Biological activities of histidine-rich peptides; merging biotechnology and nanomedicine

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Abstract
Histidine-rich peptides are commonly used in recombinant protein production as purification tags, allowing the one-step affinity separation of the His-tagged proteins from the extracellular media or cell extracts. Genetic engineering makes feasible the post-purification His-tag removal by inserting, between the tag and the main protein body, a target site for trans-acting proteases or a self-proteolytic peptide with regulatable activities. However, for technical ease, His tags are often not removed and the fusion proteins eventually used in this form. In this commentary, we revise the powerful biological properties of histidine-rich peptides as endosomolytic agents and as architectonic tags in nanoparticle formation, for which they are exploited in drug delivery and other nanomedical applications. These activities, generally unknown to biotechnologists, can unwillingly modulate the functionality and biotechnological performance of recombinant proteins in which they remain trivially attached.

Affinity protein purification
Protein engineering and production, as a widely spread methodological platform, is providing reagents for catalysis, drugs for pharmaceutical industries and in general, instruments for analytical research in structural biology, proteomics, interactomics and drug design among others. If secreted, recombinant proteins produced in prokaryotic or eukaryotic cell factories are found diluted in the medium, or alternatively, they occur as minor components of extremely complex macromolecular mixtures if retained inside the cell. Therefore, they must be purified so as achieve conveniently high concentration and purity levels required for stable storage and characterization or for efficient use in biological systems or in catalysis. The identification of peptide tags for single-step affinity-based purification, upon end terminal fusion, has provided an advantageous tool that represents a faster, easy and cost-effective alternative to multiple-step chromatographic separations [1,2]. Protein tagging allows the purification of virtually any protein without previous knowledge of its properties or those of its expected contaminants, since the capturing event relies on tag’s rather than on protein’s features.

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amino acids seem not affecting the packing of the crystals or atomic interactions, leading to the correct 3D structure determination [9]. Furthermore, the presence of purification tags might positively affect the solubility, productivity and proteolytic stability of recombinant proteins [10-14], which would provide an added value to these protein segments. These observations have prompted researchers to evaluate affinity tags not only for their usefulness as tools for downstream but also as midstream folding-assistant agents [1]. Finally, some analytical procedures to determine in vivo protein-protein interactions (such as the tandem affinity purification) are based on the presence in the model protein of affinity purification tags [15,16], relying on the assumption that these additional segments do not dramatically influence the protein’s natural capability to perform cross-molecular interactions.

**Histidine-rich peptides in Biotechnology**

Histidine-rich peptides (often H6 but also other peptide versions, Table 1) are probably the most used protein purification tags [1]. Being short sequences, His-tags do not add significant metabolic load to the protein production process and they can be easily incorporated to the protein by simple genetic engineering at the upstream level. Since the interaction between a His-tag and its ligand does not require any specific conformation, binding is feasible under either native or denaturing conditions (e.g. urea 8 M or guanidinium hydrochloride 6 M) [17] as well as under oxidizing or reducing conditions, resulting in high technological flexibility and easy scalability [18].

Immobile metal affinity chromatography (IMAC) [19] is a separation principle based on the differential and reversible affinity of transition metal ions such as Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ for histidines [20], due to the coordination bonds formed between metal ions and amino acid side chains exposed on the protein surface. Electron-donor atoms (N, S, O) present in the chelating compounds of the chromatographic support are capable of coordinating metal ions and forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds [21]. Four of the six coordination sites on the octahedral Ni$^{2+}$ center are occupied by the nitrilotriacetic acid (NTA) ligand, and the remaining coordination sites are occupied by two of the six imidazole moieties in the H6-tag. The model in which the interaction of the metal ion with His-tag locates in residues n and n+2 of the tag is confirmed by the fact that IMAC ligands can bind to His-tags consisting of consecutive as well as to alternating histidine residues.

As the chelating ligand is used to fix the metal to agarose, iminodiacetic acid (IDA) was initially employed and it is still in use today in many commercial IMAC resins. This technology was improved by the invention of the chelating ligand NTA in the 1980s [22].

