

# **Structure - function studies of human apolipoprotein A-V: a regulator of plasma lipid homeostasis\***

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**Keywords:** Apolipoprotein, antibody, circular dichroism, lipoprotein, triacylglycerol, cholesterol efflux

**Running title:** ApoA-V isolation and characterization

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\*This work was supported by grants from the American Heart Association (0255958Y) and NIH (HL64159 and HL 55493) and the Protein Engineering Network of Centers of Excellence.

**Abbreviations:**

apo = apolipoprotein

SDS = sodium dodecyl sulfate

PAGE = polyacrylamide gel electrophoresis

CD = circular dichroism

TG = triacylglycerol

HDL = high density lipoprotein

VLDL = very low density lipoprotein

TTBS = Tween - Tris buffered saline

DMPC = dimyristoylphosphatidylcholine

SDS = sodium dodecyl sulfate

PAGE = polyacrylamide gel electrophoresis

## Abstract

To investigate structure and function relations of a new member of the exchangeable apolipoprotein family that modulates plasma lipid levels, recombinant human apolipoprotein (apo) A-V was produced in *E. coli* and isolated by a combination of nickel chelation affinity chromatography and reversed-phase HPLC. Antibodies directed against apoA-V were generated and employed in immunoblotting experiments. Anti apoA-V IgG gave a strong response against recombinant apoA-V from *E. coli* and human apoA-V expressed in transgenic mice, but did not recognize human apoA-I or apoA-IV. In neutral pH buffers, at concentrations > 0.1 mg/ml, isolated lipid free apoA-V is poorly soluble. By contrast, apoA-V is soluble in 50 mM sodium citrate, pH 3.0. Far UV circular dichroism analysis and spectral deconvolution reveal that apoA-V possesses 32%  $\alpha$ -helix, 33%  $\beta$ -sheet, 16%  $\beta$ -turn and 18% random coil secondary structure conformers. Temperature-induced denaturation studies gave rise to a transition midpoint of 47.1 °C. Upon return to ambient temperature from 85 °C, apoA-V failed to recover all of the negative ellipticity present in unheated apoA-V. ApoA-V interacts with bilayer vesicles of dimyristoylphosphatidylcholine to form discoidal complexes with diameters in the range of 15 - 20 nm. However, apoA-V was a poor activator of lecithin:cholesterol acyltransferase where activity was  $8.5 \pm 1.8$  % that of apoA-I. Furthermore, apoA-V failed to support enhanced efflux of cholesterol from cAMP-treated J774 macrophages although low levels of efflux were obtained from unstimulated cells. Taken together, the results demonstrate recombinant apoA-V possesses unique structural and functional characteristics in keeping with its proposed role in modulation of plasma lipid levels.

The exchangeable apolipoproteins play a critical role in plasma lipoprotein metabolism. As many as 11 different exchangeable apolipoproteins have been identified and characterized from human plasma and these generally possess a common structural motif: the amphipathic  $\alpha$ -helix (1). Given their fundamental and essential role in plasma lipoprotein metabolism, it is generally recognized that aberrations in exchangeable apolipoprotein concentration or structure can lead to dyslipidemia and premature atherosclerosis. For example, using gene disruption techniques, it has been shown that a deficiency in apoE leads to severe hypercholesterolemia and premature atherosclerosis (2,3). On the other hand, apoA-I and apoA-IV transgenic mice were protected from diet-induced atherosclerosis (4,5).

Recently, a new member of the exchangeable apolipoprotein family was discovered. In a comparative genomics study designed to identify evolutionary conserved sequences with potential function, Pennacchio et al. (6) determined the sequence of 200 kbp of mouse DNA in a region orthologous to the human apolipoprotein gene cluster (apoA-I/C-III/A-IV) on human chromosome 11q23. By comparison of mouse and human sequence data, a region of inter-species sequence conservation was discovered approximately 30 kbp proximal to the apolipoprotein gene cluster. Further analysis revealed that this genomic interval contained a putative apolipoprotein-like gene (termed apoA-V). Further characterization of this region of the mouse sequence, as well as expressed sequence tag information, indicated the presence of four exons containing a 1, 107 bp open reading frame. The predicted 368 amino acid protein possessed sequence homology to known apolipoproteins. The highest level of homology (24 % identity and 49 % similarity) was observed with mouse apolipoprotein A-IV. Analysis of the orthologous human genomic structure revealed a similar gene structure and predicted the

presence of an open reading frame encoding a 366 amino acid protein possessing 71 % homology with mouse apoA-V.

To evaluate the physiological role of apoA-V Pennacchio et al. (6) generated transgenic mice that over-express human apoA-V protein as well as mice lacking apoA-V using gene disruption techniques. Profound effects were observed in both transgenic and knockout mice. ApoA-V transgenic mice displayed a 3-fold lower plasma triacylglycerol (TG) level compared to control littermates. By contrast, apoA-V knockout mice revealed a 4-fold higher plasma TG compared to controls. Levels of very low-density lipoprotein (VLDL) particles were increased in the homozygous knockout mice and decreased in the transgenic mice, compared to controls. In the heterozygous knockout mouse VLDL levels were intermediate between the homozygous knockout and control mouse. Using cDNA subtraction techniques van der Vliet et al (7) demonstrated that mRNA encoding a novel gene, identified as apoA-V, was up-regulated following partial hepatectomy. ApoA-V protein was secreted by the liver and was transported primarily on large high density lipoprotein (HDL). In subsequent adenoviral-mediated transfection experiments van der Vliet et al. (8) induced a 20-fold increase in apoA-V plasma concentrations that resulted in a 70% decrease in TG levels.

