Direct Biophysical Characterization of Human Apolipoprotein A-1 in ISCOMs

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Abstract □ Human apolipoprotein A-1 was formulated in "Immune Stimulating Complexes" (ISCOMs). The structure of the protein in ISCOMs was examined directly using several biophysical techniques including Fourier transform infrared (FTIR) spectroscopy, near UV circular dichroism (CD), and fluorescence spectroscopy. Amide I FTIR data indicate that human apolipoprotein A-1 displays a slightly increased α-helical content after its incorporation into ISCOMs. Near UV CD and tryptophan fluorescence data suggest that association with ISCOMs results in the tryptophan residues of the protein experiencing a relatively hydrophobic environment, motional restriction, and local electrostatic interactions. These observations are consistent with an increased order in the protein structure upon incorporation in ISCOMs. In addition, biomolecular interaction analysis (BIA), based on surface plasmon resonance (SPR) measurements, suggests that the binding affinity of human apolipoprotein A-1 to a monoclonal anti-human apolipoprotein A-1 antibody is moderately decreased (by 20%) after its incorporation into ISCOMs. This study demonstrates that these biophysical techniques can be used to noninvasively monitor integrity of or changes in secondary and tertiary structure of proteins within the ISCOM particles without the need for protein extraction.

Introduction

Monitoring the structure of a protein following its formulation is crucial to the development of a successful formulation. Unfolding or changes in protein structure during formulation may result in loss of the desired biological efficacy. Although a large number of protein and peptide drugs have been formulated in delivery systems, monitoring structural changes after incorporation in these systems remains a challenging task. Most existing techniques require extraction of protein from the delivery systems with an organic solvent or a strong detergent.[1] These harsh extraction methods can be damaging to the secondary and tertiary structures of a protein and, therefore, prevent meaningful examination of any structural perturbations that may have occurred during formulation.[1]

In this study, several noninvasive spectroscopic techniques, including Fourier transform infrared (FTIR), near UV circular dichroism (CD), and fluorescence, were employed to directly examine the structure of a formulated protein. FTIR spectroscopy is a powerful tool for studying protein secondary structure, and it can be applied to samples in different physical states.[2] This technique has recently been applied to directly examine the integrity of recombinant human growth hormone[1,4] (rhGh), bovine serum albumin[3] (BSA), and chicken egg-white lysozyme[3] in poly(lactic-*co*-glycolic acid) (PLGA) microspheres, and in PLGA films.[4] Near UV CD spectroscopy is widely used for monitoring changes in the environments of aromatic amino acids that may result from changes in the protein's tertiary structure.[5] Changes in tryptophan fluorescence spectra usually reflect changes in the local environment of tryptophan residues, which may result either from a global change or a subtle local perturbation in the tertiary structure of a protein.[6] All of these spectroscopic techniques provide direct structural information using intrinsic chromophores or fluorophores of a protein, provided that any background interference from formulation excipients or delivery vehicles is minimal or can be corrected for.

Biomolecular interaction analysis (BIA) is a functional assay that monitors biological interactions at the molecular level. It uses an optical phenomenon called surface plasmon resonance (SPR) and a continuous flow system to monitor biomolecular interactions in real time.[7] Typically, one biomolecule is immobilized on the sensor surface, while a solution containing other biomolecules flows continuously over the sensor surface. As molecules from the solution bind to the immobilized biomolecule, a signal is registered in the form of a sensorgram which is based on the change of refractive index that results from this binding.[7] Real time monitoring of intermolecular interactions in the form of sensorgrams provides kinetic information such as association rate constants and dissociation rate constants as well as equilibrium binding constants.[7]

Human apolipoprotein A-1 was used in this study as a model protein and was incorporated into immune stimulating complexes (ISCOMs), a nanoparticulate delivery system made of a phospholipid, cholesterol, and Quil A.[8] Human apolipoprotein A-1 protein is the major protein component of high-density lipoprotein (HDL) particles. It has a molecular weight of 28 kDa, and its known protein sequence is composed of 243 amino acid residues including four tryptophan residues.[9] In the solution form, it is loosely folded with a high degree of exposure of its nonpolar groups to solvent.[10] Upon interaction with lipids, it undergoes subtle rearrangements in its tertiary structure to accommodate the more nonpolar environment of lipids.[10]