The amino acid histidine shows the strongest interaction with immobilized metal ion matrices, as electron donor groups on histidine imidazole ring readily form coordination bonds with the immobilized metal. In Bia
core experiments, the dissociation rate of a H6-tagged protein to Ni-NTA was estimated to be $1 \times 10^{-6}$ to $1.4 \times 10^{-8}$ M at pH 7.0-7.4. The same study also showed that two histidines separated by either one or four other residues are the preferred binding motifs [23]. Interestingly, selection of an optimum tag by a phage-displayed library showed that tags with only two histidine residues possessed chromatographic characteristics superior to those of the most commonly used H6-tag [24]. Adsorption of a

| Protein                                      | Tag sequence | Position in the fusion protein | Tag removal | Use or biological activity                  | # of His residues | Reference or PDB entry |
|----------------------------------------------|--------------|--------------------------------|-------------|---------------------------------------------|-------------------|------------------------|
| GFP                                          | H6-N1-H6     | C                              | No          | Protein immobilization                      | 12                | [35]                   |
| N-acetiltransferase (PA4794)                 | GSS-H6       | N                              | No          | Putative antibiotic resistance protein      | 6                 | 3KKW                   |
| Toxoflavin-degrading enzyme (TxDE)            | H8           | C                              | No          | Phytotoxin degradation                      | 8                 | [36]                   |
| Affibody                                     | HEHEHE       | N                              | No          | In vivo imaging                             | 3                 | [37]                   |
| Antibody                                     | H6           | C                              | No          | Antibody Against HER3 and EGFR              | 6                 | [38]                   |
| Low-density lipoprotein receptor-related protein 6 | H8           | N                              | No          | Blood brain barrier crossing                | 8                 | [39]                   |
| eEF-2K                                       | thioredoxin- H6 | N                              | Yes         | Modulation of rate of protein synthesis     | 6                 | [40]                   |
| Human peripheral cannabinoid receptor (CB2)   | H10          | C                              | Yes         | Intracellular signal transduction          | 10                | [41]                   |
| Serine protease granzyme B (grB)              | H6           | N                              | Yes         | Apoptosis inducer                          | 6                 | [42]                   |
| Human granulocyte macrophage colony stimulating factor (hGMCSF) | H6           | N                              | Yes         | Cytokine                                   | 6                 | [43]                   |
protein to the IMAC support is usually performed in neutral or slightly basic media in which imidazole nitrogens in histidine residues are non-protonated. Elution of the target protein is achieved by protonation, ligand exchange or extraction of the metal ion by a stronger chelator, like EDTA.

**Histidine-rich peptides in Nanomedicine**

In a very different context, histidine-rich peptides are largely exploited in drug delivery as powerful endosomal escape agents, by their incorporation into drug-loaded nanoparticles or polyplexes to be internalized by target cells through endosome formation. During centripetal intracellular trafficking, most of the uptake routes converge into endocytic vesicles, where both vehicle and cargo may become enzymatically degraded when late endosomes are transformed into lysosomes [25]. Within endosomes, the imidazole group of histidine, with a pK around 6.0, gets protonated under the increasingly acid conditions. This step recruits Cl- ions with a consequent osmotic swelling and endosomal cracking (the ‘proton sponge’ model [26]), which results in the cytoplasmic release of the nanoparticle and escape from lysosomal enzymes. Histidine-rich peptides have been incorporated into polymers, liposomes and proteins (including virus-like particles, VLPs) (Table 2). In non-viral gene therapy, such histidine-empowered vehicles show up to almost $10^3$-fold transgene expression improvement when compared with their corresponding histidine-lacking, parental versions [27]. Despite other membrane-active peptides, poly-histidines remain neutrally charged at neutral pH, avoiding non specific binding to serum proteins and consequent inactivation of the particle [28].

The number of histidines and their distribution in the amino acid sequence of the tag determine endosomal escape efficiency of His-tagged vehicles and nanoparticles. Lo and Wang [27] showed that the endosomolytic activity of Tat-H10 fusion peptides could be 10-fold higher than Tat-H5 and Tat-H20 versions. Also, 10 His residues distributed at both sides of Tat peptide (C-5H-Tat-5H-C), are 10$^2$-fold more efficient than its equivalent C-Tat-10H-C. Although the optimal number of His residues is believed to be ranging between 5 and 10 (similar to the figures seen in His-based affinity tags, Table 1), the incorporation of only one His residue is sufficient to grant membrane-active properties to a drug delivery system [29]. Interestingly, although most of His-tags seem to favour intracellular targeting exclusively through proton sponge activities, LAH4