The association of apoA-V with HDL in the mouse and rat suggests that the protein possesses lipid binding properties similar to that of other exchangeable apolipoproteins. Its HDL association may also be important in interaction of the protein with membrane lipids where it may have a role in cholesterol efflux or facilitated lipid exchange reactions. This HDL-association might also be linked with an ability of apoA-V to activate lecithin:cholesterol acyltransferase (LCAT) since both apoA-I and apoA-IV have this capability. To investigate apoA-V structure and function relations, we constructed a plasmid vector for expression of

recombinant human apoA-V in bacteria. As reported here, recombinant apoA-V possesses hallmark features of an exchangeable apolipoprotein yet possesses unique characteristics that may ultimately be important to the mechanism whereby it modulates plasma lipid homeostasis.

## **Experimental Procedures**

*ApoA-V/ pET plasmid vector.* The coding sequence of human apoA-V (a kind gift of Drs. Eddy Rubin and Len Pennacchio) was amplified using synthetic oligonucleotide primers and inserted into the pBluescript KS(+) vector (Stratagene, La Jolla, CA). Manipulations of the DNA sequence were performed in this plasmid, which was propagated in *E. coli* DH5 $\alpha$  cells. DNA sequencing was performed to verify that no unwanted mutations occurred during the process of plasmid vector construction. For protein expression the apoA-V cDNA was subcloned into the pET 20b+ plasmid (Novagen, Madison, WI) to yield the pET/A-V vector. Mouse plasma samples were obtained from Dr. Len Pennacchio (Lawrence Berkeley Laboratories).

*Expression and purification of recombinant apoA-V.* ApoA-V expression and purification were performed according to Ryan et al. (9) with some modification. Briefly, *E. coli* BL21(DE3)pLysS cells bearing the pET/A-V plasmid were cultured in 500 ml NCZYM media (+50  $\mu$ g/ml ampicillin) at 37 °C. When the culture OD<sub>600</sub> reached 0.6, apoA-V synthesis was induced by the addition of isopropylthiogalactopyranoside to a final concentration of 0.5 mM. After 3 h, the bacteria were pelleted by centrifugation and disrupted by sonication. The cell lysate was centrifuged at 20,000 g for 30 min at 4 °C. The supernatant fraction was then mixed with an equal volume of phosphate buffered saline containing 6 M guanidine HCl and applied to a 5 ml bed volume Hi-Trap affinity column (Amersham Pharmacia Biotech), washed with two

column volumes of buffer (containing 3 M guanidine HCl and 40 mM imidazole) and eluted in buffer containing 3 M guanidine HCl plus 500 mM imidazole. Fractions containing apoA-V protein were pooled and dialyzed against deionized water resulting in precipitation of the protein. The sample was re-dissolved in 0.05 % trifluoroacetic acid in water (or 0.1 N HCl) and further purified on a Perkin-Elmer Series 200 high-pressure liquid chromatograph . The sample was applied to a RXC-8 Zorbax 300SB semi-preparative column and eluted with a linear AB gradient of 2% B/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions were monitored at 230 nm, and those containing apoA-V were pooled, lyophilized and stored at 20 °C.

*Antibody generation.* Purified recombinant human apoA-V (200 µg protein) was emulsified in adjuvant and injected into a goat. Four weeks later, an additional injection (200 µg protein) emulsified in adjuvant was given. Seven weeks after the initial injection, 200 ml of serum was collected. An aliquot of goat anti apoA-V serum was passed over a 5 ml Hi -Trap Protein G HP column (Amersham Pharmacia Biotech). The IgG fraction present in the serum sample was bound to the column in buffer (20 mM sodium phosphate, pH 7.0) and, after washing, eluted with 0.1 M glycine, pH 2.5 directly into tubes containing 200 µl 1 M Tris-HCl, pH 9.0. IgG recovered from the column was equilibrated in 20 mM sodium phosphate, pH 7.0. Subsequently, the IgG fraction was biotinylated by incubation for 15 min with 5 ml 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce Chemical Company) in borate buffer on a rocking platform with slow agitation. Unreacted biotin reagent was quenched with buffer supplemented with 100 mM glycine. After removal of the quenching medium, labeled IgG was dialyzed into 20 mM sodium phosphate, pH 7.0, aliquoted and stored at -80 C.

*Immunoblotting.* For immunoblotting, protein samples were run on a 4-20% acrylamide gradient, Tris-glycine SDS slab gel (Invitrogen Life Tech). Separated proteins were transferred to a 0.2 µm PVDF membrane (Bio-Rad Laboratories) using the blot module Electro-Eluter (Bio-Rad Laboratories) at 150 mA constant current for 3 h. Non specific binding sites on the membrane were blocked with 0.1% TTBS (0.1% Tween 20/ 20 mM Tris/ 150 mM NaCl, pH 7.2) overnight, at room temperature while rotating. Biotinylated apoA-V IgG (1:10,000 dilution in 0.1% TTBS) was incubated with the membrane for 60 min while rotating. After washing 3 x in TTBS, avidin-D with horseradish peroxidase conjugate (Vector Laboratories; diluted 1:30 in 0.1% TTBS) was incubated with the membrane for 60 min. Subsequently, the membrane was washed 3 x in TTBS and incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Chemical Company) in which both reagents were diluted by adding 0.5 ml in 9 ml of dH<sub>2</sub>O. The substrate was incubated for 2 - 5 min at room temperature and exposed to CL-Xposure Film (Pierce Chemical Co.) for 60 sec. Film was developed using a Kodak M35A X-OMAT processor.