In the study reported here, FTIR, near UV CD and fluorescence spectra of human apolipoprotein A-1 in solution and in ISCOMs were compared. Binding of human apolipoprotein A-1 in ISCOMs to a specific monoclonal anti-human apolipoprotein A-1 antibody was studied using BIAcore (Biacore, Inc.) and compared to binding of the free protein in solution to the same antibody. The data indicate that human apolipoprotein A-1 undergoes subtle changes in secondary and tertiary structures when incorporated into ISCOMs. Furthermore, incorporation in ISCOMs also resulted in a modest alteration in its binding affinity for the anti-human apolipoprotein A-1 antibody. These results are consistent with the reported ability of human apolipoprotein A-1 to change its structure to accommodate lipophilic environments.[10] Our findings suggest that these

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biophysical techniques can indeed be used to monitor protein structure in ISCOMs without the need for protein extraction.

Experimental Methods

ISCOMs Formation—ISCOMs were formed according to previously published method[11] with slight modifications. Briefly, 10 mg of L-α-phosphatidylcholine dipalmitoyl (Sigma) and 10 mg of cholesterol (Sigma) were dissolved in 1 mL of 20% decanoyl-*N*-methylglucamide (Mega-10, Sigma) in phosphate-buffered saline (PBS, pH 7.4). A 100 μL amount of the lipid mixture was mixed with 200 μL of 20 mg/mL Quil A (Superfos Biosector) and 600 μL of 2 mg/mL human apolipoprotein A-1 in PBS (Calbiochem). When preparing protein-free ISCOMs, PBS alone was used. The final volume was made up to 1 mL with PBS. The mixture was dialyzed against PBS over 48 h to remove the detergent. ISCOMs were formed upon detergent removal.

Sucrose Gradient Ultracentrifugation—Human apolipoprotein A-1 incorporated into ISCOMs was separated from unincorporated protein by ultracentrifugation at 200 000*g* for 18 h at 10 °C through a 10−50% discontinuous sucrose gradient. The gradient was then fractionated into volumes of 1 mL. The fractions were tested for the presence of protein with a colorimetric Lowry assay kit from BioRad, and for the presence of cholesterol with a colorimetric total cholesterol assay kit obtained from Sigma. Fractions containing both protein as well as cholesterol were pooled and washed with PBS to remove sucrose. Final protein concentration in each sample was determined by SDS−PAGE densitometry.

Dynamic Light Scattering—Hydrodynamic size distribution of the ISCOMs was determined using a dynamic light scattering (DLS) apparatus (Model 4700, Malvern Instrument) using the 488 nm line of a 5 W argon ion laser and a detection angle of 90°. The autocorrelation functions obtained from scattering measurements were fitted using the nonnegative least square (NNLS) algorithm to yield the size distribution of the ISCOMs.[12]

Transmission Electron Microscopy—Transmission electron microscopy (TEM) was used to examine the morphology of ISCOMs. Samples were stained with 2% uranyl acetate and examined under a JEOL JEM 1010 microscope.

Fourier Transform Infrared (FTIR) Spectroscopy—FTIR spectra were recorded at 25 °C with a resolution of 4 cm^{-1} on a BOMEM MB-104 FTIR spectrometer (BOMEM, Inc.), continuously purged with nitrogen and equipped with a DTGS detector. For each spectrum, 400 scans were accumulated in the single beam mode. Samples were placed in a sample cell with two CaF_2 windows separated by a 6 μm spacer. Human apolipoprotein A-1 and its ISCOM formulation were concentrated to 5 mg/mL using a 10 000 MWCO ultrafiltration device (Millipore). Proper blank spectra (PBS buffer for protein in solution and protein-free ISCOMs for protein in ISCOMs) and water vapor components were subtracted from the protein spectra using the BOMEM-PROTA (BOMEM, Inc.) software. Second derivatives of the spectra were calculated for peak identification. Secondary structure of the protein was analyzed using factor analysis[13] with the BOMEM-PROTA database[14] (BOMEM, Inc.).

