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Polymorphic distribution of proteins in solution by mass spectrometry: The analysis of insulin analogues

Maely P. Fávero-Retto [a, d, 1], Luiz Henrique Guerreiro [a, e, 1], Cássio M. Pessanha [a, 1], Leonardo C. Palmieri [a, 1], Luís Maurício T.R. Lima [a, b, c, *]

[a] Federal University of Rio de Janeiro – UFRJ, Av. Carlos Chagas Filho 373, CCS, Bss24, Ilha do Fundão, 21941-902, Rio de Janeiro, RJ, Brazil
[b] Laboratory for Macromolecules (LAMAC-DIMAV), Brazilian National Institute of Metrology, Quality and Technology – INMETRO, Av. N. Sa. das Graças, 50 – Xerém, Duque de Caxias-RJ, 25250-020, Rio de Janeiro, Brazil
[c] National Institute of Science and Technology for Structural Biology and Bioimaging (INBEB-INCT), Federal University of Rio de Janeiro, Rio de Janeiro, 21941-590, Brazil
[d] Brazilian National Cancer Institute (INCA), 20230-014, Rio de Janeiro, RJ, Brazil
[e] Department of Chemistry, Institute of Exact Sciences, Rural Federal University of Rio de Janeiro – UFRRJ, Rodovia BR 465, km 7, CEP: 23890-000, Seropédica, RJ, Brazil

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ABSTRACT

The characterization of conformational and oligomeric distribution of proteins is of paramount importance for the understanding of the correlation between structure and function. Among the bioanalytical approaches currently available, the electrospray ionization-mass spectrometry (ESI-MS) coupled to ion mobility spectrometry (IMS) is the best suited for high resolution identification with high sensitivity, allowing the *in situ* separation of oligomeric and conformational species. We tested the performance of the ESI-MS technique along with the IMS separation approach on a broad variety of insulin and insulin analogues with distinct oligomeric distribution pattern. The measurement of commercial insulin allowed the identification of species ranging from monomers to hexamers and their complexes with zinc ions. Dissimilar distribution profile for regular insulin as a function of formulation component and among the insulin analogues were observed by ESI-IMS-MS but not by ESI-MS along, crystallographic assays or size-exclusion chromatography. These data suggest the additional suitability of ESI-IMS-MS in conformational and oligomeric profiling of biomacromolecules and biopharmaceuticals. The easiness of the technique provides further motivation for its application in the characterization of both raw and formulated protein biopharmaceuticals in routine and comparability exercises.

1. Introduction

Profiling the oligomeric distribution of proteins is key in the understanding of protein function, its interactome network under a proteomic perspective, and also applicable for biopharmaceuticals. For this particular class of products, both the conformation and the distribution of oligomeric state can ultimately determine the absorption rate from the site of administration of the biopharmaceutical, affecting the pharmacokinetics and ultimately the pharmacodynamics [1]. In particular for biosimilar/biopharmaceuticals follow-on products, an extensive characterization in all quality aspects is highly desirable in order to maximize quality, safety and efficacy [2–6].

According to the Food and Drug Administration/United State Pharmacopeia-Substance Registration System (FDA/USP-SRS):

"A substance is any matter that has a discrete existence, irrespective of origin, which may be biological or chemical." [7]. In addition, *"a substance is defined by what something is, and is not defined by how it is made or how it is used. Substance definitions are based on molecular structure or other immutable properties of a given material."* [7].

Abbreviations: ESI-IMS-MS, Electrospray ionization-ion mobility spectrometry-mass spectrometry; MALDI, matrix-assisted laser desorption and ionization; TOF, time of flight; HDMS, high definition mass spectrometry; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering.

* Corresponding author. Federal University of Rio de Janeiro – UFRJ, Av. Carlos Chagas Filho 373, CCS, Bss24, Ilha do Fundão, 21941-902, Rio de Janeiro, RJ, Brazil. Fax: +55 21 3938 6639.
E-mail address: Mauricio@pharma.ufrj.br (L.M.T.R. Lima).
[1] Fax: +55 21 3938 6639.

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In the context of this definition, a biopharmaceutical product must be characterized not only according to its chemical identity, but also based on its molecular structure, including higher order assembly, regardless of the manufacturing process.

The characterization of oligomeric states for protein can be performed by using a variety of bioanalytical techniques. However, the use of routine strategies such as size exclusion chromatography (SEC), electrophoretic mobility, dynamic light scattering (DLS), small angle x-ray scattering (SAXS) among others are limited by their resolution, concentration of the analyte and the composition of the solution [8,9], which in conjunction makes the analysis of biopharmaceuticals in its final, formulated product a challenging task. In particular, the experimental protocol for performing the bioanalytical measurements should be the closer as possible to the original formulation, since variations can result in changes of conformation and/or the assembly order [10]. Sedimentation velocity-analytical ultracentrifugation (AUC) is a powerful technique that allows the estimation of oligomeric states under varying solution conditions (including the finished formulated drug product), although it could not clearly separate them, such in the case of overlapping monomers and dimers of lispro and aspart [11]. Mass spectrometry (MS) techniques have been applied to the characterization of biomacromolecules, both the elucidation of chemical identity and routine analytical procedures. Among the MS techniques, the electrospray ionization mass spectrometry (ESI-MS) display distinctive advantages, due to its high resolution and its application for quantitative analysis. However, unidimensional ESI-MS does not allow the accurate determination of multiple oligomeric distribution of proteins due to overlapping of species with equivalent oligomeric order/charged states ratio. A solution to this issue would rely in using higher resolution and sensitivity and/or a separation technique in combination to the ESI-MS. The ion mobility spectrometry (IMS) has been used for the characterization of chemical and structural isomer, both for small and macromolecules [12]. This technique has been extensively validated by the use of proteins with well-characterized supramolecular complexes (n-mer) varying from monomers, dimers up to dodecamers, including myoglobin (n = 1), transthyretin (n = 1, 2, 4), insulin (n = 1, 2, 6), β2-microglobulin (n = 2,3,4) and *trp* trp RNA binding protein (n = 11) confirming the preservation of oligomeric and conformational states in the desolvated gas phase [13–18]. In brief, the IMS technique consist in passing a mixture of molecules through a pressurized gas chamber, resulting in a separation of molecules based on their transverse collision cross-section and resistance against the gas. The combined ESI-IMS-MS technique results thus in both separation of a complex mixture and a subsequent identification of the ion species.