*Analytical procedures.* Protein concentrations were determined using the bicinchoninic acid assay (Pierce Chemical Co.) using bovine serum albumin as standard. SDS-PAGE was performed on 4 - 20 % acrylamide slab gels run at 30 mA constant current for 1.5 h. Gels were stained with Coomassie Brilliant Blue R-250 and the relative mobility of protein samples compared to that of known standards (Bio-Rad low MW standards). ApoA-V solubility studies were conducted at various pH values employing 50 mM sodium citrate, sodium phosphate or Tris (plus 150 mM NaCl) within the range of pH 2.5 to 9.5.



*Circular dichroism spectroscopy.* Far UV circular dichroism (CD) measurements were performed on a Jasco J-720 spectropolarimeter using Jasco J-700 Hardware Manager Software (v 1.10.00) on a Pentium computer (Win98). The spectropolarimeter was routinely calibrated using a 0.06% (w/v) aqueous solution of ammonium d-10 camphor sulphonate at 290.5 nm. Scans were performed using a 0.02 cm path length cuvette and a protein concentration of 21.5  $\mu$ M. The sample chamber was maintained at a constant 25 °C using a Lauda RM6 refrigerated recirculating water bath. Temperature denaturation scans were performed using a 0.10 cm path length cuvette in a Jasco Peltier-type thermostatic cell holder and a protein concentration of 6.64  $\mu$ M. All protein samples were dissolved in 50 mM sodium citrate, pH 3.0. The concentrations of apoA-V stock solutions were determined hydrodynamically by fringe count in a Beckman XLI analytical ultracentrifuge according to Babul and Stellwagen (10). Baseline correction, noise reduction and ellipticity calculations for the scans were done using Jasco J-700 for Windows Standard Analysis Software (v 1.20.00). The values are shown as mean residue molar ellipticity ( $\text{mdeg cm}^2 \text{dmol}^{-1}$ ) calculated as:

$$[\theta] = (\text{MRW})(\theta_{222})/10 lc$$

where  $[\theta]$  is mean residue ellipticity, MRW is the mean residue weight (taken to be 113.403).  $(\theta_{222})$  is the measured ellipticity at 222 nm in millidegrees,  $l$  is the cuvette path length in cm and  $c$  is the protein concentration in mg/mL. Deconvolution was performed on the scans using the Contin program of Provencher and Glöckner (11).

*Analytical Ultracentrifugation.* Sedimentation equilibrium experiments were conducted at 30 °C in a Beckman XL-I Analytical Ultracentrifuge using interference optics, as described by Laue

and Stafford (12). Aliquots (110  $\mu$ l) of sample solution were loaded into 6-sector CFE sample cells, allowing 3 concentrations of sample to be run simultaneously. Runs were performed at a minimum of 2 different speeds and each speed was maintained until there was no significant difference in  $r^2/2$  versus absorbance scans taken 2 hours apart to ensure that equilibrium was achieved. The sedimentation equilibrium data was evaluated using the NONLIN program, which incorporates a nonlinear least-squares curve-fitting algorithm described by Johnson et al. (13). This program allows the analysis of both single and multiple data files. Data can be fit to either a single ideal species model or models containing up to four associating species, depending on which parameters are permitted to vary during the fitting routine. The protein's partial specific volume and the solvent density were estimated using the SEDNTERP program, which incorporates calculations detailed by Laue et al. (14).

*ApoA-V interaction with phospholipid.* Bilayer vesicles of dimyristoylphosphatidylcholine (DMPC) were prepared by extrusion as described elsewhere (15) in 50 mM sodium citrate, 150 mM NaCl, pH 3.0. Vesicles were incubated with apoA-V (1:1 w/w) at 24 °C for 16 h. Following incubation the sample was adjusted to 1.31 g/ml by the additional of solid KBr (2.5 ml final volume), transferred to a 5.1 ml centrifuge tube, overlaid with 2.5 ml saline and centrifuged at 275,000 g for 5 h in a Beckman VTi 65.2 rotor. The tube contents were fractionated (0.5 ml) and those containing phospholipid and protein were combined, dialyzed into phosphate buffered saline and stored at 4 °C. The size distribution of DMPC-apoA-V complexes was evaluated by non-denaturing gradient PAGE as described by Nichols et al. (16). Morphology of complexes was determined by negative stain electron microscopy as previously described (17).