Near UV Circular Dichroism (CD)—Near UV CD (in the wavelength range of 250−350 nm) spectra were obtained using a Jasco J-715 spectropolarimeter. Samples at a protein concentration of 0.3 mg/mL were placed in a rectangular quartz cell of 0.1 or 1 cm path length. The cell holder was maintained at a temperature of 25 °C. A bandwidth of 2 nm and a scan speed of 20 nm/min were used for each spectrum. For each sample, four spectral scans were averaged. Proper background spectra (PBS or protein-free ISCOMs) were recorded and subtracted to obtain background-corrected protein spectra.

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded using a Perkin-Elmer LS50B fluorometer. Samples were excited at 295 nm, and tryptophan fluorescence was monitored between 305 and 400 nm using excitation and emission slits of 5 nm. Blank spectra (PBS or protein-free ISCOMs) were subtracted to obtain background-corrected tryptophan fluorescence spectra. Spectra were then corrected for wavelength-dependent monochromator and photomultiplier tube responses.

Biomolecular Interaction Analysis (BIA)—Binding interactions between human apolipoprotein A-1 (in solution or in ISCOMs) and a specific antibody were determined using a BIAcore 3000 biosensor system (Biacore, Inc.). Rabbit anti-mouse antibody (Biacore, Inc.) was immobilized on a research grade CM5 sensor chip in 10 mM sodium acetate, pH 4.5, using the amine-coupling kit (Biacore, Inc.). Mouse monoclonal anti-human apolipoprotein A-1 antibody (Calbiochem) was captured at a concentration of 10 μg/mL. Human apolipoprotein A-1 in solution or in ISCOMs was diluted in HEPES buffer for kinetic binding study. The samples were allowed to flow over the captured antibody for binding. Dissociation of the protein from the antibody was achieved by continuously flowing HEPES buffer over the sensor chip. All measurements were carried out at 25 °C using a flow rate of 20 μL/min. The sensor chip surfaces were regenerated with 20 μL of 10 mM glycine/HCl, pH 2, after each measurement.

The resulting sensorgram data were fitted with the 1:1 Langmuir binding model[7,15] using the BIAevaluation 3.0 software (Biacore, Inc.), yielding values for association and dissociation rate constants (k_a and k_d, respectively). Binding affinities of the antibody for human apolipoprotein A-1 in solution and in ISCOMs were expressed as dissociation constants K_D, where $K_D = k_d/k_a$.

