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Use of circular dichroism spectroscopy in determining the conformation of a monoclonal antibody prior to its incorporation in an immunoliposome

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Abstract

Attachment of antibodies to liposomes endows target specificity to liposomes for a certain cell or organ that express the targeted antigenic determinant. These so-called immunoliposomes hold high promise as targeted drug carriers. One approach of immunoliposome preparation involves conjugating antibodies to hydrophobic anchors (e.g. fatty acids or phospholipid molecules) for incorporation into the liposome membrane. Often, these conjugation reactions are harsh and may result in undesirable chemical and structural changes in the antibody molecule. This necessitates confirmation of the target specificity of the derivatized antibody prior to its incorporation into the liposome. Our approach to this problem is to utilize circular dichroism spectroscopy, which can detect subtle structural differences in proteins with high reproducibility and accuracy in relatively short period of time. In addition, circular dichroism is a non-destructive technique. In this study, we demonstrate the ability of circular dichroism to confirm the conformation of a model antibody, HYB-241, conjugated to N-glutarylphosphatidylethanolamine, prior to its mixing with dioleoylphosphatidylethanolamine/dioleoylphosphatidic acid to form a target-sensitive immunoliposome. © 1997 Elsevier Science B.V.

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1. Introduction

The use of liposomes as a carrier system for delivering drugs or macromolecules to cells has been extensively studied [1,2]. Among their many advantages, liposomes can be readily conjugated with antibodies directed against cell-specific antigens, comprising one strategy for site-specific drug delivery [3]. One approach for immunoliposome preparation involves chemically coupling the antibody molecule to hydrophobic anchors, e.g. fatty acids or phospholipids, followed by insertion into the liposomal membrane [3,4]. Unfortunately,

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these conjugation reactions can be quite harsh and may result in undesirable chemical and structural changes in the antibody molecule, even to the extent of rendering the antibody inactive. Therefore, it is important to confirm the structural integrity of the derivatized antibodies prior to their incorporation into liposomes.

One of the most sensitive methods for detecting changes in protein structure is circular dichroism (CD) spectroscopy. While CD spectroscopy does not provide site-specific information, it is a very powerful tool for assessing the secondary and tertiary structure of proteins and peptides [5,6]. Through deconvolution of the far-UV ($\lambda \sim 180–250$ nm) region of the CD spectrum, CD can provide semi-quantitative estimates of secondary structural composition of a protein. This is done by reconstructing the experimental spectrum in terms of basis spectra that represent the various types of secondary structures (e.g., alpha helix, beta sheet, beta turn, etc.) using a least squares fitting procedure. The relative contribution of each basis spectrum is proportional to the amount of the given secondary structure present in the overall structure. For example, if the basis spectrum for the alpha-helix contributes 40% to the best fit of the experimental spectrum, then 40% of the secondary structure of the protein is helical. The analyses are performed on spectra that have been normalized for differences in concentration, number of residues, and sample pathlength, leading to spectra reported in mean residue ellipiticity rather than the raw signal in millidegrees. The details of deconvolution of protein CD spectra have been previously described [7,8].

Meanwhile, the near-UV ($\lambda \sim 350–250$ nm) CD spectrum arises from signals from the side chains of aromatic amino acids (Phe, Tyr, and Trp) and disulfide bonds, allowing characterization of the tertiary structure or even aggregation. Since the near-UV CD active chromophores are usually distributed throughout the molecule, changes in their intensity, position, and sign indicate local structural rearrangement, that is, loss or alteration of tertiary structure. Similarly, if the near-UV CD spectrum exhibits changes as a function of peptide concentration, it can be surmised that adjacent peptide molecules are interacting and perturbing the CD spectrum of the monomeric species. This has been demonstrated for leuprolide, a luteinizing hormone releasing hormone analog [9].

Nevertheless, the greatest strength of CD is its ability to detect as little as a five to ten percent change in overall globular structure using a protein concentration as low as 1 mg ml^{-1}. In addition, CD is noninvasive and nondestructive. Furthermore, CD has the potential to monitor antibody secondary structure, both free in solution and after incorporation into the liposomes, provided the liposomes are relatively small ($<$ 50–100 nm) and light scattering is minimal [10].