*Lecithin:cholesterol acyltransferase activity assays.* The ability of apoA-V to activate lecithin:cholesterol acyltransferase (LCAT) was examined using recombinant LCAT obtained from CHO-K1 cells stably transfected with human LCAT cDNA (a kind gift of Dr. John Parks). Cholesterol esterification was assessed using the exogenous substrate procedure described by Chen and Albers (18). Proteoliposomes composed of either apoA-V (or apoA-I) were prepared using egg-yolk phosphatidylcholine (PC) and unesterified cholesterol (1:250:12.5, mole ratios, respectively) containing trace amounts of [<sup>14</sup>C]cholesterol as previously described (19). Proteoliposomes were exposed to increasing concentrations (0.2-1.0 µg) of purified LCAT protein to measure initial reaction rates in incubation mixtures containing human serum albumin (0.5 %), β-mercaptoethanol (5 mM), and Tris (20 mM) buffered saline EDTA (2.7 mM). Incubations were performed at 37 °C for 0.5 h. Lipids were extracted with hexane and separated by TLC using toluene as the mobile carrier solvent. Results were expressed as a percentage of the initial [<sup>14</sup>C]cholesterol converted to [<sup>14</sup>C]cholesteryl ester.

*Cellular cholesterol efflux.* J774 macrophages were used to assess the capacity of apoA-V to promote cholesterol efflux. Cells were plated at an initial density of 1x10<sup>6</sup> cells/ml/well in 24 well culture plates and allowed to grow (48 h) to confluent monolayers in RPMI medium containing 1% fetal bovine serum and 1 µCi/ml/well of [<sup>3</sup>H]cholesterol as described previously (20). The cAMP analogue 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (0.3 mM) was used to up-regulate the ABCA1 transporter (21). Recombinant human apoA-I was used

as a control. Lipid-free apoA-V (25µg/ml) and apoA-I (25µg/ml) were added to serum-free RPMI and filter sterilized (0.45 µm) before additions to cells. Filtration did not remove any apoA-V (judged by Western-blot analysis) indicating apoA-V solubility in serum free medium at this concentration. Aliquots of efflux medium were removed at various times and cellular debris removed by centrifugation (1000 x g, 8 minutes at 4 ° C). Radioactivity in medium supernatant was quantified and efflux results were expressed as a percentage of the cellular [<sup>3</sup>H]cholesterol released into the medium as a function of time.

## Results

*Expression and isolation of apoA-V.* The cDNA encoding human apoA-V was introduced into the pET 20b expression vector immediately downstream from an in frame His-Tag encoding sequence. When the fusion protein was expressed under standard conditions developed for recombinant human apolipoprotein A-I (9) we noted that the yield of recombinant protein was quite low. Whereas significant amounts of apoA-V were present in the crude bacterial pellet, intact protein was not recovered in the eluate from the nickel chelation affinity chromatography column. Investigation of this phenomenon revealed that recombinant apoA-V bound to the nickel chelation column is subject to degradation when the column contents are exposed to buffer lacking guanidine HCl. Because the original protocol employed included removal of guanidine HCl prior to protein elution with imidazole, poor yields of apoA-V were observed. Two major breakdown products were characteristically observed using this protocol, an 11 kDa band and a 30 kDa band. On the other hand, when 6 M guanidine HCl was included in all buffers used during this chromatography step, the yield of intact apoA-V was substantially improved. Further

purification by reversed phase HPLC resulted in an apoA-V preparation that gave rise to a single major band when analyzed by SDS-PAGE (**Figure 1**). Using this protocol, yields of recombinant apoA-V were 3-5 mg per liter of bacterial culture.

An antibody to recombinant human apoA-V was generated and **Figure 2** depicts a Coomassie Blue stained gel and a corresponding immunoblot. No reactivity was observed in either control mouse plasma or apoA-V knockout mouse plasma. On the other hand, in the case of human apoA-V transgenic mouse plasma, the antibody recognized a single band corresponding to the molecular weight of apoA-V. In addition, the immunoblot contained different amounts of isolated recombinant apoA-V as well as two other members of the human apolipoprotein family, apoA-I and apoA-IV. Whereas anti apoA-V IgG recognized as little as 1 ng apoA-V, no reactivity was observed toward apoA-I or apoA-IV at 100 ng sample loads. These results indicate that the antibody is specific for apoA-V.

*ApoA-V properties.* Whereas apoA-V was soluble in 50 mM sodium citrate, pH 3.0, it was insoluble in neutral pH buffers at concentrations > 0.1 mg/ml. To characterize the solubility properties of apoA-V, the effect of solution pH on the solubility of apoA-V was determined. It was observed that between the pH limits 3.5 and 9.0 apoA-V is largely insoluble. The poor solubility of apoA-V around neutral pH values may be attributed to its pI of 6.2. Sequence analysis indicates that apoA-V does not possess predicted transmembrane segments and lacks consensus sites for N-linked glycosylation or lipid modification. Thirty percent of the amino acids in mature human apoA-V possess charged side chains at neutral pH (49 Glu/Asp) and 41 (Arg/Lys) that, together with the 14 His present, are relative proportions that are similar to those

found in other apolipoproteins. It is conceivable that apoA-V maintains solubility in plasma through interaction with lipoproteins.

*CD spectrum of apoA-V.* A far UV CD spectrum of apoA-V is depicted in **Figure 3**. The spectrum reveals molar ellipticity minima at 208 nm and 222 nm, indicative of the presence of  $\alpha$ -helix secondary structure. Deconvolution of the spectrum with the Contin program of Provencher and Glöckner (11) indicated that, at pH 3, apoA-V is comprised of 32 %  $\alpha$ -helix, 33 %  $\beta$ -sheet, 16  $\beta$ -turn and 18 % random coil. Thus, unlike most members of the exchangeable apolipoprotein family, apoA-V displays significant amounts of  $\beta$  structure.