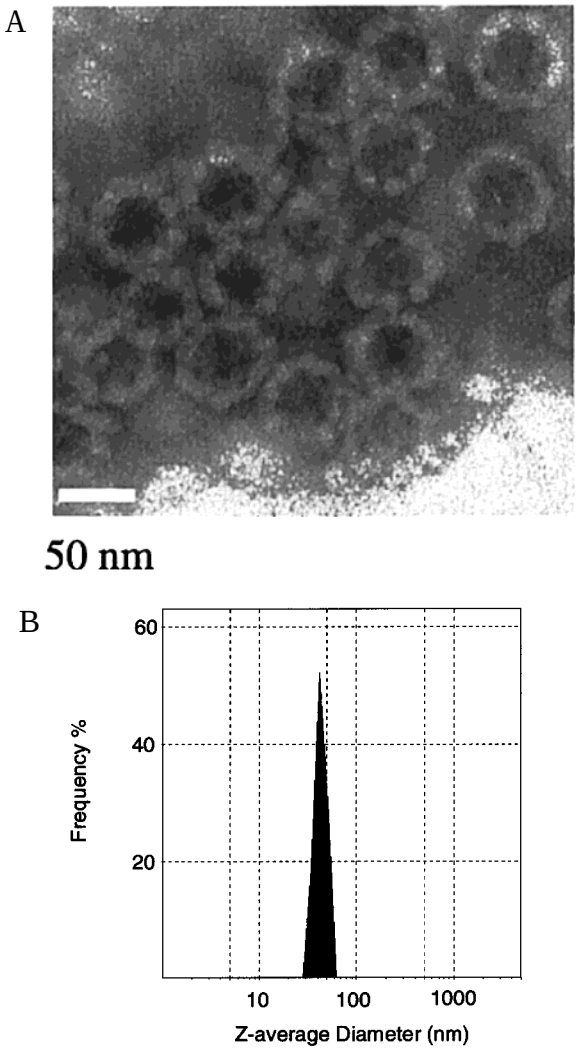

Figure 1—(A) Negatively stained transmission electron micrograph of apolipoprotein A-1 ISCOMs. (B) Size distribution of human apolipoprotein A-1 ISCOMs as measured by dynamic light scattering (DLS).

Results and Discussion

ISCOMs Characterization—*A. Size*—TEM indicated that human apolipoprotein A-1 ISCOMs had the characteristic cagelike appearance[8] (Figure 1A). DLS measurement showed that the ISCOMs had a *z*-average hydrodynamic diameter of 50 nm with a narrow unimodal size

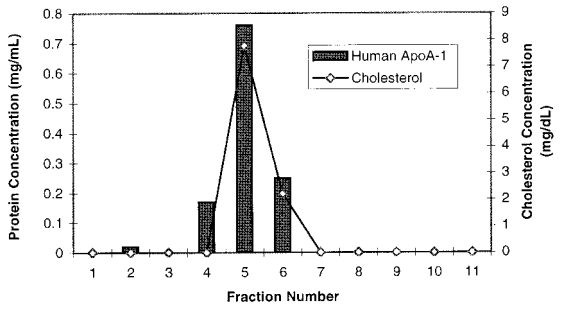

Figure 2—Sucrose gradient centrifugation of human apolipoprotein A-1 in ISCOMs. The gradient was fractionated into 1 mL fractions and each fraction was analyzed for the presence of cholesterol and protein.

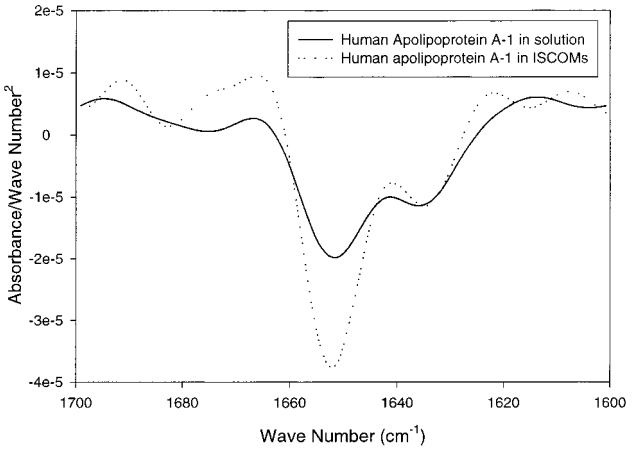

Figure 3—Amide I region of second derivative FTIR spectra of human apolipoprotein A-1 in solution and in ISCOMs. Proper blank spectra have been subtracted as described in the text.

distributions (polydispersity index = 0.08) (Figure 1B). This particle size is consistent with the size estimated from the TEM image shown in Figure 1A.

B. Protein Incorporation—The percentage of human apolipoprotein A-1 incorporated into the ISCOMs was determined using sucrose gradient ultracentrifugation. The ISCOMs showed up as a narrow band on the gradient after ultracentrifugation. Fractionation of the gradient indicated that close to 90% of the protein was associated with cholesterol after ultracentrifugation. Since cholesterol is an essential component of ISCOMs, the data suggested that approximately 90% of the total protein added was incorporated into the ISCOMs (Figure 2).

