B. Size comparison and structure of the Tris-NTA ligand (I), along with common labeling approaches (radiolabeling with Technetium 99m (II) or Tris-NTA-based labeling with gold nanoparticles (III), fluorescent dyes (IV) and quantum dots (V)). C. Schematic representation of direct techniques to study subcellular localization of his-tagged proteins *in vitro* (I), co-localization of different his-tagged proteins *in vitro* (II), or protein bioaccumulation in *in vivo* models (III).

making it appropriate for applications in biological interfaces (Wegner et al., 2016). The conventional procedure to immobilize His-tagged proteins involves an earlier step of surface functionalization with NTA, followed by the incorporation of the divalent cation, typically Ni^{2+} (Liu et al., 2010). In such process, a deep consideration of surface pretreatment, metal leaching and unwanted dissociation of the His-rich tag from the divalent cation-NTA pair is suited (Ravikumar et al., 2018). In this regard, a plethora of procedures are emerging to solve these issues whilst maintaining the benefits of His-tagged immobilization. For instance, alternative strategies involve the use of multiple NTA groups (Lata et al., 2005), longer His-rich tags (Lata et al., 2005), different chelators (e.g. chitosan (Ravikumar et al., 2018)), acbztacn (Johnson and Martin, 2005), 1-acetato-4-benzyl-triazacyclononane (Wollenberg et al., 2014), iminodiacetatic acid (Zhao et al., 2017), NiO (Jia et al., 2016) and polydopamine (Yang et al., 2015)) or the conversion to different metal oxidation states (namely Fe^{3+} (Pellis et al., 2017) and Co^{3+} (Wegner et al., 2016)). In this last case, the process requires a first-step of complexation between histidines and a Co^{2+} ion, followed by a subsequent Co^{2+} ion oxidation into Co^{3+}, resulting in a tight immobilized complex.

4.1. Biosensing

In biosensors, His-tagged proteins have been broadly used in optical (Auer et al., 2017; Avsar et al., 2020; Faccio et al., 2016; Horta et al., 2020; Lata et al., 2005; Ravikumar et al., 2018) and electrochemical (Dai et al., 2018; Povedano et al., 2020; Vallina-Garcia et al., 2007; Zhang et al., 2014) devices and also in piezoelectric transducers (Baltus et al., 2007; Li et al., 2016; Nam et al., 2013; Wegner et al., 2016). Optical biosensors, mainly based on Surface Plasmon Resonance (SPR) or Biolayer Interferometry (BLI), are extensively employed to detect the interaction of an immobilized His-tagged protein with its ligand, due to a change in the light properties (Fig. 3A). For example, Horta and co-workers have recently developed an optical-fiber-based SPR biosensor to detect autoantibodies in autoimmune diseases, by immobilizing His-tagged antigens on Co^{3+}-NTA Au-coated probes (Horta et al., 2020). A similar approach, involving Co^{3+}-His complexes, has been used for immobilizing virus-like particles in a BLI biosensor that detects norovirus antibodies from human serum samples (Auer et al., 2017). Moreover, the chemokine receptor CXCR4 has been successfully immobilized for studying its interaction with nanobody-Fc ligands, which represents an important progress in the study of G protein-coupled receptors involved in a broad range of diseases (Boonen et al., 2020). Electrochemical biosensors tend to be sensitive, fast, simple, cheap, and easy-to-miniaturize devices that detect a change in electrical properties in the media (Dai et al., 2018; Ley et al., 2011). Their use is specially promising in point-of-care facilities, but their susceptibility to electrochemical interferences such as pH or ionic strength changes must be faced. His-tagged proteins in electrochemical biosensors have been used for the detection of nucleic acids (Dai et al., 2018; Povedano et al., 2020), biomarkers (Ta et al., 2016), toxins (Vallina-Garcia et al., 2007), immunoglobulins (Kang et al., 2017; Mikula et al., 2018) or pesticides (Zhang et al., 2014). The quartz crystal microbalance (QCM) is a simple, label-free, real-time, and efficient tool for the detection of biomolecular