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**Table 2 Representative examples of His-rich peptides used in nanomedicine as endosomolytic agents.**

| Type of construct | Tag sequence | Experimental model | # of His residues | References |
|------------------|--------------|--------------------|------------------|-----------|
| pDNA/siRNA +peptide | CHK6HC CHBK3HC CH6K6HC | In vitro, HepG2, COS 7, and CHO cells; 10X more expression than w/o histidine | 2-12 | [44] |
| pDNA + peptide +lactosylated polylysine | HSWyG (23 aas; GLFHAIHFIHGGWHGLIHGWYG) | In vitro, HepG2, B16 and Rb-1 cells, 93-2150X more expression than control, with serum. | 5 | [45] |
| MS2 VLPs-peptide | In Hep3B cells mediate endosomal escape that doesn’t occur without the peptide. | 5 | [46] |
| pDNA+ PEG-H5WYG | CHO cells; increase expression 2-5 fold. | 5 | [47] |
| Lipopeptide + pDNA | Lau/PalCK3H2 | In vitro, COS 7 cells, similar results to PEI, lipofectamine | 2 | [48] |
| pDNA + peptide | LAH4 (26 aas; KKKALALHLHALHLHALHLALA) | In vitro, human hepatocarcinoma cells, 10x more expression than lipofectamine. | 4 | [49] |
| Chitosan-CH + pDNA | Chitosan-CH Chit. KH dendron | In vitro, HEK293 cells, increases expression up to 50-fold over chitosan alone. | 1 | [50] |
| Chit. 4 gen KH dendron + pDNA | Chitosan dendron improves escape over Chitosan-CH | 1 | [51] |
| pDNA + peptide | Tat-H10 C-H5-Tat-H5-C | In vitro, in U251, H4, T98G and C6 cell lines, up to 7000-fold improvement. In vivo, in rat intrastriatum injection | 10 | [27] |
| FuGENE lipid+ peptide + pDNA | In vitro in 5 different cell lines, significant improvement over pDNA+ peptide alone | 10 | [52] |
| CM-PLH+PbAE+pDNA | Polymer CM-PLH | In vitro, in HEK293 and B16-F10 cells, and in vivo, i.v. mice injection; higher transfection efficiency over PbAE alone | 1 | [53] |
| STR-CH2R4H2C +pDNA | STR-CH2R4H2C | In vitro, COS-7 cells, improves lipofectamine levels of expression. | 2 | [54] |
| [KK]KKHKK[K[K]KFG2] + pDNA | KHKKHKKK | In vitro, NIH 3T3, T-47D and COS-1 cells; expression is detected in 15-41% of cells, but not quantitated; no serum. | 24 | [55] |

Abbreviations: aas, amino acids; pDNA, plasmid DNA; PEI, poly (ethylene imine); aas, aminoacids; Lau, lauryl; Pal, palmitoyl; chit. 4 gen, chitosan 4 generation; CM-PLH, carboxymethyl poly l- histidine; PbAE, poly β-amino ester; STR, stearoyl; FGF-2, fibroblast growth factor-2.
(KKALLALHLAHLALHLALLKKA) is believed to interact with the endosomal membrane at pH 7, due to its amphipathic nature, and disrupt its physical integrity upon subsequent protonation [30].

On the other hand, H6 has been recently found involved in intermolecular protein-protein interactions, leading to the self-assembling of C-terminal His-tagged building blocks into nanoparticles, useful as vehicles in non viral gene therapy [31,32]. The nature of such organizing interactions is yet to be completely understood, and might involve other specific amino acids such arginines. Since the resulting nanoparticles seem to be more efficient in transgene expression when formed at slightly acidic pH values [32], its seems reasonable to speculate that protonated, overhanging H6 tails interact with anionic protein areas of side building blocks and also with DNA. A deeper understanding of the interactions promoted by His-rich peptides could offer tools for the rational engineering of nanoparticle formation and DNA encapsulation, both issues of critical relevance in biotechnological medicine [33,34].

