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Comparison of predicted extinction coefficients of monoclonal antibodies with experimental values as measured by the Edelhoch method

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ABSTRACT

Pace et al. (1995) [1] recommended an equation used to predict extinction coefficient of a protein. However, no antibody data was included in the development of this equation. The main objective of this study was to therefore investigate how the predicted value of the extinction coefficient is comparable to the experimentally determined extinction coefficient of antibodies measured by the Edelhoch method. We have measured the extinction coefficients (ε) of 13 IgG1 monoclonal antibodies (mAbs) in phosphate buffer at pH 7.2. The maximum variability in the experimentally measured extinction coefficient of a given mAb molecule was found to be about 2%. Experimentally determined extinction coefficients of all mAbs were found to be lower than the predicted value, with the maximum difference found to being 4.7%. The highest and lowest values of experimental extinction coefficient among the thirteen IgG1 monoclonal antibodies obtained were 230525.9 M^{-1} cm^{-1} (i.e. 1.55 $(mg/ml)^{-1}$ cm^{-1}) and 191,411.6 M^{-1} cm^{-1} (i.e. 1.29 $(mg/ml)^{-1}$ cm^{-1}). A difference of <3% (with respect to mean value) was observed between the experimental and predicted values of the extinction coefficient. A comprehensive analysis and interpretation of the comparison of the predicted and experimentally determined extinction coefficient by the Edelhoch method is discussed in terms of structural characterization and accessible surface area (ASA).

1. Introduction

Accurate determination of protein concentration is essential for studies involving characterization, functional assays, and during any quantitative assessment of protein–protein and protein–ligand interaction parameters such as binding constant, stoichiometry, specific activity of enzymes, etc. In the past, the Lowry method (a colorimetric based method) was commonly used to measure protein concentration and proved to be useful during protein purification [2]. Later, Bradford developed a relatively more efficient protein-dye binding method to measure protein concentration [3]. However, the most commonly used method used to accurately measure protein concentration is ultraviolet (UV) spectroscopy using the Beer–Lambert law. This method requires knowledge of the absorbance (generally at 280 nm), pathlength of the cuvette, and an accurate value of the extinction coefficient (ε) for a protein at a given wavelength. Absorbance is a directly measured quantity and pathlength is easily known. In order to determine an accurate value of the extinction coefficient (ε), it is essential to accurately measure the protein concentration. Four commonly used methods to measure protein concentration are, (i) amino acid analysis [4], (ii) Kjeldahl nitrogen determination [5], (iii) dry weight method [6], and the (iv) Edelhoch method [7,8]. Pace et al. (1995) [1] has demonstrated that the most accurate method to experimentally measure protein concentration, and hence extinction coefficient, is the Edelhoch method. For proteins with no tryptophan or tyrosine residues, the Scopes method [9] can be used to measure protein concentration by measuring absorbance at 205 nm. An approximate estimate of the amount of protein in a crude extract can be determined by assuming an absorbance value of 1.3 for 1 mg/mL at 280 nm [10].

Pace et al. (1995) [1] also recommended that one can predict the extinction coefficient of a protein (particularly those containing tryptophan) in water with high accuracy (within ~2%) by using the

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above Eq. (1). However, no antibody data was included in the development of this Eq. (1). The extinction coefficient of a protein will depend on the environment of three chromophores; tryptophan, tyrosine, and cystine (disulfide bond) and in globular proteins, these chromophores are buried by 87%, 76% and 92% respectively [1,11]. Structural characterization and accessible surface area (ASA) of different antibodies were determined to understand the comparison of the predicted and experimental values of extinction coefficients by the Edelhoch method.

2. Materials and methods

2.1. Materials

Purified humanized IgG1 monoclonal antibody (mAb) was prepared by Eli Lilly and Company, Branchburg, NJ. Guanidinium chloride (GdmCl) was purchased from M.P. Biomedicals, LLC. All other chemicals were of molecular biology/analytical grades. The composition of the buffer (PBS) used here was 10 mM sodium phosphate, 145 mM sodium chloride pH 7.2.

2.2. Methods

2.2.1. Calculation of extinction coefficient from sequence

Using a detailed analysis of 116 experimentally determined molar extinction coefficient (ε) values of 80 globular proteins, Pace et al. (1995) [1] recommended that one can predict the extinction coefficient of a protein (particularly tryptophan-containing proteins) in water with high accuracy (within ~2%) by using the following equation,

$$\varepsilon_{280}(\mathrm{M^{-1}\,cm^{-1}}) = 5500 \times (\#Trp) + 1490 \times (\#Tyr) + 125 \times (\#(S\text{-}S)) \quad (1)$$

where *#Trp*, *#Try*, and *#(S-S)* are number of tryptophan, tyrosine residues and disulfide bonds respectively. In the development of Eq. (1), no antibody data was considered.