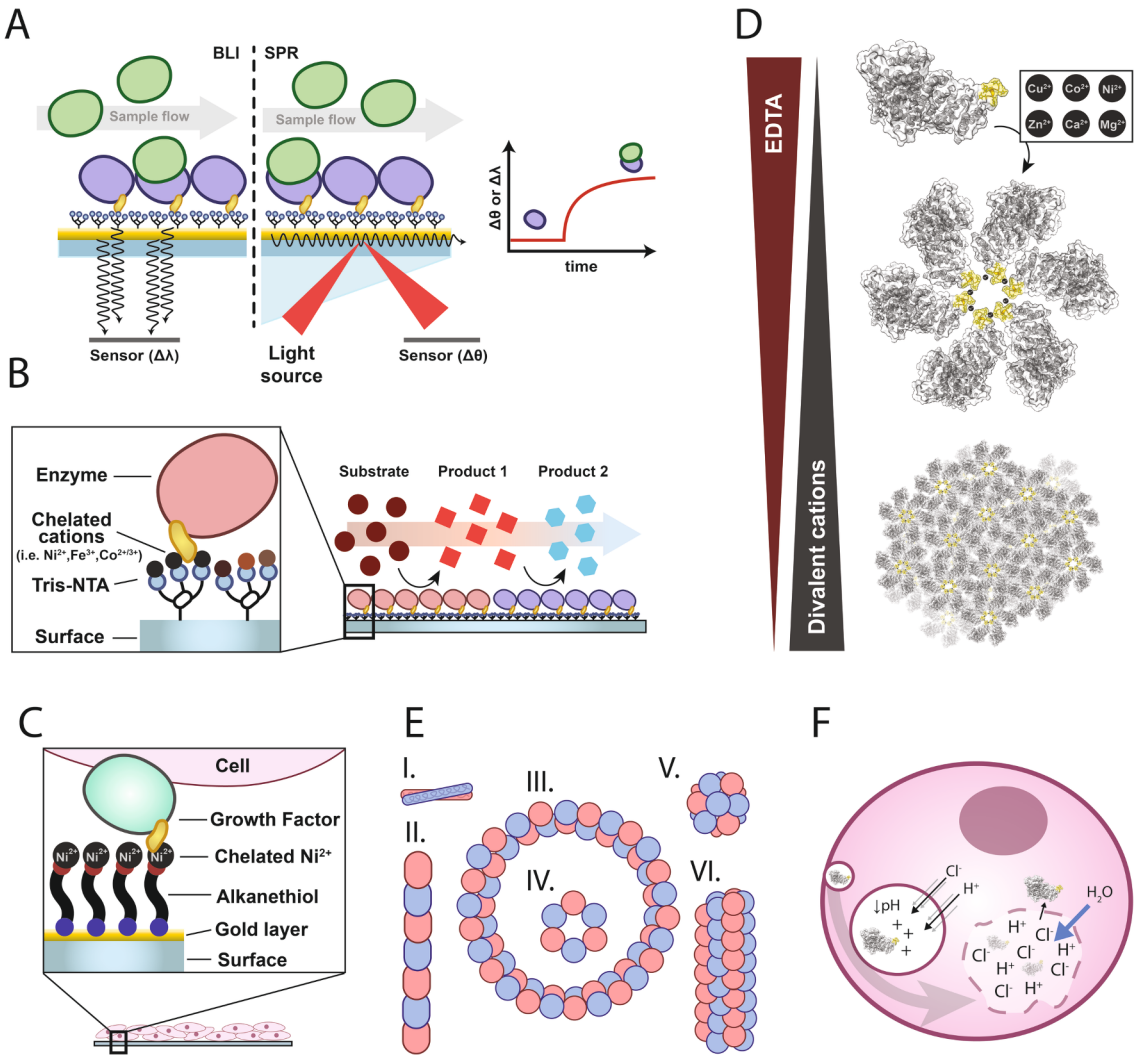

Fig. 3. A. Schematic representation of biolayer interferometry (left) and surface plasmon resonance (right) in his-tag based biosensing. B. Schematic disposition of immobilized enzymes for a sequential biocatalysis in which the product of the first reaction acts as substrate for the second reaction. Common cations used in immobilization are Ni^{2+}, Fe^{3+}, Co^{2+} or Co^{3+}. C. His-tagged protein coating as a bioengineering strategy to enhance cell growth. D. Schematic representation of how histidine residues promote protein assembly and secretory granule-like structures with the addition of divalent cations. E. Representation of the wide range of nanoscale protein materials that can be assembled by histidine coordination with metal cations such as coiled coils (I), nanowires (II), nanorings (III), toroids (IV), nanocapsules (V) and nanotubes (VI). F. Proton sponge effect; illustration of how the decrease in pH in endosomes in presence of his-tagged proteins results in the endosomal disruption and subsequent protein liberation.

interactions that is based in the measurement of the frequency decrease caused by a mass increase (upon interaction) in the functionalized surface (Nam et al., 2013). Its major drawback is the lack of sensitivity for small molecules. In a recent report it has been demonstrated the feasibility of QCM to explore the binding properties of a GPCR to its immobilized partner, which provides the basis for studying other similar interactions through this technique (Avsar et al., 2020). Finally, mycotoxin recognition peptides have been also immobilized over Zn^{2+} adsorbed chitosan foam via His-rich tag for the development of hapten biosensors (Soleri et al., 2015).