Direct Estimation of Protein Secondary Structure in ISCOMs by FTIR Spectroscopy—Protein-free ISCOMs gave minimal spectral interference in the region of interest, i.e., the amide I region (1600−1700 cm^{-1}). Figure 3 shows the amide I region of the second derivative FTIR spectra of human apolipoprotein A-1 in solution and in ISCOMs. Human apolipoprotein A-1 is known to contain a high percentage of α-helices.[9,16] This was clearly reflected in the prominent absorption band observed at 1656 cm^{-1} for human apolipoprotein A-1 in solution (Figure 3). The presence of some β-sheets was also seen from the band at 1634 cm^{-1}. The assignments of these amide I IR bands to defined secondary structures are based on a previously published report.[17] For the protein formulated in ISCOMs, an increase in absorption intensity was observed for the 1656 cm^{-1} absorption band. This observation is consistent with previously published findings that human apolipoprotein A-1 displays increased amount of α-helices when it is in HDL particles and bound to lipids, as compared to being in a lipid-free solution environment.[9,16]

Table 1—Secondary Structure Components of Human Apolipoprotein A-1 in Solution or in ISCOMs. Results Are Presented as Mean ± sd of Three Independent FTIR Measurements

	helix (%)	sheet (%)	bend and turn (%)	coil (%)
apolipoprotein A-1 in solution	44 ± 5	16 ± 2	17 ± 2	23 ± 3
apolipoprotein A-1 in ISCOMs	56 ± 5	10 ± 2	15 ± 2	20 ± 2

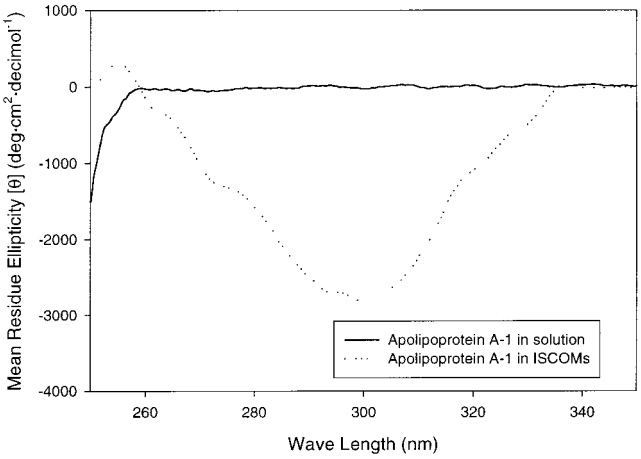

Figure 4—Near UV CD spectra of human apolipoprotein A-1 in solution and human apolipoprotein A-1 in ISCOMs. Proper blank spectra have been subtracted as described in the text.

Factor analysis was used to generate quantitative information on protein secondary structure from the IR absorption spectra.[13,14] This method predicts the secondary structure of an unknown protein using the (BOMEM-PROTA database of proteins with known crystal structures.[13,14] The results for human apolipoprotein A-1 in solution and in ISCOMs obtained from factor analysis are shown in Table 1. These values represent averages of results from three independently measured spectra. A modest increase in the amount of α-helices and a slight decrease in the amount of β-sheets were observed after apolipoprotein A-1 was incorporated into ISCOMs. These estimates are in qualitative agreement with the second derivative spectra in Figure 3.

It should be noted that far UV CD spectroscopy could not be used reliably to estimate secondary structure content of the protein in ISCOMs due to large interferences from the lipids and Quil A in ISCOMs.

Near UV Circular Dichroism—Near UV CD spectra for human apolipoprotein A-1 in solution and human apolipoprotein A-1 in ISCOMs are shown in Figure 4. When free in solution, human apolipoprotein A-1 did not show any near UV CD signal at the concentration of 0.3 mg/mL used, at either 0.1 or 1 cm optical path length, suggesting motional freedom of its aromatic side-chains. When incorporated into ISCOMs, human apolipoprotein A-1 displayed a broad CD signal with the spectral maximum at 300 nm and a shoulder at 295 nm. This indicates that the tryptophan residues experience motional restriction when the protein is in ISCOMs. It should be noted that apolipoprotein A-1 contains four tryptophan residues, and the individual contribution of each to the near UV CD or fluorescence spectrum (below) may or may not be equal. These data, therefore, reflect the environment of one or more, but not necessarily of all, of the tryptophans in the protein.

