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Single-Molecule Fluorescence Studies of Glycosylated and Aglycosylated Antibodies

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ABSTRACT Antibodies are Y-shaped, flexible proteins whose structures can be studied using Förster Resonance Energy Transfer (FRET) at the single-molecule level. Dye molecules must be attached to these proteins so as to carry out FRET studies of antibodies. In order to label the binding sites of an antibody, dye molecules were attached to a small molecule, or hapten, which the antibody binds to. Evidence for this binding was provided by ultraviolet-visible (UV-Vis) spectroscopy. To label the stem region of a humanized immunoglobulin G (IgG) antibody, the DNA for this antibody was mutated to introduce a cysteine residue to which dyes can be attached. In this research, the DNA was sequenced and checked to provide the desired sequence for protein production.

INTRODUCTION

Immunoglobulin G (IgG) antibodies have structural conformations that can be studied using Förster Resonance Energy Transfer (FRET) at the single-molecule level. An antibody is a very flexible protein composed of four chains (two heavy and two light) and has a Y-shaped structure as shown in Figure 1. The lower portion contains a constant region (Fc fragment) that is composed of similar amino acids found in all antibody molecules whereas the upper portion is the "variable region" (two Fab fragments) with varying amino acids that results in antibodies that specifically bind different antigen molecules. The stem region contains carbohydrates, or sugar molecules, that provide shape to the Fc region. Previous studies have shown that if the sugars are removed, the antibody is unable to elicit an immune response[2]. The purpose of this research is to examine the distribution of antibody structures with and without the sugars present in order to study whether different conformations are present, what happens to conformations once the carbohydrates are removed, and if there is a preferred conformation. The IgG antibody used in this research is the 39C2 catalytic aldolase antibody.

FRET was the chosen technique to determine the distances between areas of interest such as between the two portions of the Fc region near the carbohydrate groups on the antibody and between the antigen-binding sites. FRET requires the use of two dye molecules, a donor and an acceptor. These dye molecules can covalently bond to macromolecules and are used for fluorescence spectroscopy, as they absorb and fluoresce visible light. Once the donor molecule is excited, it can transfer energy to the acceptor molecule which proceeds to fluoresce. The closer the acceptor is to the donor, the more likely it is that energy transfer will take place. Therefore, the relative amounts of donor and acceptor fluorescence can be used to calculate the distance between the donor and acceptor molecules, resulting

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in the ability to determine structural information about the protein[2] (Figure 2). Single molecule FRET can be used to obtain a histogram of the distances between the donor and acceptor sites on the antibody[3,4]. Single molecule spectroscopy was the method chosen as it examines one molecule at a time rather than multiple molecules at once.

In order to study the distance between the antigen-binding sites of an antibody, dye molecules were attached to a hapten, or a small molecule that specifically adheres to the binding site of an antibody. This dye-hapten conjugate was then reacted with the antibody to observe the degree of binding. FRET experiments were performed on this system, but no significant energy transfer was observed. So as to attach dye molecules to the Fc region, manipulation of the DNA of a humanized (part mouse, part human) IgG antibody was necessary. The DNA sequences for the light and heavy chains of the humanized IgG antibody were checked for unwanted mutations. A satisfactory sequence has been obtained and submitted to a company for the production of the antibody. The labeling of the antigen binding sites and the examination of the humanized IgG DNA sequence are both described below.

METHODS

PROTEIN SEQUENCE ANALYSIS

From previous research, the Stratagene QuikChange Lightning Site Directed Mutagenesis Kit was used to introduce a cysteine point mutation into the CH2 region of the Fc region. The cysteine point mutation allows for dye attachment, crucial for FRET spectroscopy analysis. The sequences were checked using the CLC Sequence Viewer (Figure 3). Literature references for the plasmids were provided so as to determine the desired amino acid sequences for the light and heavy chains of the IgG antibody by comparison[5-7].

DYE ATTACHMENT

The carboxylic acid form of the hapten was previously reacted with reagents to generate the succinimidyl ester form of the hapten. The product was reacted with the donor and acceptor dye molecules (AlexaFluor® 568 and Cy 5.5®). Thin layer chromatography (TLC) was used to establish a desired solvent system, found to be a 60:40 mixture of methanol and ethyl acetate. A ThermoScientific NanoDrop 1000 UV-Visible Spectrometer was used to study the concentration of the samples and to determine the percent of antibody binding sites labeled with the dye-hapten. The UV-Visible Spectrometer displayed a peak at 318 nm which indicates an enaminone, the product of the hapten binding to the antibody, is present.

RESULTS

The protein sequences were analyzed and the desired DNA and amino acid sequences for the light (plasmid 5.3) and heavy (plasmid 6.4) chains of the humanized IgG antibody were obtained and sent to Creative BioLabs for protein production. There were no unwanted mutations in the light chain. However, the heavy chain is the area in which the DNA mutation was executed and the new sequence includes the cysteine point mutation into the CH2 region of the Fc region. Figure 4 presents the desired sequence for the heavy chain. There were sequence errors found in the variable heavy chain that will be fixed by Creative BioLabs.

