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Biophysical study of bevacizumab structure and bioactivity under thermal and pH-stresses

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ABSTRACT

The evaluation of the structural stability and bioactivity of monoclonal antibodies (mAb) is a crucial step in the development of mAb therapeutic based products, since immunogenicity needs to be avoided. In the present work, a study was carried out to understand the changes on the structure and bioactivity of mAbs induced by different pH and temperature values. Structural changes of bevacizumab were monitored using fluorescence spectroscopy, circular dichroism (CD) and Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). The secondary and tertiary structural content was monitored at six different pH values and at room temperature, upon heating up to 85 °C and upon cooling down to 20 °C. Furthermore, the temperature induced conformational changes were continuously monitored from 20 °C to 85 °C using fluorescence spectroscopy and circular dichroism, allowing to monitor the melting temperature of the protein at different pH values. The results showed that the thermal denaturation of bevacizumab was irreversible at all pH value. The conformational changes induced by pH were higher at extreme pH values (5, 9 and 10) than neutral pH. Thermal stability studies showed that pH 6 was the pH that confer bevacizumab the highest structural stability. These studies were confirmed by in vitro studies, where bevacizumab's bioactivity was measured by cell viability/proliferation at all pH values at room temperature, and it was found a higher bioactivity for pH 6. Biophysical and biological studies were correlated in order to understand the importance of the modifications in bevacizumab structural content on its bioactivity. However, a decrease in bevacizumab's bioactivity was observed for pH 8, 9 and 10. Overall, this work demonstrated the usefulness of the spectroscopy techniques for estimating the stability of therapeutic mAb during formulation development.

1. Introduction

Soon after the first FDA-approved therapeutic monoclonal antibody (mAb) in 1986, antibody-based therapy has received a remarkable interest in the treatment of various diseases, with approximately 50 mAbs being used as therapeutics molecules (Weiner, 2015). Their ability to bind with specificity and affinity to antigenic epitopes made them relevant, not only in the therapeutic, but also in diagnosis. For this reason, persistent investigators continue to explore the characteristics and potential of mAbs. Among all of the approved therapeutic mAb, the most common immunoglobulin used in therapeutics is immunoglobulin G1 (IgG1) (Elvin et al., 2013). Bevacizumab is a humanized IgG1 anti-angiogenic mAb used in the treatment of age-related macular degeneration (Li et al., 2012), retinal neovascularization (Abrishami et al., 2009), and several cancers (Penson et al., 2015; Zhang et al., 2016). Bevacizumab acts against vascular endothelial growth factor (VEGF), avoiding the interaction with their receptor. For that reason, this mAb is considered as anti-angiogenic drug, preventing the tumour angiogenesis and growth vascular endothelial cells (ECs) (Ferrara and Davis-Smyth, 1997).

Despite the high quality of current mAbs formulations, difficulties can emerge during their formulation, storage, and administration. These difficulties are usually related with physical and chemical instabilities, which can lead to denaturation and/or aggregation (Oliva et al., 2015). The aggregation of mAbs may potentially induce immunogenicity in patients, leading, in extreme cases, to anaphylactic reactions when mAbs are administrated (Schellekens, 2010). Indeed, the presence of aggregates in mAbs formulation is considered a critical

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quality attribute (CQA) for the FDA approval (Rathore and Winkle, 2009). Therefore, the understanding of how pH and temperature may affect the structural stability of mAb is essential for practical applications. A new challenge in antibody-based therapy is the encapsulation and conjugation of mAbs into nanocarriers (Cardoso et al., 2012). During the formulation process of new biopharmaceuticals products, it is common the use of aggressive conditions such as heat, extreme pH, sonication and the use of organic solvents. Therefore, it is an important to understand in which range of pH and temperature it is possible to manipulate the mAb, to avoid their aggregation and denaturation. It is known that these phenomena can lead to stability issues, an increase of immunogenicity and bioactivity loss of the mAb.

Exclusion-high performance liquid chromatography (SEC), dynamic light scattering (DLS), field flow fractionation (FFF), and mass spectrometry were some of the analytical techniques used to identify and quantify insoluble aggregates in any mAb formulation (Beck et al., 2013; Berkowitz et al., 2012). However, aggregates can also be detected through monitoring structural changes assessing the state of folding/unfolding of mAbs. The aggregation of mAb is associated with significant changes in their secondary and tertiary structure (Joshi et al., 2014; Vermeer and Norde, 2000). Structural modifications can be monitored by spectroscopic techniques, such as, attenuated total reflectance-Fourier transform infrared (ATR-FTIR), circular dichroism (CD) and fluorescence spectroscopy (Lin et al., 2015).

ATR-FTIR spectroscopy is a rapid and non-invasive technique used to evaluate and quantify changes in the secondary structure of proteins and to investigate protein folding and unfolding (Matheus et al., 2006). Algorithms such second-derivative, area overlap (AO) and spectral correlation coefficient (SCC) are used to quantify the changes in the mAb secondary structure and to detect the onset of aggregates. Additionally, ATR-FTIR is still sensitive to intermolecular β-sheet aggregation, being an excellent tool to monitor the aggregation processes (Cerasoli et al., 2014).