Our goal in this study is to demonstrate the utility of CD spectroscopy to confirm the conformation and, consequently, the specificity of the derivatized antibody prior to its incorporation to form a target-sensitive immunoliposome (TSIL) [11–14]. The immunoliposome system used here is composed of a mixture of dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylethanolamine (DOPE), and an appropriate amount of antibody conjugated to a hydrophobic anchor molecule such as the fatty acid or lipid molecule [14]. The negatively charged lipid, DOPA, serves as a bilayer stabilizer for DOPE, which normally exists in hexagonal (H_{II}) nonbilayer configuration under physiological conditions [15]. The hydrophobic anchor-linked antibody is present on the liposomal membrane for the purpose of specific recognition of the target. In this design, target-specific binding of the immunoliposome will induce bilayer destabilization, resulting in a site-specific release of the liposomal contents [10]. Utilizing a CD-confirmed structurally intact hydrophobic anchor-linked anti-*p*-glycoprotein antibody, HYB-241, a calcein-loaded TSIL was prepared and evaluated. Release of calcein was found to be time-dependent and target-specific. These results demonstrate the ability of CD spectroscopy to confirm the structural integrity of a chemically-modified antibody.

2. Experimental

2.1. Materials

DOPE and DOPA were purchased from Avanti Polar Lipids (Alabaster, AL). HYB-241 (MW 150 kDa), a mouse antihuman *p*-glycoprotein monoclonal IgG_1 antibody, was kindly provided by Hybritech, Inc. (San Diego, CA). This particular antibody was previously described by Meyers et al. [16]. All other chemicals were from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

2.2. Cell culture

The multidrug-resistant (MDR) human breast adenocarcinoma cell line MCF 7, which heavily expresses MDR *p*-glycoprotein [17], was a gift from Dr Vasilis Vasiliou (School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO). MCF 7 cell line was maintained in minimum essential medium alpha medium (MEM α) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 / 95% air at 37°C.

2.3. Derivatization of antibody with *N*-glutarylphosphatidylethanolamine (NGPE)

HYB-241 monoclonal antibody or nonspecific mouse polyclonal IgG antibody (referred to as IgG hereafter) was conjugated to *N*-glutarylphosphatidylethanolamine (NGPE) using a previously described method [14] with slight modification. Briefly, 0.067 μmol of NGPE was dissolved in 1.0 ml of 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (5 mM MES, 0.15 M NaCl, pH 5.5) containing 0.1 M *N*-octyl-D-gluco-pyranoside. To this solution, 0.2 ml each of 0.25 M 1-ethyl-3(3″-(dimethyl-amino)propyl)carbodiimide and 0.1 M *N*-hydroxy-sulfosuccinimide dissolved in MES were added. The resulting mixture was incubated at room temperature for 10 min. The pH of the mixture was then adjusted to 7.5. To 1.4 ml of this mixture, 1.05 mg of HYB-241 monoclonal antibody or IgG antibody was then added (10:1 molar ratio between NGPE and HYB-241 monoclonal antibody) and the resulting mixture was incubated at 4°C for 12 h with frequent mixing. The resulting HYB-241-NGPE or IgG-NGPE conjugate was then dialyzed against three changes of 3 l of 0.01 M Hepes buffer (pH 7.8) containing 0.15 M NaCl (referred to as Hepes buffer hereafter) to remove *N*-octyl-D-gluco-pyranoside and other excess reagents. Typically, this procedure would result in more than 90% of the antibody to undergo conjugation reaction [18].

2.4. CD measurement and spectra analysis of HYB-241 and NGPE-conjugated HYB-241

Aviv Model 62 DS spectrometer (Lakewood, N.J.) was used to measure the CD spectra of HYB-241 or NGPE-conjugated HYB-241. Briefly, HYB-241 or NGPE-conjugated HYB-241 was added to 1 ml of Hepes buffer. The far-UV CD and near-UV CD spectra were recorded at 25°C using a 0.1-cm and a 1.0-cm pathlength quartz cell, respectively. Spectra were background corrected, smoothed, and converted to mean residue ellipticity.