Fluorescence Spectroscopy—Figure 5 shows the tryptophan fluorescence spectra for human apolipoprotein A-1 in solution and in ISCOMs. The observed fluorescence maximum at 345 nm suggests that, on the average,

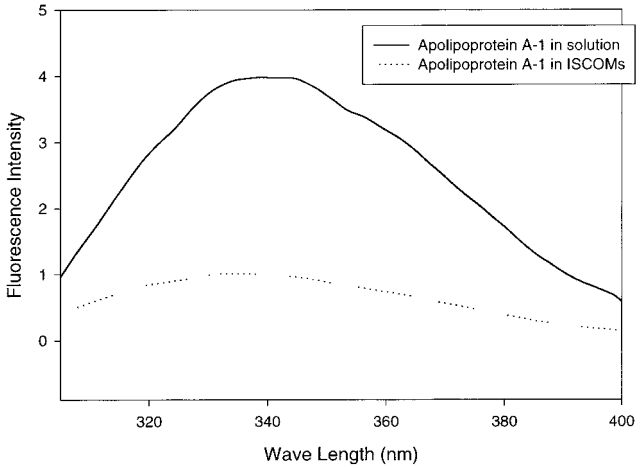

Figure 5—Fluorescence spectra of human apolipoprotein A-1 in solution and human apolipoprotein A-1 in ISCOMs. Proper blank spectra have been subtracted as described in the text.

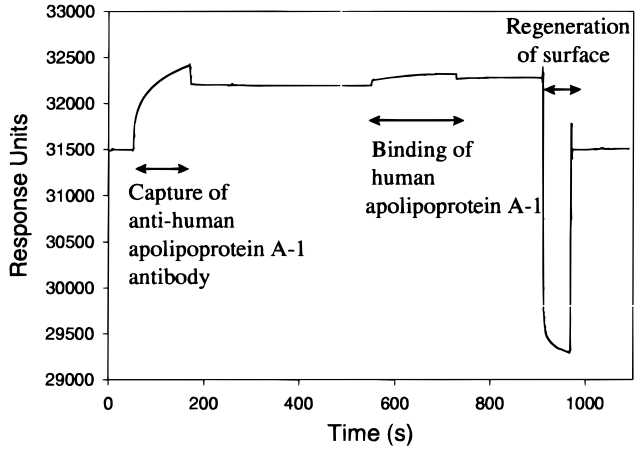

Figure 6—Sensorgram illustrating capture of a monoclonal anti-human apolipoprotein A-1 antibody, binding of human apolipoprotein A-1, and surface regeneration steps in kinetic analysis.

tryptophan residues in the free protein are partially exposed to water. This is consistent with previously published data suggesting that, in solution, human apolipoprotein A-1 has a high degree of exposed nonpolar groups.[10] After incorporation into ISCOMs, a blue-shift of the fluorescence maximum to 335 nm was seen, suggesting that ISCOMs provide a more hydrophobic environment for the tryptophan residues, possibly via interaction with the phospholipid or cholesterol in the ISCOMs. The quenching of fluorescence observed upon incorporation of the protein in ISCOMs may be a result of electrostatic interactions with the lipids or Quil A, which are in close proximity to the contributing tryptophan(s). Alternatively, the protein in ISCOMs may experience sterically allowed neighboring group interactions.

Biomolecular Interaction Analysis (BIA)—The sensorgram in Figure 6 illustrates the BIA data collection process. A mouse monoclonal anti-human apolipoprotein A-1 antibody was immobilized onto the sensor chip surface through binding to the rabbit anti-mouse antibody covalently bound to the chip surface. Human apolipoprotein A-1 in solution or in ISCOMs was then flowed over the chip surface and allowed to bind to the anti-human apolipoprotein A-1 antibody. Finally, the chip surface was regenerated before the next cycle would begin.