4.2. Biocatalysis

In biocatalysis, immobilization enhances enzymatic stability and facilitates product recovery and enzyme reusability. His-rich tag-based immobilization has been used for many different reactions in different supports. For example, EnginZyme® has developed Fe^{3+}-chelated glass carriers with controlled porosity in which His-tagged enzymes can be successfully immobilized and perform their activities (Pellis et al., 2017). Also, H6-tagged β-galactosidase has been self-immobilized using a molar excess of Zn^{2+} as mechanically stable microscale granules, a format that allows reusability and that enhances the operational stability of the enzyme (Sanchez et al., 2021). On the other hand, there is a growing tendency to synchronize purification and immobilization of His-tagged enzymes in one-step procedures, avoiding the initial on-column step and directly working with complex protein mixtures derived from cell lysates (Wang et al., 2017). Britton and collaborators developed a continuous-flow bioreactor with a 10-min synchronized purification and immobilization strategy (Britton et al., 2017). Moreover, they were able to produce a multi-step bioreactor that allows successive transformations by controlling spatial distribution of each enzyme throughout the bioreactor surface (Fig. 3B). The same principle has been applied in semi-continuous bioreactors (Plz et al., 2020). Magnetic nanoparticles have been widely used for such dual purpose for both intracellular and extracellular proteins obtaining great enzyme reusability indexes after several batches (Yang et al., 2015; Zhao et al., 2017; Zhou et al., 2017). Peschke and co-workers have exploited the use of magnetic beads in a microfluidic packed-bed bioreactor with His-tagged loaded ketoreductase (Peschke et al., 2019). The use of miniaturized reactors may be notably important in the future development of

sustainable production processes.

4.3. Bioengineering

His-tagged protein immobilization is also useful for bioengineering (Fig. 3C). For example, the Fibroblast Growth Factor (FGF) was immobilized onto the surface of a Ni^{2+} chelated glass-plate to enhance *in vitro* proliferation of human mesenchymal stem cells (Shakya et al., 2020). The successful outcome can be directly attributed to the structural integrity of the FGF domain in the fusion protein. As it has been done for biosensors, Ni^{2+} has been replaced by Co^{3+} to stabilize the bond between His-rich tag and NTA. Di Russo and co-workers proved that such replacement results in the development of a higher and broader range of E-cadherin adhesion forces, leading to extended cell spreading and colony organization (Di Russo et al., 2019). This principle is highly relevant for further development of functionalized surfaces for cell culture.

5. Site-specific conjugation

The functionalities of proteins or protein nanomaterials can be tuned by the conjugation of drugs or different organic molecules. As a representative example, in the Antibody Drug Conjugates (ADCs) the therapeutic molecule is covalently attached to a protein nanocarrier for its targeted delivery. Usually, protein nanomaterials are also linked to different organic molecules such as Polyethylene Glycol (PEG) to improve their pharmacokinetic properties, stability or immunogenicity. In all cases, site-directed conjugation is very convenient, not only because it would allow molecular binding in a desired and controlled position of the protein, but also since such strategies generate more homogeneous products with low batch-to-batch variability and with higher stoichiometric control. In this sense, engineering of histidine-rich motifs within the protein also plays an important role as site-specific conjugation targets.

5.1. Divalent transition metal ion coordination

As the most explored approach, many molecules chelated to divalent transition metal ions such as Ni^{2+} or Zn^{2+} have been attached to different nanomaterials (Table 1). This is done by the generation of ternary complexes with the unpaired electrons in imidazole rings of specific histidine residues as previously described. In this sense, NTA-modified DNA molecules have been conjugated to proteins such as Green Fluorescent Protein (GFP) (Goodman et al., 2009) or alkaline phosphatase (Shimada et al., 2008) using Ni^{2+} ion coordination as a molecular glue. This strategy has been also used to attach different types of molecules such as monophospharyl lipid A adjuvants containing nanolipoprotein particles (MPLA:NiNLP) to His-tagged foot-and-mouth disease virus capsids (Rai et al., 2016), or NTA-modified quantum dots to progeny virus capsids (Zheng et al., 2016). Further, NTA-modified gold-polymer nanoparticles have been also conjugated to His-tagged antifreeze peptides to generate new active nanomaterials (Wilkins et al., 2019).

5.2. Templated addition of alkynyl group or lysine conjugation

Hexahistidine tags can be also used as templates for a first-step temporal cation-coordinated molecular binding and subsequent covalent bonding with a neighboring histidine residue within the protein. In this sense, templated alkylation of an imidazole residue in a H6 tag can be achieved using Baylis-Hillman esters tethered to NTA in presence of divalent cations. Here, Ni^{2+}-coordinated ternary complex is generated between NTA and two adjacent histidine residues. This event brings the reactive double bond of the Baylis-Hillman ester into the vicinity of another free imidazole ring for its covalent binding and subsequent breaking of the previous NTA bond. This methodology allows the successful derivatization of recombinant proteins with a reactive alkynyl group that undergo subsequent cycloaddition of molecules (Liu and Melman, 2013).