2.2.2. Determination of protein concentration

Concentration of a protein is most commonly measured using ultraviolet (UV) spectroscopy and the Beer–Lambert law:

$$A_\lambda = \varepsilon_\lambda c l = \log_{10}\left(\frac{I_0}{I}\right) \quad (2)$$

where A_λ and ε_λ are the absorbance and molar extinction coefficient ($\mathrm{M^{-1}\,cm^{-1}}$) of a protein at a given wavelength (generally 280 nm) respectively. c is the protein concentration in molar unit, l is the pathlength of the cuvette in cm, and I_0 and I are the intensities of incident and transmitted light respectively. Determination of accurate protein concentration requires an accurate value of the extinction coefficient. In addition, light scattering correction should be taken into consideration when measuring protein concentration [1,12]. A protein molecule does not have an intrinsic chromophore that absorbs above 310 nm. Therefore, any significant absorbance above 310 nm results from light scattering caused by aggregates or large particles. In the presence of light scattering, the observed absorbance will be elevated. According to Rayleigh light scattering, if the size of the scattering objects is smaller than the incident wavelength, Intensity of scattering light at a given wavelength ($I_{S,\lambda}$) $\propto \lambda^{-4}$

Therefore, one can write,

$$\frac{I_{S,\lambda_1}}{I_{S,\lambda_2}} = \left(\frac{\lambda_2}{\lambda_1}\right)^4 \quad (3)$$

I_{S,λ_1} and I_{S,λ_2} are the intensities of scattered light at wavelengths λ_1 and λ_2 respectively. So using Eq. (3), one can write [12],

$$A_{280,\mathrm{corrected}} = A_{280,\mathrm{observed}} - 1.706 \times A_{320,\mathrm{observed}} \quad (4)$$

We have used Eq. (4) for light scattering correction in all the experimental data presented here. However, other forms of equations for light scattering correction can also be used as discussed by Maity et al. [12].

2.2.3. Experimental determination of extinction coefficient using Edelhoch method

Only the side chains of tryptophan, tyrosine, and cystine (i.e. disulfide bond content) of a protein contribute to absorbance above 275 nm [13]. In 1967 [7], Edelhoch demonstrated that the absorption spectrum of an unfolded protein in 6M guanidium chloride (GdmCl) is very similar to the absorption spectrum of a solution containing an equi-residue concentration of the model compounds of tryptophan, tyrosine, and cystine. Therefore, Edelhoch assumed that ε values of tryptophan, tyrosine, and cystine of an unfolded protein in 6M GdmCl are highly comparable to the ε values of the model compounds of tryptophan, tyrosine, and cystine in 6M GdmCl.

Extinction coefficients of mAb-1 to mAb-8 have been determined in 10 mM sodium phosphate pH 7.2, and those of mAb-9 to mAb-13 have been determined in PBS pH 7.2 (10 mM sodium phosphate, 145 mM sodium chloride pH 7.2). The Edelhoch method used to determine extinction coefficient is outlined below:

(1) The extinction coefficient of an unfolded protein at 280 nm in 6M GdmCl solution is calculated using the following equation [1],

$$\varepsilon_{280}(\mathrm{6M\,GdmCl}) = 5685 \times (\#Trp) + 1285 \times (\#Tyr) + 125 \times (\#S\text{-}S) \quad (5)$$

(2) A solution of approximately 6.8 M GdmCl, 10 mM sodium phosphate pH 7.2 (or PBS pH 7.2) was prepared. The concentration of the GdmCl solution was determined from refractive index measurements using the following equation [12,14,15],

$$C = 57.147 \times (\Delta N) + 38.68 \times (\Delta N)^2 - 91.60 \times (\Delta N)^3 \quad (6)$$

Here, C is the molar concentration and ΔN is the difference between the refractive index of the denaturant solution and the buffer solution at the sodium D line.

(3) mAb-1 to mAb-8 were buffer exchanged into 10 mM sodium phosphate pH 7.2 and mAb-9 to mAb-13 were buffer exchanged into PBS pH 7.2 using a PD-10 desalting column.

(4) An appropriate volume of buffer-exchanged stock protein was taken into the buffer in which the extinction coefficient was to be determined so that the absorbance of the protein solution became close to 1 at 280 nm. The absorbance spectrum of the sample was recorded in the wavelength range of 250–320 nm.

(5) The same dilution of the buffer-exchanged stock protein was prepared into GdmCl solution so that the final concentration of GdmCl was 6M. The sample was then stored at room temperature for 6 h, followed by which the absorbance spectrum was recorded in the wavelength range of 250–320 nm.

(6) The extinction coefficient of a mAb at 280 nm in 6M GdmCl was calculated using Eq. (5) as mentioned in step #1. The measured absorbance at 280 nm of the unfolded protein sample (step #5) was corrected for light scattering contribution using Eq. (4). The protein concentration was then calculated using the