Figure 7 shows the antibody binding regions of the sensorgrams for human apolipoprotein A-1 in solution

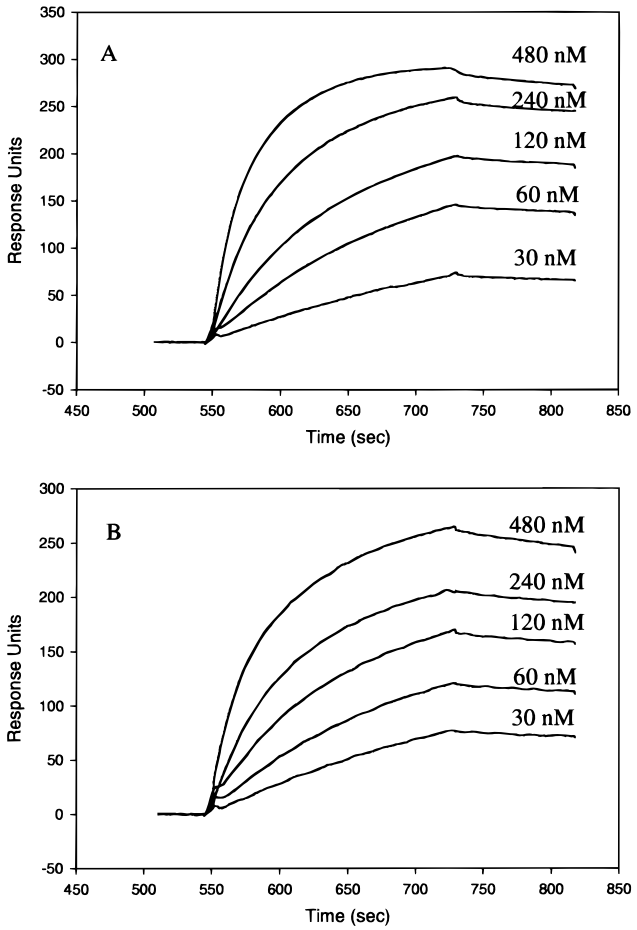

Figure 7—Effects of varying concentrations of human apolipoprotein A-1 on its binding kinetics to immobilized monoclonal antibody. (A) Human apolipoprotein A-1 in solution. (B) Human apolipoprotein A-1 in ISCOMs.

Table 2—Affinity and Rate Constants for Human Apolipoprotein A-1 in Solution and Human Apolipoprotein A-1 in ISCOMs. A Total of Three Measurements Were Made and the Results Are Presented as Mean ± sd

	$k_a \times 10^4$ ($M^{-1}s^{-1}$)	$k_d \times 10^{-4}$ (s^{-1})	K_D (nM)
human apolipoprotein A-1 in solution	11.5 ± 0.1	6.3 ± 0.1	5.7 ± 0.1
human apolipoprotein A-1 in ISCOMs	7.3 ± 0.1	4.8 ± 0.1	6.6 ± 0.1

(Figure 7A) and for human apolipoprotein A-1 in ISCOMs (Figure 7B). Five different protein concentrations were tested in each case. It can be seen that as the protein concentration was increased (either in the case of protein in solution or that of protein in ISCOMs), binding response increased correspondingly. When protein-free ISCOMs were tested, no response was seen (data not shown).

The sensorgram data in Figure 7 were fitted using the BIAevaluation 1:1 Langmuir binding model[7,15] to yield binding affinity and rate constants. A total of three evaluations were made for each sample, and the mean results are summarized in Table 2. After incorporation into ISCOMs, human apolipoprotein A-1 showed a 1.6-fold and a 1.3-fold decrease in k_a and k_d, respectively, for the monoclonal antibody. This translated to a 1.2-fold decrease in the binding affinity (or a 1.2-fold increase in K_D) for this interaction upon incorporation of the protein in ISCOMs. It is not known whether this slightly decreased antibody

affinity is the result of reduced accessibility of the antibody to ISCOM-associated protein compared to the free protein in solution.