As a similar approach, the DNA-template protein conjugation strategy (DTPC) allows the initial direction of an NTA-modified oligonucleotide to a His-tagged protein. This serves then as a template for complementary N-hydroxysuccinimide (NHS)-bearing molecule conjugation to a lysine residue, in the close proximity of the histidine-rich tag (Rosen et al., 2014).

5.3. Bis-alkylation of histidine residues

A more sophisticated His-rich tag specific conjugation methodology relies on the *bis*-alkylation of two histidine residues located close together in the target protein by sulfone-containing molecules, without the need of any metal chelator. Following this strategy, molecules such as PEG have been successfully attached to different His-tagged proteins using PEG-*bis*-sulfone or PEG_{10}-mono-sulfone molecules. Moreover, this type of conjugation was successfully achieved using different types of polyhistidine tags containing both continuous (H2, H6, H8) or discontinuous (-HGH or -HGHGHG) histidine residues (Cong et al., 2012; Peciak et al., 2019).

5.4. Generation of cyclic ketone

The use of an electrophile molecule such as 2-cyclohexanone has also allowed cation-free site-selective modification of surface-accessible histidine residues on a protein. This Michael addition generates a cyclic ketone able to form an oxime by the addition of aminooxy-derivative probes. Using this methodology, different types of molecules including nuclear magnetic resonance (NMR) tags, fluorescent dyes or biotin have been successfully attached to a His-tagged protein (Joshi and Rai, 2019).

5.5. Histidine acylation

Finally, in a similar approach, specific acylation of an optimized amino-terminal histidine tag ($GHHH_n$) has been also reported using D-gluconic acid δ-lactone (GDL) and 4-methoxyphenil esters. This His-rich tag specific acylation methodology has allowed the introduction of small reactive groups such as azides in different proteins including GFP, maltose-binding protein (MBP) or the small ubiquitin-like modifier protein (SUMO) for further reaction with DBCO-functionalized molecules or direct conjugation of biotin (Martos-Maldonado et al., 2018).

6. Refined protein assembling and functional protein aggregation

Artificial but largely bioinspired nanostructured materials have been developed based on protein building blocks, with high precision and structural complexity, integrating order and dynamics into the system (Hamley, 2019; Korpi et al., 2020). Among the different protein-based assembling approaches, simple fusion protein engineering permits the incorporation of peptidic cross-interactors and the facile synthesis of *de novo* multimeric nanomaterials. This is done by bottom-up fabrication approaches that ensure a control over the range of biochemical (e.g. specificity, bioactivity or biodegradability) and physical (e.g. small size, conformation or multivalency) properties (Mendes et al., 2013). His-rich tags in recombinant fusion proteins have been empirically tested to construct regular, stable and highly versatile nanocarriers with pseudospherical architectures (Lopez-Laguna et al., 2019), highly suited for precision intracellular drug delivery (Falgas et al., 2020; Pallares et al., 2020). One of the most exploited chemical mechanisms that drives the assembling process based on H6 tags is the ion-based gluing (Lopez-Laguna et al., 2020). In this regard, divalent cations (e.g. Ni^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, and Ca^{2+}) are able to simultaneously coordinate more-than-one histidine residues from overhanging histidine tails,

promoting building block recruitment and adequate protein organization at the nanoscale (Fig. 3D). This process, usually renders materials ranging between 10 and 350 nm, which are efficiently disassembled by EDTA (Lopez-Laguna et al., 2020). The gluing process takes place when the respective ion interacts with the nucleophilic electron pair from δ1 nitrogen of the histidine imidazole ring and accommodates at least 2 near-in-space building blocks into the final oligomeric structure. These interactions could also take place when carboxyl groups from asparagine and glutamine amino acid residues are close by (Erthal et al., 2016; Lopez-Laguna et al., 2019). When envisaged for biomedical applications, engineering ionic doses should be appropriately adjusted to avoid possible systemic toxicity as stated in the recommended dietary allowance (RDA) (Lopez-Laguna et al., 2020). A huge variety of different H6-tagged materials have been lately constructed (Table 1) with different morphologies and pointing to different applications (Fig. 3E).