CD spectroscopy is based on the difference in the absorption of left-handed and right-handed polarized light by chiral groups, which are present in mAbs (Kelly et al., 2005). This technique allows to evaluate the secondary structure composition through Far-UV CD spectra (190–260 nm), tertiary structure fingerprint through Near-UV CD spectra (260–320 nm) and mAb folding/unfolding (Kelly and Price, 2000). Secondary structural changes occurring during protein unfolding can be monitored with CD spectroscopy (Kelly et al., 2005).

The fluorescence of aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine) in proteins can be monitored by fluorescence spectroscopy, being tryptophan the most relevant fluorescent probe used to investigate conformational changes in proteins due to its higher extinction coefficient and quantum yield (Royer, 2006) and due to the fact that tryptophan (Trp) emission is modulated by the accessibility and polarity of the solvent. Fluorescence spectroscopy allows to detect protein conformational changes, solvent accessibility changes and to determine the melting temperature of proteins. Protein conformational changes detected by alterations in fluorescent emission are modulated by solvent polarity (Abbas et al., 2013). When buried in an apolar environment, Trp displays an emission maximum (λ_{max}) around 320 nm. However, when exposed to polar solvents such as water, its emission maximum (λ_{max}) is around 350 nm. According to the solvent polarity, blue (lower wavelengths) and red shifts (higher wavelengths) on λ_{max} will be observed. Therefore, Trp is an intrinsic protein probe that allows to monitor protein conformational changes.

The aim of this study was to retrieve information about the thermal and pH structural stability of bevacizumab using ATR-FTIR, CD, and fluorescence spectroscopy, and to understand in which way the pH and temperature influence the denaturation phenomena and the bioactivity of the mAb. Thermal scanning using CD and fluorescence spectroscopy was carried out in order to monitor bevacizumab's thermal stability as a function of pH. On the other hand, in order to understand the bevacizumab bioactivity at room temperature and different pH values, proliferation studies with human umbilical vein endothelial cells (HUVEC) were made.

2. Materials and methods

2.1. Materials

The mAb used was Bevacizumab (Avastin®), which was kindly provided by Genentech Inc., South San Francisco, CA. Bevacizumab was stored at 4 °C and was provided with 51 mM sodium phosphate pH 6.2, 60 mg/mL trehalose dihydrate and 0.04% polysorbate 20 at pH 6.2. For the buffers preparation, sodium phosphate dibasic dehydrate, sodium phosphate monobasic monohydrate, sodium carbonate, and sodium bicarbonate were purchased from Merck (Darmstad, Germany). All buffers were filtered through 0.22 μm nylon membrane filter and degassed. Ludox® HS-40 colloidal silica was acquired from Sigma-Aldrich (St. Louis, MO, U.S.A.) for time-resolved fluorescence studies. For the cell culture, HUVEC were purchased from ScienCell (Carlsbad, CA, U.S.A.). The reagents for cell culture were acquired from Sigma, such as M199 medium, Fetal Bovine Serum (FBS), gelatin solution 1%, heparin sodium, endothelial cell growth supplement (ECGS), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

2.2. Sample preparation

A stock solution of bevacizumab was prepared at pH 5, pH 6, pH 7.4, pH 8, pH 9 and pH 10 from a commercial solution 25 mg/mL Avastin® pH 6.2. The pH values were chosen taking into account the possible pH used in a formulation process. Buffer solutions were prepared at concentrations used for real-time storage and drug delivery, considering the composition of Avastin®. The pH 7.4 was used to simulate the pH of blood in order to understand the behaviour of bevacizumab structure in the blood. Stock solutions of bevacizumab at 75 μg/mL, 100 μg/mL, 1.5 mg/mL and 3 mg/mL were prepared for steady-state fluorescence spectroscopy, CD spectroscopy, time-resolved fluorescence spectroscopy and FTIR spectroscopy, respectively. Different concentrations were used considering the sensitivity and resolution of these techniques.

2.3. Circular dichroism spectroscopy

The secondary structural content of bevacizumab at different pH values and different temperatures was evaluated by CD spectroscopy. The measurements were performed using a Jasco J815 CD Spectrophotometer (Jasco Incorporated, Easton, U.S.A.) and the lamp housing was purged with nitrogen. The spectra were monitored from 200 to 260 nm using a bandwidth of 1 mm, a data integration time of 2 s, a data pitch of 0.5 nm, and a scanning speed of 50 nm/min. The sample volume required was 0.4 mL because a 0.1 cm cell was used. All spectra were the average of 8 scans, leading to 9.6 min of acquisition time for each sample. The CD spectrum of the buffer was subtracted from the sample spectrum and smoothed using 10 points Savitzky-Golay function before conversion to absolute CD values. The mean residual ellipticity (MRE) was determined using a mean residual weight (MRW) of 112 for the bevacizumab according to the following equation:

$$[\varnothing]_{mrw,\ \lambda} = \theta_{\lambda} \times \frac{\mathrm{MRW}}{10 \times d \times c} \quad (1)$$

θ is the observed ellipticity (in degrees) at wavelength λ, d is the path length (cm), and c is the concentration (g/mL) (Joshi et al., 2014). The bevacizumab concentration was calculated by UV absorption at 280 nm in a NanoDrop ND-1000 UV–vis spectrophotometer (Thermo Scientific, Willmington, DE, U.S.A.), using a molar extinction coefficient of 243,340 M^{-1} cm^{-1}, at 280 nm for 1 mg/mL. The spectra of