Conclusions

Protein production scientists are usually unaware of the potent biological properties of histidine-rich peptides for which they are used in nanomedicine (essentially endosomal disruption but also cross-molecular electrostatic interactions), which are activated at acidic pH. The same (or very similar) His-rich peptides used in affinity chromatography, incorporated into different type of nanoparticles (most ranging from H2 to H10, Table 2) to favour intracellular trafficking in drug delivery [25], are equally employed as affinity tags for separation and often not removed after protein purification (Table 1). These remaining agents might render unexpected protein behaviour, when exposed to cells or nucleic acids, and specific measures for control should be implemented in biological assays when technical obstacles make His-tag removal inconvenient upon protein purification.

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Authors’ contributions

NFM and JLC have equally contributed to this manuscript. All authors have read and approved the final version.

Competing interests

The authors declare that they have no competing interests.

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References

1. Waugh DS: Making the most of affinity tags. Trends Biotechnol 2005, 23:316-320.
2. Kemp ME, Sondek J: Overview of affinity tags for protein purification. Curr Protoc Protein Sci 2004, Chapter 9, Unit 9.9.
3. Arnau J, Lauritzen C, Petersen GE, Pedersen J: Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. Protein Expr Purif 2006, 48:1-13.
4. Waugh DS: An overview of enzymatic reagents for the removal of affinity tags. Protein Expr Purif 2011, 80:285-293.
5. Li Y: Self-cleaving fusion tags for recombinant protein production. Biotechnol Lett 2011, 33:869-881.
6. Fong BA, Wood DW: Expression and purification of ELP-intein-tagged target proteins in high cell density E. coli fermentation. Microb Cell Fact 2010, 9:77.
7. Wood DW, Wu W, Belfort G, Derbyshire V, Belfort M: A genetic system yields self-cleaving inteins for bioseparations. Nat Biotechnol 1999, 17:889-892.
8. Wu WY, Mee C, Calilano F, Banki R, Wood DW: Recombinant protein purification by self-cleaving aggregation tag. Nat Protoc 2006, 1:2257-2262.
9. Carson M, Johnson DH, McDonald H, Brouillette C, Delucas LJ: His-tag impact on structure. Acta Crystallogr D Biol Crystallogr 2007, 63:295-301.
10. Wang Z, Li H, Guan W, Ling H, Wang Z, Mu T, et al: Human SUMO fusion systems enhance protein expression and solubility. Protein Expr Purif 2010, 73:203-208.
11. Sun QM, Chen LL, Cao L, Fang L, Chen C, Hua ZC: An improved strategy for high-level production of human vasostatin1-120-180. Biotechnol Prog 2005, 21:1048-1052.
12. Tang W, Sun ZY, Pannell R, Gurevich Y, Liu JN: An efficient system for production of recombinant urokinase-type plasminogen activator. Protein Expr Purif 1997, 11:279-283.
13. Nallamsetty S, Waugh DS: Solubility-enhancing proteins MBP and NusA play a passive role in the folding of their fusion partners. Protein Expr Purif 2006, 45:175-182.
14. Stefan A, Conti M, Rubboli D, Ravagli L, Presta E, Hochkoeppler A: Overexpression and purification of the recombinant diphertheria toxin variant CRM197 in Escherichia coli. J Biotechnol 2011, , 156: 245-252.
15. Li Y: The tandem affinity purification technology: an overview. Biotechnol Lett 2011, 33:1487-1499.
16. Li Y: Commonly used tag combinations for tandem affinity purification. Biotechnol Appl Biochem 2010, 55:73-83.
17. Hochuli E, Bannwarth W, Dobeli H, Gentz R, Stuber D: Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent. Nat Biotechn 1988, 6:1321-1325.
18. Block H, Kubicek J, Labahn J, Roth U, Scharf P: Production and comprehensive quality control of recombinant human Interleukin-1beta: a case study for a process development strategy. Protein Expr Purif 2008, 57:244-254.
19. Porath J, Carlsson J, Olsson L, Belfrage G: Immobilized-metal ion affinity chromatography. A new approach to protein fractionation. Nature 1975, 258:598-599.
20. Porath J: Immobilized metal ion affinity chromatography. Protein Expr Purif 1992, 3:263-281.
21. Gabrec-Porekar V, Menart V: Perspectives of immobilized-metal affinity chromatography. J Biochem Biophys Methods 2001, 49:335-360.