*ApoA-V stability properties.* To assess its stability properties in solution, the effect of temperature on the ellipticity of apoA-V was determined (**Figure 4**). As the temperature was increased from 5 to 37 °C a small decrease in negative ellipticity was noted. However, above 37 °C, there was a much sharper temperature dependent decrease in molar ellipticity, with nearly complete loss of structure occurring at 60 °C. Between 60 °C and 85 °C, further losses in molar ellipticity were small. The general features of the temperature melt profile are consistent with a single native  $\rightarrow$  unfolded transition with a midpoint of 47.1 °C. To evaluate the reversibility of temperature induced unfolding of apoA-V, CD spectral scans were obtained before, during and after a temperature melt (**Figure 5**). The results obtained revealed that, although apoA-V recovers from the melt, it is unable to regain 100% of the molar ellipticity present in unheated apoA-V.

*ApoA-V self-association.* To investigate apoA-V self association properties, sedimentation equilibrium experiments were performed in 50 mM sodium citrate, pH 3.0 at speeds of 8,000 and 12,000 rpm and loading concentrations of 1.50, 2.70, and 5.88 mg/ml. A significant dependence on rotor speed and loading concentration was observed in the calculated apparent average molecular weights, ranging from 330,000 at 8,000 rpm to 68,000 at 12,000 rpm. It was not possible to obtain a good fit to any of the self-association models when fitting all of the data sets globally using the NonLin program. Under these conditions we conclude that apoA-V undergoes non-specific aggregation at the concentrations used in these experiments. Further runs were performed with the inclusion of different concentrations of NaCl added to the buffer with no effect on the association / aggregation phenomenon.

*ApoA-V binding to phospholipid.* Upon incubation with bilayer vesicles of DMPC at a 1:1 weight ratio, apoA-V interacts with the phospholipid to form a discrete population of lipid particles (**Figure 6**). Native gradient PAGE analysis of the isolated lipid particles revealed a Stokes diameter in the range of 15-20 nm. Negative stain electron microscopy of the particles corroborated the size measurements and revealed disc shaped particles that stack to form rouleaux. This result is similar to results obtained with other exchangeable apolipoproteins-and is consistent with observations that apoA-V is found in association with HDL.

*LCAT activation and cholesterol efflux properties of apoA-V.* To evaluate the ability of apoA-V to serve as an activator of LCAT, purified apoA-V was used to prepare a proteoliposome substrate that was exposed to physiological concentrations of purified LCAT. Compared with apoA-I, apoA-V was a poor activator of LCAT, where activity was  $8.5 \pm 1.8\%$  (n=3) that of

apoA-I. In studies of apolipoprotein mediated cholesterol efflux, apoA-I was used as a positive control. ApoA-I is known to stimulate the efflux of cholesterol from cells through interaction with the ABCA-1 transporter. Up-regulation of ABCA1 by cAMP maximizes cholesterol efflux. As shown in **Figure 7**, apoA-V-mediated cholesterol efflux was unaffected by cAMP treatment of the cells and efflux levels were low. ApoA-I, on the other hand, shows the expected increase in cellular cholesterol efflux in the presence of cAMP. The data suggest that cholesterol efflux to apoA-V occurs through an ABCA1-independent mechanism.

## Discussion

With the advent of the human genome project and the ability to compare large tracts of sequence across species, new opportunities for discovery of potentially important gene products exists. An excellent example of this concept emerged with the discovery of a previously unknown member of the exchangeable apolipoprotein family, apoA-V. Whereas genetic studies illustrate the important role played by apoA-V in plasma lipid metabolism, particularly TG metabolism (6,22,23), questions remain about the mechanism whereby apoA-V exerts its biological effects. The goal of determining how apoA-V influences plasma TG levels requires additional knowledge of the protein itself. In fact, previously, human apoA-V has not been isolated and little is known of its structure, stability, lipid interaction properties or potential regulatory effects.

Human apoA-V is synthesized with a cleavable signal peptide, comprising the first 23 amino acids of the protein sequence. This signal sequence directs the protein toward a secretory pathway, resulting in the appearance of apoA-V in plasma. To improve our understanding of



apoA-V structure and function relationships, we created a vector construct for expression of human apoA-V in *E. coli*. Following optimization of the expression and purification protocol, yields of recombinant apoA-V in the range of 3-5 mg/L bacterial culture were obtained. Recombinant apoA-V was used as an antigen to generate polyclonal antibodies in a goat. In characterization studies it was shown that goat anti human apoA-V IgG is specific for apoA-V and thus, will likely be useful in future studies designed to quantitate apoA-V levels in human plasma samples and correlate these with TG levels. Whereas preliminary studies with human serum revealed specific reactivity against apoA-V, the present anti human apoA-V IgG did not detect mouse apoA-V (Figure 2, wild type mouse serum lane), suggesting either a lack of epitope conservation or the amounts of apoA-V present are below the limits of detection.