In this sense metal-controlled protein dimers have been generated upon zinc binding to designed histidine residues (Maniaci et al., 2019), which in some particular cases such as CheA/W chemoreceptors can further assemble into ternary complexes to generate functional arrays (Haglin et al., 2017). The bioengineering of strategically placed metal-coordinating histidine residues has also allowed the generation of synthetic coiled coil structures (Tunn et al., 2018). Going still further, the combination of specifically oriented metal coordinating histidines with additional intrinsic non-covalent interactions has allowed the generation of more sophisticated nanostructures. These include linear nanowires of different lengths (Zhang et al., 2012), circular nanorings whose diameters can be regulated by tuning the strength of the non-covalent interactions (Bai et al., 2013), or even multi-responsive triblock copolypeptides that self-assemble as 20 nm diameter nanocapsules (van Eldijk et al., 2016). Other sophisticated structures, generated through metal-histidine interaction, also include helical nanotubes, whose widths can be controlled by solution conditions (Brodin et al., 2015), stimuli-responsive interfacial films (Dexter et al., 2006) or toroid nanoparticles (e.g. toxin-based (Diaz et al., 2018; Serna et al., 2020a), venom-based (Serna et al., 2020c) GFP-based (Cespedes et al., 2018; Pallares et al., 2020; Unzueta et al., 2020) or human nidogen-based (Alamo et al., 2021), either being intrinsically toxic or delivering coupled antitumoral drugs (e.g. floxuridine or monomethyl auristatin E). Finally, in a step further regarding structural complexity, functional secretory-like granules at the micro scale have been generated also through controlled protein aggregation via H6 tag–divalent cation coordination. These artificial aggregates permit the slow release of a protein drug in a prolonged manner, then acting as a sustained drug release systems aimed at reducing the need of repeated doses (Chen et al., 2019; Jiang et al., 2011; López-Laguna et al., 2021; Sanchez et al., 2020; Serna et al., 2020b).

7. Systemic administration for therapeutic applications

When proteins with histidine tails are meant to be used for biomedical purposes, it is essential to know whether histidine tails could change the behavior of therapeutic proteins when they are applied *in vivo*. It is especially critical to study whether histidines play a role in protein biodistribution as well as subcellular localization. To this purpose, different clarifying studies have been carried out, the results of which are described below.

7.1. Biodistribution

Because of their universal use as purification tags, the potential impact of His-rich tails in the biodistribution of systemically administered proteins and their implication in drug delivery have been carefully explored in different experimental settings. In these studies, the role of the amino acid composition of the histidine-rich tail has been evaluated, using intercalated hydrophobic or hydrophilic amino acids with different charges and upon an H-X-H-X-H-X arrangement (Hofstrom et al., 2013), which still allows an efficient purification through immobilized metal affinity chromatography (Knecht et al., 2009). Besides this, the influence of the position of the tag at the carboxy or amino terminal end of the protein has been studied (Hofstrom et al., 2013; Hofstrom et al., 2011; Tolmachev et al., 2010; Vorobyeva et al., 2019) by using radioactive labeling, to determine their organ biodistribution in mouse models. The resulting data indicated that variations in the body localization of the materials do not rely exclusively on the histidine tail, but also on the presence of charges and hydrophobic patches on the scaffold protein and the way they are distributed (local vs. overall) on the protein surface (Hofstrom et al., 2011; Lindbo et al., 2016; Tolmachev et al., 2010). Even though the different studies are not comparable due to the use of different chimeric scaffold proteins such as affibodies (Altai et al., 2016; Hofstrom et al., 2011; Mitran et al., 2015; Tolmachev et al., 2010), nanobodies (Singh et al., 2014), scFvs (Casey et al., 1995), toxins (Altai et al., 2016), uniCAR target modules (Jureczek et al., 2019), ADAPT scaffolds (Lindbo et al., 2016), DARPIN scaffold (Vorobyeva et al., 2019) and also due to different linker chemistry for radioactive tagging (Lindbo et al., 2016), it can be globally inferred that hydrophobic domains and positively charged areas favors liver uptake (Altai et al., 2016; Hofstrom et al., 2013; Mitran et al., 2015; Singh et al., 2014; Tolmachev et al., 2010). In this sense, His-based tails like (HE)3, hydrophilic and negatively charged, could help to counterbalance the hydrophobicity and positive charges of the area (Hofstrom et al., 2013; Hofstrom et al., 2011; Mitran et al., 2015; Tolmachev et al., 2010), while (HT)5, uncharged, gets mostly cleared by kidneys (López-Laguna et al., 2019). Also, poly-histidine tails at the amino terminal end show, in general, a low level of nonspecific organ uptake, especially in kidney (Hofstrom et al., 2013; Hofstrom et al., 2011; Vorobyeva et al., 2019). In contrast, other studies show that the histidine tail does not affect significantly the biodistribution of the protein (Casey et al., 1995; Jureczek et al., 2019; Lindbo et al., 2016), indicating again that changes of the protein fate in the body depend more on the overall distribution of charged and hydrophobic amino acids of the His-tagged protein than on the mere presence and nature of the His-rich tag (López-Laguna et al., 2020). Of course, all these concepts and considerations have important implications in the therapeutic applications of His-tagged proteins.