2.5. Preparation of target-sensitive immunoliposome

Target-sensitive immunoliposomes containing HYB-241 monoclonal antibody or control immunoliposomes containing non-specific IgG antibody were prepared using a previously described procedure [14] with slight modification. Briefly, 8 μmol of DOPE and 2 μmol of DOPA were mixed and dried under a stream of N_2 and vacuum desiccated for no less than 2 h. The lipid mixture was then hydrated with 1 ml of Hepes buffer containing 525 μg NGPE-conjugated HYB-241 monoclonal antibody or 525 μg NGPE-conjugated non-specific IgG antibody and 50 mM calcein. The mixture was incubated at 4°C for 24 h with occasional mixing, followed by a brief period of sonification (5 min) in a bath sonicator. After an additional 8 h of incubation at 4°C, another sonification (5 min) was performed. Immunoliposomes were separated from unencapsulated calcein and unbound antibody by gel chromatography with a Bio-Gel A 15 M column

(BioRad, Hercules, CA). The peak immunoliposomes fractions, eluted with Hepes buffer, were then pooled and measured for their protein and lipid contents. Protein analysis was performed using BioRad microplate protein assay protocol. Lipid analysis was by the method of Barlett [19]. The final ratio of antibody/lipid in the pooled HYB-241 immunoliposomes and non-specific IgG immunoliposomes was approximately 1:4830 and 1:4000 (mol/mol), respectively. These ratios are comparable to previously reported values and provide a reasonable high level of fluorescence quenching (see below) [14].

2.6. Fluorescence quenching measurements

Calcein fluorescence of the liposomes was measured using a Shimadzu RF-1501 spectrofluoromter with $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm. Lipid concentration in the cuvette was approximately 0.5 μM in Hepes buffer containing 1 mM EDTA. Percent fluorescence quenching was calculated using the following formula:

$$\% \text{ quenching} = (1 - F_0/F_t) \times 100$$

where F_0 and F_t are the fluorescence of the liposome samples before and after the addition of 0.15% Triton X-100, respectively. The calcein fluorescence quenching was about 64.2 and 59.9% for HYB-241 immunoliposomes and non-specific IgG immunoliposomes, respectively.

These levels are comparable to previously reported values [14].

2.7. Target-induced lysis of immunoliposome

Confluent MCF 7 cells in 22.6-mm 12-well plates were used in these experiments and each experiment was performed in duplicate at room temperature. Prior to the experiments, the growth medium of MCF 7 cells was removed and the cells were washed twice with Hepes buffer. Then, 0.5 ml of Hepes buffer containing calcein-encapsulated target-specific and non-specific immunoliposomes (incubation buffer) was added to the targeted cells. After incubating the immunoliposomes with the cells for various time intervals, 0.02 ml of the incubation buffer was removed from each well and diluted for fluorescence measurement. For inhibition experiments, the inhibiting free antibodies were added 30 min earlier prior to the addition of the calcein-containing HYB-241 immunoliposomes. The total calcein fluorescence in the incubation buffer was measured after the addition of Triton X-100 to a final concentration of 0.15%. The percentage of calcein release was calculated using the following formula:

$$\% \textit{ release} = (F - F_0)/(F_t - F_0) \times 100$$

where F_o and F are the calcein fluorescence before and after incubation of the immunoliposomes with the targeted MCF 7 cells, respectively, and F_t is the total fluorescence after lysis of liposomes with 0.15% Triton X-100 [14].

3. Results and discussion

One approach to attaching a targeting antibody to the liposome involves chemically cross-linking the antibody to hydrophobic anchor (e.g. fatty acid or phospholipid molecule) for insertion into the liposome membrane [3,4]. Since the specificity of an antibody is a direct function of its overall globular structure (both at the secondary and tertiary structure levels), CD spectroscopy of the derivatized antibody, which yields secondary (far UV CD) and tertiary (near UV CD) structural information about a protein, [5,6] should be able to detect structural alterations.

Fig. 1 displays the far-UV ($\lambda \sim 180$–250 nm) CD spectra of HYB-241 before and after derivatization with NGPE. Besides the weak features near 230 nm, which arise from the L_a transition of tryptophan and tyrosine side chains, the exhibition of an intense negative band near 215 nm in the far-UV CD spectra of both the non-derivatized and derivatized antibodies is characteristics of other antibodies. This negative band indicates a high beta sheet content [6] ($\sim 50\%$). In addition, deconvolution of the spectra also suggests that a relatively small amount ($\sim 10\%$) of random structure is present in both antibodies. The similarities in secondary structure characteristics between the non-derivatized and derivatized antibodies suggest

that no major changes in the secondary structure occur upon derivatization. Quantitative analysis of the spectra, using SELCON [20], further supports our claim that the secondary structure composition of the derivatized antibody is indeed unchanged after chemical modification.