In studies of apoA-V properties, results obtained indicate the lipid-free protein is poorly soluble between the pH limits 3.5 and 9 (at concentration > 0.1 mg/ml). This solution behavior suggests human apoA-V may exist in association with lipoproteins in the plasma compartment, consistent with its observed plasma distribution in animal models (6,7). Based on the fact that apoA-V is a member of the apoA-I/CIII/A-IV gene family whose protein products belong to the exchangeable apolipoprotein family, studies were conducted to characterize apoA-V in the lipid-free state. Far UV CD spectroscopy of apoA-V in buffer at pH 3 revealed the presence of different secondary structure elements including,  $\alpha$ -helix,  $\beta$ -sheet and  $\beta$ -turn. This result, together with the relatively large size of apoA-V, suggests apoA-V may possess more than one structural domain. Examination of its amino acid sequence reveals an unusual Ala<sub>292</sub>-Pro-Pro-Pro-Gly<sub>297</sub> segment in the C-terminal region of the protein. It is conceivable that the 47 residues beyond this tetraproline segment adopts an independent fold or may represent the 18 % of apoA-V that exist as random coil in the lipid free state. Future studies of this peptide in

isolation or a C-terminal truncated apoA-V may provide insight into the structural and / or biological role of this region of apoA-V. In the case of human apoA-I and apoE, their C-terminal regions are known to play a key role in lipoprotein binding and self-association in the absence of lipid (24,25). In a similar manner, apoA-V self associates in the absence of lipid. As with other apolipoproteins, this may be a mechanism to sequester lipid-binding sites on the protein from exposure to the aqueous milieu. At present it is unknown which regions of apoA-V are responsible for self-association or lipid interaction. In the case of other exchangeable apolipoproteins, such as apoE, lipid interaction induces a conformational change in the protein wherein hydrophobic sites in the protein are exposed to the lipid surface (26). The present bacterial expression system is ideally suited for experiments designed to dissect which portions of apoA-V that are responsible for self-association and lipid interaction and these studies are in progress.

In the case of human apoA-I or insect apolipoprotein III (27), it has been proposed that lipid interaction is facilitated by adoption of a loosely folded conformation in buffer. Secondary structure elements in both these proteins are stabilized by lipid association, and this may represent a driving force for their intrinsic lipid surface seeking property. Apolipoprotein III displays a temperature induced denaturation transition midpoint of 52 °C (28) while the corresponding value for apoA-I is 54 °C (29). Thermal denaturation experiments with apoA-V revealed a transition midpoint of 47.1 °C, consistent with a loosely folded structure in solution. Unlike human apoA-I or insect apolipoprotein III, apoA-V was unable to fully recover 100 % of its secondary structure elements following temperature-induced denaturation. Thus, apoA-V may possess a more complex tertiary fold, a portion of which is irreversibly altered, upon exposure to elevated temperature. At present it is not known if similar results will be observed with

chaotrope-induced unfolding of apoA-V. In light of the fact that guanidine HCl exposure is employed in the protocol used for isolation of recombinant apoA-V, detailed characterization of the ability of apoA-V to recover a fully native conformation following such perturbations will be important to understand the biological role of this protein.

The physico-chemical properties of lipid-free apoA-V suggest that the protein has lipid-binding activity. This was indeed demonstrated by apoA-V's ability to form lipid-protein complexes with DMPC. The complexes are similar to those formed with other exchangeable apolipoproteins and suggest that apoA-V binds to the lipid surface of lipoproteins, particularly HDL (7). Because apoA-V is transported on HDL and has structural similarity to other apolipoproteins such as apoA-IV and apoA-I that are known activators of LCAT, it is conceivable that it can serve to activate this enzyme. As reported herein, however, it is evident that this is not the case, suggesting that the structural motif required for LCAT activation is not present in this protein.

One of the characteristics of exchangeable apolipoproteins, including apoA-I and apoA-IV, is the ability to stimulate the efflux of cholesterol from cells via the ABCA-1 transporter. However, the present study indicates that this is not the case for apoA-V. By contrast, apoA-V recruits low levels of cholesterol from cells, apparently by an ABCA1 independent mechanism. Thus, apoA-V appears to have detergent-like properties that allow it to remove membrane lipids in a non-specific manner.

The fact that apoA-V is present in very low concentrations in plasma leaves open the question as to its role in lipid metabolism, despite convincing studies in mice that show a correlation between apoA-V and TG levels. The availability of a recombinant apoA-V and a specific antibody will be useful for future studies in correlating apoA-V and TG levels in mice

and humans and by allowing one to assess how apoA-V functions to modulate TG levels. In summary, we have developed a bacterial expression system for production of milligram quantities of human apoA-V. The recombinant protein was isolated and characterized in terms of its solution properties, secondary structure content and thermal stability. In addition, we have generated an antibody directed against apoA-V that provides a potentially useful reagent for detection and quantification of apoA-V. Given the existing correlation between apoA-V and plasma TG in mouse models (6,8), human populations studies designed to assess the significance of the correlation between apoA-V concentrations and plasma TG are possible. Because of the strong correlation between elevated plasma TG and cardiovascular disease, knowledge of how apoA-V modulates plasma TG levels may be of therapeutic significance.