7.2. Intracellular trafficking

His-rich tags have been exploited as endosomal escape enhancers to deliver proteins or nucleic acids to the cytosol due to their ability to trigger osmotic swelling and endosomal disruption (Ferrer-Miralles et al., 2011). Since the pKa of their lateral chain imidazole group is around 6.0, histidine residues undergo protonation state change from neutral to positive when endosomal lumen becomes acidic. In consequence, not only H^+ protons enter the lysosomes, but Cl^- anions also do it likewise in order to equilibrate charges, which generates an osmotic pressure that ends up with membrane disruption, namely the proton sponge effect (Fig. 3F). Following this mechanism, His-tagged proteins can promote higher cytosolic delivery of their cargo molecule (e.g. nucleic acids and other drugs (Ferrer-Miralles et al., 2011)) into the cytosol. Also, based on their selective protonation at acidic pH, histidines has resulted attractive in the design of novel endosomolytic peptides (Boeckle et al., 2006; Paray et al., 2021). In this regard, the number of histidine residues, their distribution in the primary sequence and the endosomal vesicle size and membrane leakiness, has been described to affect this process (Ahmad et al., 2019; Kichler et al., 2007; Lo and Wang, 2008; López-Laguna et al., 2020; Vermeulen et al., 2018). (Munsell et al., 2016; Smith et al., 2019). Therefore, His-rich tag-dependent proton sponge effect can be considered a lysosome-specific membrane disruption system, although its real impact in promoting endosomal burst remains still controversial

8. Bottlenecks associated to polyhistidine tags

Because of the chemical versatility of His-rich tags, a plethora of different functional materials and surfaces have been constructed following bottom-up rational approaches. In this sense, although no intrinsic signs of cytotoxicity has been reported in the literature for polyhistidine tags, its potential influence over the protein structure, stability or function should be precisely considered when designing devices for biotechnological or biomedical applications (Zhao and Huang, 2016). Interestingly, while B-factor values (reflecting thermal motion) were usually slightly higher in His-tagged proteins, no significant structural effects have been generically observed neither in both resolution or R-factor (reliability factor) when considering a broad spectrum of crystallographic 3D protein structures with or without fused His tags (Carson et al., 2007). Similar results were obtained in recombinant GFP-based fusion proteins, in which H6 was placed in either the amino or carboxy termini or in a permissive solvent-exposed loop of the GFP barrel. In all cases, protein fluorescence remained intact and purification took place without any technical issues (Voltà-Durán et al., 2019).

8.1. Conformational impact

However, the influence of H6 tags on structural propensities and internal dynamics of small peptides might be more noticeable. While small peptides are highly mobile with no unique preferred spatial conformation, the addition of certain peptides such as His-based tags, triggers small chemical shifts and changes relaxation properties, being translated into long-range effect on overall structural propensities (Bräuer et al., 2019). This possibility was demonstrated when studying the His-rich tag effects on zinc finger proteins, in which the addition of those peptides either at their amino or carboxy-terminal positions promoted changes in their native or complexed-with-zinc CD spectra, unfolding profiles under urea degeneration tests, and protein functionality (Zhao and Huang, 2016). Alike influences were observed in disulfide bond structures but not in the binding behavior of the rat corticotropin-releasing factor receptor 2a in a His-rich tag carrying version (Klose et al., 2004). In this regard, while a huge catalogue of proteins should not be nearly affected by His-rich tag fusion, enzymes appear as a special and particular group sensitive to small structural changes by either creating or depleting functional scenarios. In this context, the carboxy-terminal tagging of tropinone reductase enzyme impaired its enzymatic activity while the amino-terminal tagged version remained fully active (Freydank et al., 2008). In agreement with that, *in silico* modelling of the protein structure showed an interference of the H6-tag with the enzyme active site by steric and electrostatic interactions only when placed at the carboxy terminal (Freydank et al., 2008). Similar scenarios were also described in L-lactate dehydrogenase, in the molybdoenzyme YedY, in secretory leukocyte protease inhibitors (SLPIs) and in hormone-sensitive lipases (HSL), in which the carboxy-terminal (Halliwell et al., 2001; Munadziroh et al., 2020; Sabaty et al., 2013) or the amino terminal (Wang et al., 2019) tagging resulted in reduced activities. The H6 tag fusion can also elicit a modulatory influence on the enzymatic activity of certain proteins and be used therefore as an advantage, as observed in the catalytic capacity of the nuclease domain of the Colicin E7 metallonuclease (NColE7) from E. coli, an enzyme used by *E. coli* to kill competing bacteria under stress conditions (Abd Elhameed et al., 2020). In this regard, the interference of the oligohistidine peptide tag with the Zn^{2+} containing active center was used for the regulation and fine tuning of the enzymatic activity. From another point of view, scanning fluorimetry profiles displayed both beneficial and deleterious effects on the thermal stability of the protein, depending on the presence or absence of a H6 in the amino-terminus (Booth et al., 2018) possibly due to an occasional architectonic rearrangement of the final protein structure. Mild and case-associated modulation of protein solubility have been also occasionally observed upon His-rich tag fusion (Woestenenk et al., 2004).