In order to determine if the tertiary structure of the antibody was altered by the modification procedure, the near-UV ($\lambda \sim 350$-250 nm) CD was measured. In this region, CD signals arise from aromatic side chains and disulfides, each reporting about the local environment around the chromophore. As indicated in Fig. 2, chemical modification was not found to perturb the near-UV CD spectrum significantly. The intensity is nearly unchanged and all of the vibronic fine structure is retained. The weak shoulder near 291 nm and the more intense bands at 284 and 278 nm indicate that the near-UV CD is dominated by tyrosine contributions, with little intensity due to tryptophan. Also, there is little intensity beyond 295 nm, suggesting disulfides make only a modest contribution to the spectrum. The overall intensity of ~ -80 deg cm^2 dmol^{-1} is typical of intact antibodies [21,22]. Observation of well defined vibronic fine structure means that certain tyrosines exist in relatively rigid conformations within the protein, indicating a stable folded

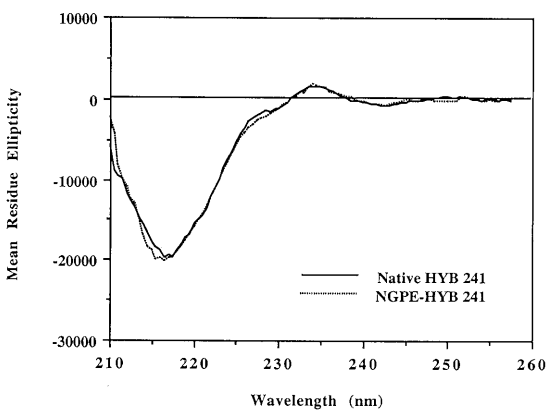

Fig. 1. Far-UV CD spectra of HYB-241 before (——) and after (· · ·) modification with NGPE: The protein concentration was 0.74 mg ml^{-1} for non-derivatized HYB-241 and 0.66 mg ml^{-1} for derivatized HYB-241. Sample temperature was 25°C and the pathlength was 0.1 cm.

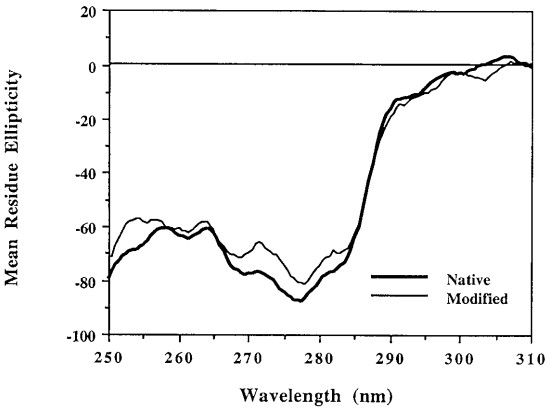

Fig. 2. Near-UV CD spectra of HYB-241 before (——) and after (——) modification with NGPE: The protein concentration was 0.415 mg ml^{-1} for non-derivatized HYB-241 and 0.193 mg ml^{-1} for derivatized HYB-241. Sample temperature was 25°C and the pathlength was 1.0 cm.

structure even after chemical modification. Taken together, both the far-UV and near-UV CD data suggest that the antibody structure is intact after attachment of the lipid anchor. Therefore, specificity for the intended target is anticipated.

While it is possible that the binding site of the antibody could be altered without affecting the overall fold of the protein, it is expected that retention of the native-like structure, as revealed by CD spectroscopy, should correlate with a high level of retained activity. In the case of a TSIL, this refers to the ability of the derivatized antibody to bind to its targeted antigen and trigger the immunoliposomes to undergo target-induced release of its encapsulated contents. In order to determine if the derivatized antibody causes target-induced destabilization of the TSIL, the target sensitivity of calcein-loaded TSILs prepared with NGPE-conjugated HYB-241 antibody was examined.

As indicated in Fig. 3, addition of calcein-loaded HYB-241 TSIL to MCF 7 cells results in a release of calcein from the liposomes. This release is time-dependent and reached a level of 40% in about 60 minutes. It seems the target-specific antibody is required for target-induced destabilization of the immunoliposomes. This assertion is supported by two lines of evidence. Firstly, addition

of calcein-loaded immunoliposome prepared with NGPE-conjugated nonspecific IgG antibody to MCF 7 cells did not result in any time-dependent release of calcein (Fig. 3). Since some cells are known to induce liposomal leakage by a non-specific mechanism [23], this result also eliminates any of such possibility that the release seen in Fig. 3 is due to cell-induced liposomal leakage. If release of calcein from HYB-241 TSIL is due to a non-specific event, one would expect similar levels of calcein release from immunoliposomes prepared with non-specific IgG antibodies. The other proof of target-induced release can be seen from competition studies. If the release of calcein from HYB-241 TSIL is the result of target-induced destabilization of the immunoliposomes, the release should be inhibited by free HYB-241. Preincubation of the MCF 7 cells with a 55-fold excess of free HYB-241 monoclonal antibodies results in significant reduction of calcein release (Fig. 4). On the other hand, preincubating the cells with non-specific IgG antibodies did not result in any inhibition of calcein release from HYB-241 TSIL.