Conclusion

FTIR spectroscopy suggests that human apolipoprotein A-1 experiences small changes in its secondary structure content upon incorporation in ISCOMs. Tryptophan fluorescence and near UV CD data indicate the protein's tryptophan residues, on the average, experience a more hydrophobic and motionally restrictive environment in ISCOMs compared to that in aqueous solution. BIA results suggest that the binding affinity of the protein to a monoclonal antibody is slightly reduced upon incorporation into ISCOMs. Results from these measurements suggest that the protein largely maintains its folded structure in ISCOMs, with its side chains engaged in local interactions with components of the ISCOM matrix. We conclude that a combination of biophysical techniques, such as those employed here, can provide complementary information on the secondary and tertiary structures of a protein formulated in ISCOMs. For other delivery systems, problems of interference will need to be carefully evaluated for each technique.

References and Notes

1. Yang, T.; Dong, A.; Meyer, J.; Johnson, O. L.; Cleland, J. L.; Carpenter, J. F. Use of infrared spectroscopy to assess secondary structure of human growth hormone within biodegradable microspheres. *J. Pharm. Sci.* **1999**, *88* (2), 161−165.
2. Middaugh, C. R.; Mach, H.; Ryan, J. A.; Sanyal, G.; Volkin, D. B. Infrared spectroscopy. *Methods Mol. Biol.* **1995**, *40*, 137−56.
3. Fu, K.; Griebenow, K.; Hsieh, L.; Klibanov, A. M.; Langer, R. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. *J. Controlled Release* **1999**, *58*, 357−366.
4. Carrasquillo, K. G.; Costantino, H. R.; Cordero, R. A.; Hsu, C. C.; Griebenow, K. On the structural preservation of recombinant human growth hormone in a dried film of a synthetic biodegradable polymer. *J. Pharm. Sci.* **1999**, *88* (2), 166−173.
5. Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.* **1974**, 113−175.
6. Cantor, C. R.; Schimmel, P. R. Biophysical chemistry. Part II. Techniques for the study of biological structure and function. W. H. Freeman and Company: San Francisco, CA, 1980.
7. *BIAtechnology handbook;* Biacore, Inc.: Piscataway, NJ.
8. Morein, B.; Sundquist, B.; Hölglund, S.; Dalsgaard, K.; Osterhaus, A. ISCOMs, a novel structure for antigen presentation of membrane proteins from enveloped viruses. *Nature* **1984**, *308*, 457−460.
9. Gursky, O.; Atkinson, D. Thermal unfolding of human high-density apolipoprotein A-1: Implications for a lipid-free molten globular state. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2991−2995.
10. Osborne, J.; Lee, N.; Powell, G. Solution properties of apolipoproteins. *Methods Enzymol.* **1986**, *128*, 375−387.
11. Kazanji, M.; Laurent, F.; Péry, P. Immune responses and protective effect in mice vaccined orally with surface sporozoite protein of *Eimeria falciformis* in ISCOMs. *Vaccine* **1994**, *12* (9), 798−804.
12. Koppel, D. E. Analysis of macromolecular polydispersity in intensity correlation spectroscopy: The method of cummulants. *J. Chem. Phys.* **1972**, *15*, 4814−4820.
13. Wi, S.; Pancoska, P.; Keiderling, T. A. Predictions of protein secondary structures using factor analysis on Fourier transform infrared spectra: effect of Fourier self-deconvolution of the amide I and amide II bands. *Biospectroscopy* **1998**, *4* (2), 93−106.
14. *Prota FTIR protein analyzer user's guide;* Bomem, Inc.: Quebec, Canada.
15. Hinshelwood, C. N. *The kinetics of chemical change;* Clarendon Press: Oxford, 1940.
16. Atkinson, D.; Small, D. M. Recombinant lipoproteins: Implications for structure and assembly of native lipoproteins. *Annu. Rev. Biophys. Biophys. Chem.* **1986**, *15*, 403−456.
17. Susi, H.; Byler, D. Resolution-enhanced Fourier transform infrared spectroscopy of enzymes. *Methods Enzymol.* **1986**, *130*, 290−311.

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