**Acknowledgements.** We thank Drs. Len Pennacchio and Eddy Rubin for providing human apoA-V cDNA and aliquots of apoA-V transgenic and knockout mouse plasma. We also thank Dr. John Parks for providing LCAT, Dr. Richard Weinberg for apoA-IV and Les Hicks for the sedimentation equilibrium analysis. The authors thank Barbara-Jean Nitta for assistance with antibody production. The expert animal care provided by the U.C. Davis Goat facility is gratefully acknowledged. Finally, we thank Drs. Vasanthy Narayanaswami and Paul Weers for many helpful discussions.

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## Figure legends

**Figure 1. Reversed-phase HPLC of recombinant human apoA-V.** Protein was loaded onto a Vydac C8 column and eluted with a gradient of water/ acetonitrile (0.05 % trifluoroacetic acid) gradient from 0 - 100 % acetonitrile. *Inset:* SDS-PAGE analysis of recombinant apoA-V. Lane 1) molecular weight standards; Lane 2) 10 µg apoA-V.

**Figure 2. Immunoblot analysis of apoA-V.** Proteins were separated on 4-20 % acrylamide gradient gels and A) stained with Commassie Blue or B) transferred to a PVDF membrane and probed with biotinylated anti-apoA-V IgG. Sample load on the immunoblot: mouse plasma (0.5 µl); apoA-V (1 ng and 2 ng, see arrows); apoA-I (100 ng) and apoA-IV (100 ng).

**Figure 3. Far UV CD spectroscopy of apoA-V.** Spectra were collected at a protein concentration of 0.271 mg/ml at 25 °C.

**Figure 4. Temperature induced denaturation of apoA-V.** Data are presented as mean residue molar ellipticity collected at 222 nm every 0.1 °C from 5 °C to 85 °C.

**Figure 5. Recovery of ellipticity after temperature melt.** Far UV CD scans (from 250 to 215 nm) of apoA-V were collected before, during and after the temperature melt. Initial scan at 25 °C (●); after cooling to 5 °C and return to 25 °C (○); after heating to 85 °C and return to 25 °C (○); after an additional 30 min. equilibration at 25 °C (▼).



**Figure 6. Characterization of apoA-V-DMPC complexes.** Left panel) native gradient PAGE analysis of apoA-V-DMPC complexes prepared as described in Experimental Procedures; lane 1) apoA-V-DMPC complexes; lane 2) molecular size standards. Right panel) negative stain electron micrograph of apoA-V-DMPC complexes (magnification, 18 mm = 100 nm).

**Figure 7. Efflux of cellular cholesterol to apolipoproteins.** J774 mouse macrophages were pre-labeled with [<sup>3</sup>H]cholesterol and incubated with apoA-I (left panel) or apoA-V (right panel) in the absence (squares) and presence (circles) of cAMP analogue. Lipid-free recombinant apoA-I or apoA-V were added to cells at a final concentration of 25 µg/ml. Values reported are the mean ± SD of 3 separate experiments each performed in triplicate.