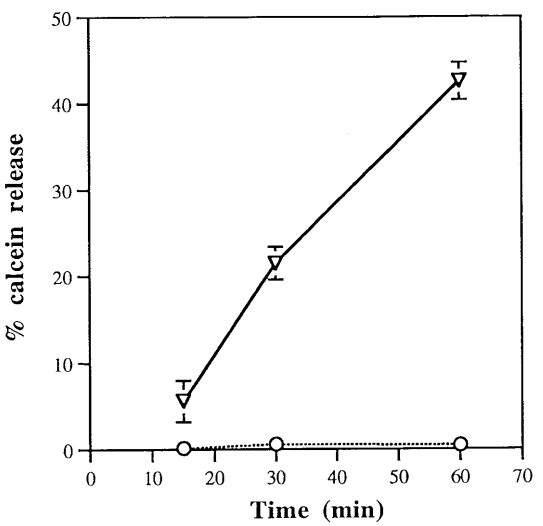

Fig. 3. Effect of specificity of NGPE-conjugated antibody on the target-induced lysis of immunoliposomes: 300 μM calcein-loaded HYB-241 immunoliposomes (▽) or calcein-loaded non-specific IgG immunoliposomes (○) were incubated at room temperature with MCF 7 cells. At indicated time interval, the percent release of calcein was determined. Each point represents the mean ± S.D. for two separate experiments.

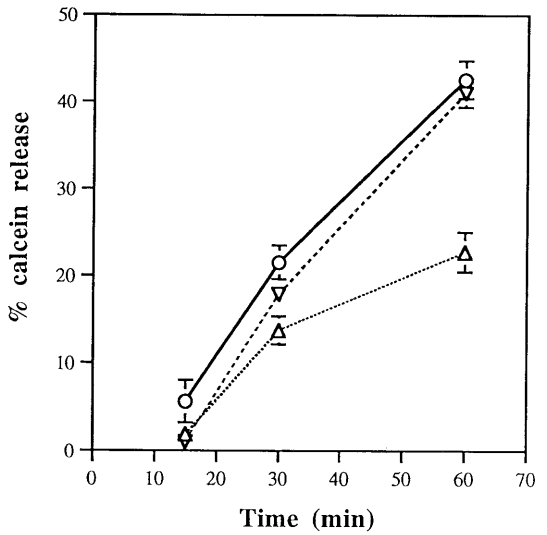

Fig. 4. Effect of free antibody on the target-induced lysis of immunoliposomes: MCF 7 cells were either preincubated with (HYB-241 Δ; non-specific IgG ▽) or without free antibody (○) for 30 min prior to addition of 300 mM calcein-loaded HYB-241 immunoliposomes. At indicated time interval, the percent release of calcein was determined. Each point represents the mean ± S.D. for two separate experiments.

Thus, only direct competition for the surface antigen results in diminished calcein release. These data indicate that HYB-241 was chemically modified without loss of potency or specificity, and the antibody is active after TSIL formation as well.

4. Conclusions

Our results demonstrate that the conditions used in the present experiment to conjugate monoclonal antibody to its hydrophobic anchor molecule does not induce major structural changes to the antibody as measured by far- and near-UV CD spectroscopy. Furthermore, it is shown that retention of native-like structure of the derivatized antibody appears to correlate with its biochemical activity. However, it is important to note that retention of native-like structure may not always mean that the binding site of a chemically modified protein is still intact. In case when unequivocal information on the binding site is

needed, it is important to use more sensitive techniques such as enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA). Nevertheless, the findings reported in this paper thus suggest the potential of utilizing CD spectroscopy to provide rapid and reliable information on the secondary and tertiary structure of chemically modified proteins. This can be extremely useful during the optimization of the chemical modification procedures when the goal is not to alter the structure of the protein significantly and to preserve the overall binding capacity of the modified proteins.

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