The variability of the already presented conformational scenario opens up to the idea of using small peptide linkers to reduce the conformational impact of fusion his-rich tags and to ensure an adequate conformational structure thus refining the design of bioactive fusion proteins. In this sense, three major categories of linkers can be found according to their physicochemical or functional properties (flexible, rigid or in vivo cleavable), which can be selected depending on the particular scientific needs. In fact, these linkers could not only contribute to minimize the conformational impact of introduced tags over the protein but also improve the biological properties of the tagged protein by increasing their production yields, by achieving desirable pharmacokinetic profiles or by improving their biological activity. In this regard, flexible linkers appear as the most appropriate ones to reduce the conformational impact of the histidine tag, as they confer sufficient mobility and flexibility to such tag. Flexible linkers are generally composed by small and either polar (e.g. Serine; Ser or Threonine; Thr) or non-polar (Glycine; Gly) amino acid residues. Their small size allows the protein sequence being sufficiently mobile, and their polarity contribute to protein stability in aqueous solution not only by forming hydrogen bonds with water molecules, but reducing the unfavored interactions between other protein regions. The most commonly used flexible linkers include (Gly-Gly-Gly-Gly-Ser)n or (Gly-Ser)n sequences. In this sense, the n value could be adjusted for an optimized separation between functional domains, or to maintain necessary interdomain interactions. Although Gly and Ser are the most used amino acids, Thr and Alanine (Ala) could be also introduced to maintain sufficient structural flexibility and Lysine (Lys) and Glutamine (Glu) to improve sequence solubility. In all cases, the rational design should be supported by experimental data, ensuring the optimization of the linker sequence, in order to fit with the particular needs linked to the recombinant protein (Argos, 1990; Chen et al., 2013).

8.2. Immunogenicity

On the other hand, immunogenicity assessment is paramount to ensure the safe and effective development of therapeutic proteins (Ishii-Watabe et al., 2018), since mitigation or full ablation of immunological-based adverse events such as anaphylaxis, cytokine release syndrome, and cross-reactive neutralization of endogenous proteins is mandatory (Lau and Dunn, 2018; Tourdot and Hickling, 2019). Indeed, immunogenic responses correspond to the most common non-desired side effects of proteins in clinics. In this scenario, protein-based drugs are currently transforming the pharmaceutical industry, displaying an unpreceded potential specially in precision medicines (Sanchez-Garcia et al., 2016; Serna et al., 2018) and promoting the development of highly effective treatments (Lagasse et al., 2017). Then, immunogenicity of potential tags used in production might be a critical issue (Tourdot and Hickling, 2019). From here, a dichotomy appears; while a large catalogue of manufactured proteins with clinical potential are His-tagged, a public concern shows up in which the use of H6 tag is discouraged by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) due to its potential non-desired immune response, although no official specifications are publicly indicated. In this context, alternative histidine-rich tags have been designed and tested with the aim of solving these regulatory concerns. For that, humanized versions, alternative to the traditional H6, were constructed derived from BLAST-ed human proteins namely H3A (HAAHAH), H5T (HTHTHTHTH) and H5E (HEHEHEHEH). Interestingly, all humanized His-tag versions maintained their protein production, purification and functional properties, displaying a huge potential to be used in humanized biomedicines (López-Laguna et al., 2020).

From a technical point of view, part of the scientific community considers H6 sequences as safe tags, due to its small size (0.84 kDa), absence of electric charge (non-interactive material), and low cell