Insulin is a pancreatic hormone known for its assembly in varying oligomeric species ranging from monomers to hexamers or even multihexamers [1,19–21]. This hormone comprises two polypeptide chains A and B connected by disulfide bounds. The B-chain can transit between a tense (T) and a relaxed (R) conformations, in respect to an increase in α-helical content in the B1 to B8 aminoacid region, which is driven by binding to phenolic compounds [22,23]. These conformation can interplaying with an intermediary conformation named *frayed* R (R^f), with the B1–B3 segment in an extended conformation [10,20,24,25]. Since the oligomeric state of insulin influences its absorption [1], the detailed characterization of this polymorphic distribution in the final formulated product or as close as possible of the finished product condition is likely to be of uber importance.

In a recent study, IMS has been used extensively in the characterization of insulin, both regular and aspart [16]. In zinc/phenol-containing solution, the authors have identified both monomers and hexamers in regular and aspart insulin, while dimers were only found in regular insulin. In contrast, in zinc-free solutions only monomers and dimers were found in regular insulin and only monomers in aspart insulin. These data clearly show a tight dependence on formulation condition and protein construct on the oligomeric distribution. In fact, in a subsequent work from our group using IMS we have reported dimers of aspart insulin sampled directly from the commercial product [10,20,24,25]. These gathered data indicate the high sensitivity of oligomeric protein distribution of insulin upon the formulation conditions. A large repertory of insulin analogues are currently available, in which changes in primary structure (i.e., aminoacid sequence) or post-purification chemical modifications (such as myristoylation) were introduced in order to achieve dissimilar properties in comparison to the regular (wild-type, original human sequence) insulin, such as modulation of the oligomeric distribution [19], resulting in varying pharmacokinetic (PK) profiles. The aspart (Asp^{28B}) and the LysPro ($Lsy^{28B}Pro^{29B}$) insulin analogues were designed to favor a lower molecular assembly and reach an ultrafast acting PK profiling [26,27]. The glargine ($Gly^{21A}Arg^{31B}Arg^{32B}$) insulin shows a slow absorption from the subcutaneous deposit due to its distinct isoelectrical point compared to regular insulin [28]. The detemir (des-Thr^{30B}; $Lys^{29B\text{-myristoyl}}$) also shows a protraction action compared to regular insulin due to the preference for assembly into hexamers and di-hexamers and its association to serum albumin after absorption [1,29].

In face of the currently available portfolio of insulin, both regular (obtained by dissimilar downstream methods and from three dissimilar expression systems though at similar final formulation composition) and insulin analogues with unrelated mutations and/or chemical modification, we decided to evaluate the performance of IMS against a series of human insulin products, behaving as an unique set to confront the performance of ESI-IMS-MS profiling approach of the oligomeric distribution of proteins. The regular insulin in the absence of formulation components was also investigated, allowing the evaluation of the specificity of IMS in the perception of the effect of such additives on the conformation and oligomeric distribution of insulin.

2. Materials and methods

2.1. Chemicals

The human insulin free from formulation components, named insulin (S), was obtained in powder form free from Sigma–Aldrich (Cat. 91077C, lot No 12A213-B; São Paulo, Brazil). The formulated human insulin (100 U/mL = 600 μM), regular and analogues, were purchased from local pharmacy and stored at 4 °C–8 °C until use. The product were identified as follow:

Regular (H) (Humulin R®; 3.5 mg/mL = 100 U/mL; lot #C020193, #A560347A),
Regular (N) (Novolin® R; 3.5 mg/mL = 100 U/mL; lot #TS62953, #TS62987);
Regular (I) (Insunorm® R; 3.5 mg/mL = 100 U/mL; lot #I550080, #I550005);
Aspart (A) (NovoRapid®; 3.5 mg/mL = 100 U/mL; lot #XS62590);
LisPro (L) (Humalog®, 3.5 mg/mL = 100 U/mL; lot #A797851C, #A696780);
Detemir (D) (Levemir®, 14.2 mg/mL = 100 U/mL; lot #BP50791);
Glargine (G) (Lantus®, 3.638 mg/mL = 100 U/mL; lot #1F248A).

Solution of insulin in the absence of formulation excipients (such as metacresol and glycerol) was prepared at 6 mg/mL in 2 mM HCl using purified insulin from powder form (Sigma–Aldrich), as directed in the Product Information (Sigma–Aldrich Cat. 91077C).