Once the protein sequences were analyzed, UV-Vis spectroscopy was used to determine if there is evidence for dye labeling. It was found that approximately 85% of the binding sites of the antibody were labeled with a dye molecule. As evidenced by Figure 5, there is a substantial peak around 568 nm signifying the AlexaFluor® 568 donor dye fluorophore and a peak at about 680nm, characteristic of the Cy 5.5® acceptor dye fluorophore. There is also an evident peak at 318nm, denoting the enaminone formed which is the product of the hapten binding to the antibody. FRET experiments were performed on this sample; however, substantial energy transfer was not observed and further experiments are necessary.

CONCLUSION

The results demonstrate it was possible to optimize dye attachment to the 38C2 antibody and UV–Visible spectroscopy provides evidence that the antibody was labeled with the AlexaFluor® 568 donor dye fluorophore and the Cy 5.5® acceptor dye fluorophore. Furthermore, the light and heavy DNA sequences were checked for any unwanted mutations and the desired sequences were found and sent to create the protein sequence. Based on this and previous research, it will be possible to label the purified protein with the dye molecules and to perform single-molecule FRET spectroscopy. FRET will examine any possible antibody conformations.

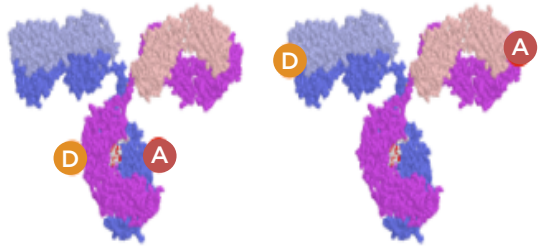

FIGURE 2

Illustrations of FRET, in which a donor molecule (D) undergoes distance-dependent energy transfer to an acceptor molecule (A), causing A to fluoresce. The amount of fluorescence observed from D and A can be used to determine the distance between the two molecules. The antibodies shown are from the protein data bank, Harris, L. J.; Larson, S. B.; Hasel, K. W.; McPherson, A. *Biochemistry*, 1997, 36, 1581.

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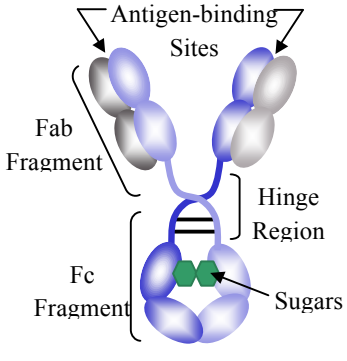

FIGURE 1

The structure of an immunoglobulin G (IgG) molecule. The regions of the antibody that correspond to the Fab (upper, antigen-binding fragment), the Fc (lower, crystallizable, constant fragment), and the hinge region are also shown. The location of the sugars, which are bound to the Fc fragment, is indicated.

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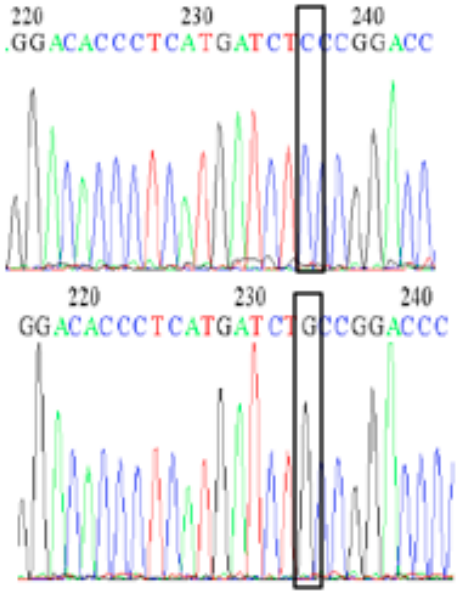

FIGURE 3

Example of a section of the protein sequence. Evidence of a cysteine point mutation in which cysteine was changed to guanine.

C_H2 sequence:

GCACCTGAACTCCTGGGGGGACCGTCA
GTCTTCCTCTTCCCCCCAAAACCCAAG
GACACCCTCATGATCTGCCGGACCCCT
GAGGTCACATGCGTGGTGGTGGACGTG
AGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTG
CATAATGCCAAGACAAAGCCGCGGGAG

FIGURE 4

The desired DNA sequence of 6.4 plasmid for the C_H2 region of the heavy chain which contains the cysteine point mutation.

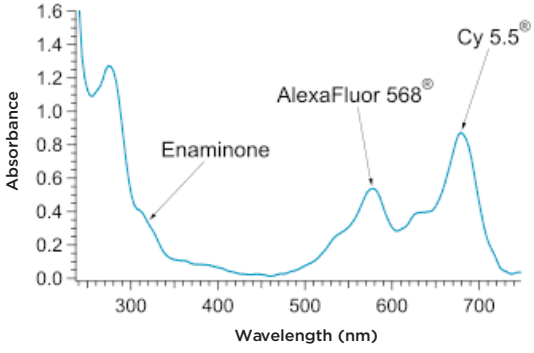

FIGURE 5

Illustration of the UV-Vis sprectroscopy of the dye-hapten to determine percent of binding sites occupied on the 38C2 antibody. Peaks for the enaminone, donor and acceptor dye fluorophoes are evident.

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