toxicity and immunogenicity (Loughran et al., 2017). This is of course in addition to the very convenient potential of His-rich segments as purification tags, at low costs and under mild imidazole-promoted elution and native or denaturing conditions (Lau and Dunn, 2018; Loughran et al., 2017), making feasible the industrial production of protein drugs. Notably, protein BLASTs also shows coincidences in more-than-six consecutive histidine segments found in human proteins, hinting that human immune system is not expected to react when administering H6-tagged formulations (López-Laguna et al., 2020). Besides this conceptual consideration, there is no huge quantity and detailed experimental facts describing H6 immunogenicity, neither *in vivo* nor *in vitro*. In addition, His-rich tags have been included in multiple vaccine candidates against malaria (Angov et al., 2003; Dutta et al., 2002; Otsyula et al., 2013), proceeding as far as phase IIb in clinical trials without apparent safety concerns in vaccinated children (Jin et al., 2017). A similar scenario was observed in tandem phase I studies evaluating reactogenicity, safety and immunogenicity of His-tagged antigens (*Plasmodium falciparum* surface protein; $MSP1_{42}$) administered intramuscularly with AS01 as adjuvant (Otsyula et al., 2013). On the other way around, the amino-terminal His-tagged *Streptococcus pneumoniae* surface protein SP08456 promoted undesired immunogenic responses when administered intramuscularly, in comparison to its non-tagged version for bacteria immunization in Balb/c mice (Singh et al., 2020). In short, there are not experimental concluding remarks that support or preclude the use of His-tagged drugs into clinics.

8.3. Catalytic activity of imidazole rings

The addition of His-tags in recombinant proteins for purification or other purposes may not be as innocuous as previously thought. Imidazole rings appear to be reactive species able to catalyze specific enzymatic reactions, and so changing the resulting properties of the His-tagged protein material.

In this sense, the catalytic function of the imidazole ring has been reported since 1956 in several scenarios, in which different types of polymers, nanoparticles, peptide bundles and nanofibers have been involved (Bai et al., 2011; Chadha and Zhao, 2013; Giusti et al., 2014; Guler and Stupp, 2007). Indeed, this chemical group has been described as able to catalyze the transformation of p-nitrophenyl acetate hydrolysis (p-NPA) (Denmark et al., 1990), as well as, having a capital role in aldol-based and RNA cleavage reactions (Anslyn and Breslow, 1989; Breslow, 1994; Kirby and Marriott, 1995; Markert et al., 2009; Scheffler and Mahrwald, 2012). Its chemical versatility (especially when conforming the side chain of the histidine amino acid) lies in its amphoteric nature. Thus, the imidazole ring is able to lose a proton via its pros nitrogen atom or to accept a proton via its tele nitrogen atom at pHs close to 7 (Schneider, 1978). Because of that, histidine residues are suitable for both nucleophilic and base catalysis reactions, in addition to the traditional metal coordination functions.

Therefore, due to this intrinsic chemical reactivity of the imidazole ring, His-tags might be not biologically inert when fused to a protein. Among the different experimentally described scenarios, the His-tag can present esterase activity as reported in a GFP-H6 protein model, being able to convert p-NPA to p-nitrophenol and acetic acid in a linear concentration dependent way, and in an aqueous PBS-based solution (Schoonen et al., 2017). This esterase catalytic activity was also observed with other esterase substrates such as p-nitrophenyl butyrate (p-NPB), polyethylene glycol MW 800-modifed p-NPA (p-NPA-PEG) and carboxyfuorescein diacetate (CFDA). In addition, an increase in His-tag-mediated esterase activity was observed upon assembling of an His-tagged cowpea chlorotic mottle virus (CCMV), showing that the bigger the protein-based structure, the higher the activity. The activity increase upon protein assembling could be associated with a cooperative effect between close-in-space imidazole rings of the His-tag from each protein building block. Besides, this plausible cooperative effect between adjacent imidazole rings was discarded, as very small activity changes were observed at alternative increasing number-of-histidines His-tags, namely H-His-NH_2, H-His6-NH_2 and H-His12-NH_2. This was indicative of the irrespective influence of the number of close histidines into the final catalytic capacity of the tag. Finally, in a further step, the addition of a His-tag into an enzyme structure can generate additional functions (dual-active enzymes) as reported in a His-tagged phenylacetone monooxygenase (His6-PAMO) able to hydrolyze p-nitroacetophenone to p-NP (via p-NPA), apart from its intrinsic natural oxidoreductase activity (from phenylacetone to benzyl acetate). Interestingly, this gain of function was inhibited in presence of a molar excess of $NiCl_2$, which was able to specifically bind to the imidazole ring and block its catalytic reactivity (Schoonen et al., 2017).

Because of that, the catalytic propensity of his-tags must also be considered when designing novel protein-based materials, to address undesired scenarios of gain of function.

9. Concluding remarks

Although short histidine-rich peptides were initially developed as tags for recombinant protein purification, their pleiotropic nature has facilitated their progressive and expansive adaptation as functional and structural agents in multiple biotechnological and biomedical fields that are sustained by protein-based materials. They range from catalysis to drug development, but include dozens of different refined uses such as designing advanced smart micro- and nano-scale materials, tools for precision medicines or for fine diagnosis approaches. Such innovative applications open exciting possibilities to tailor proteins as powerful devices with unexpected regulatable properties, as purpose-tailored functional devices.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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