Figure 1

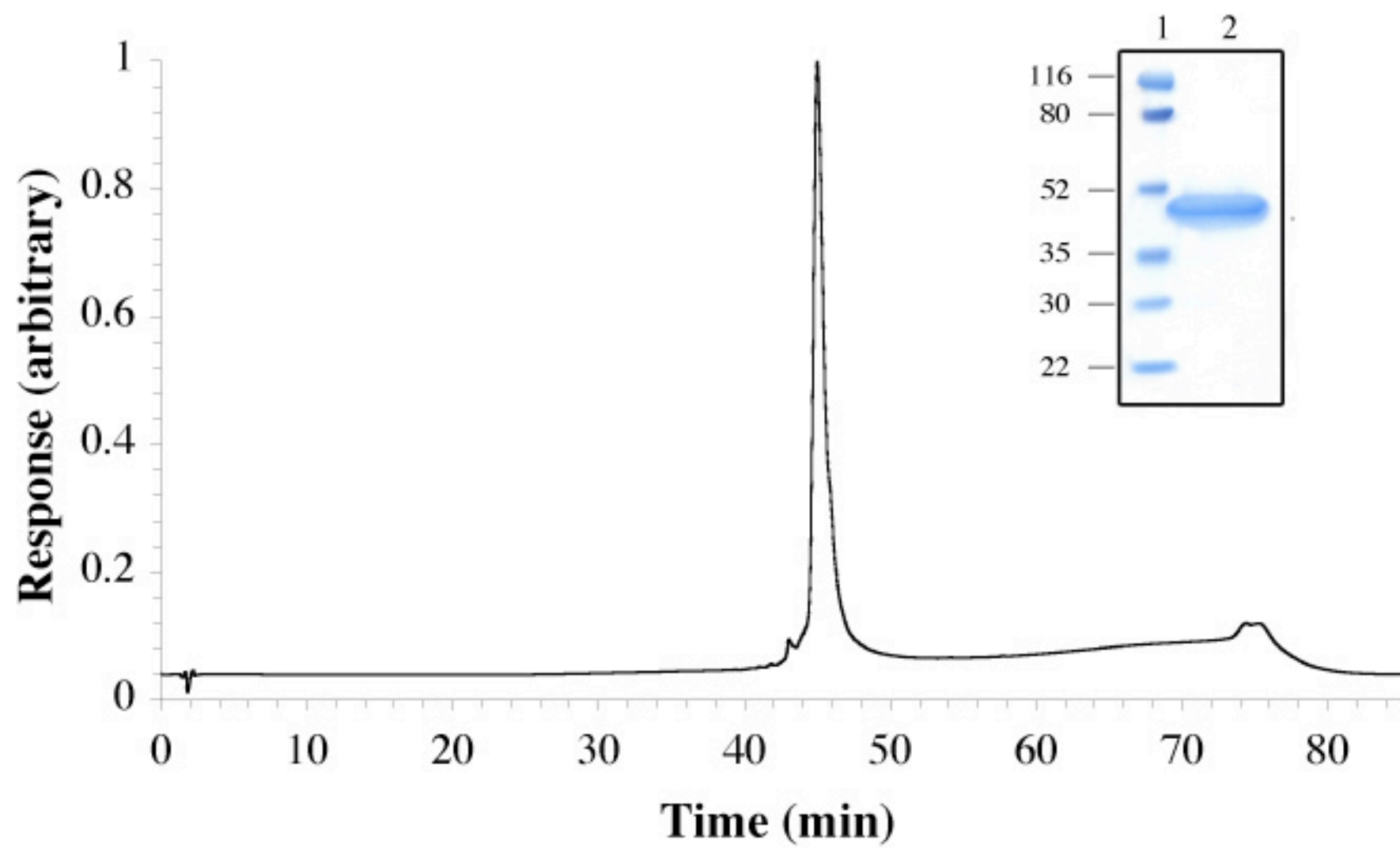


Figure 2

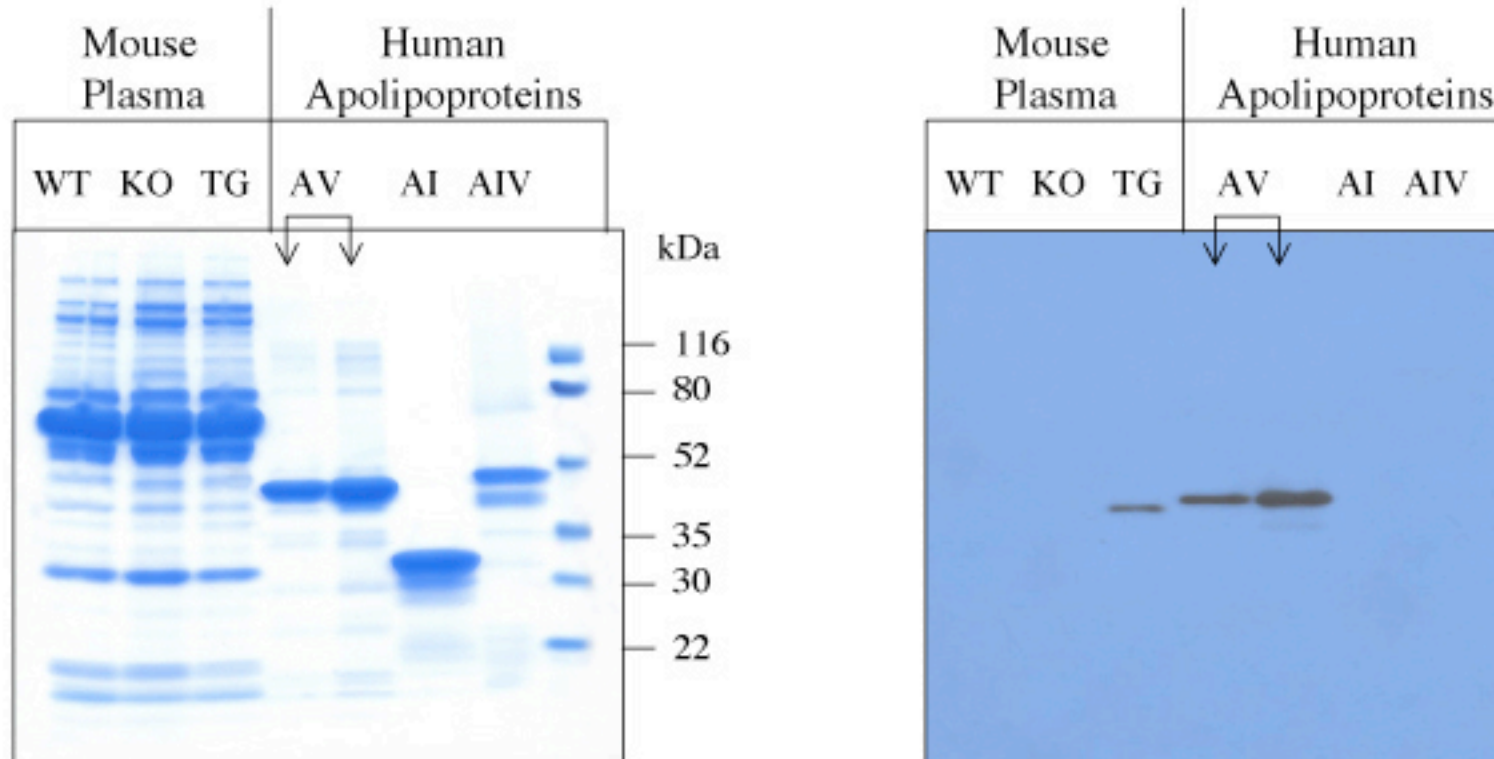


Figure 3

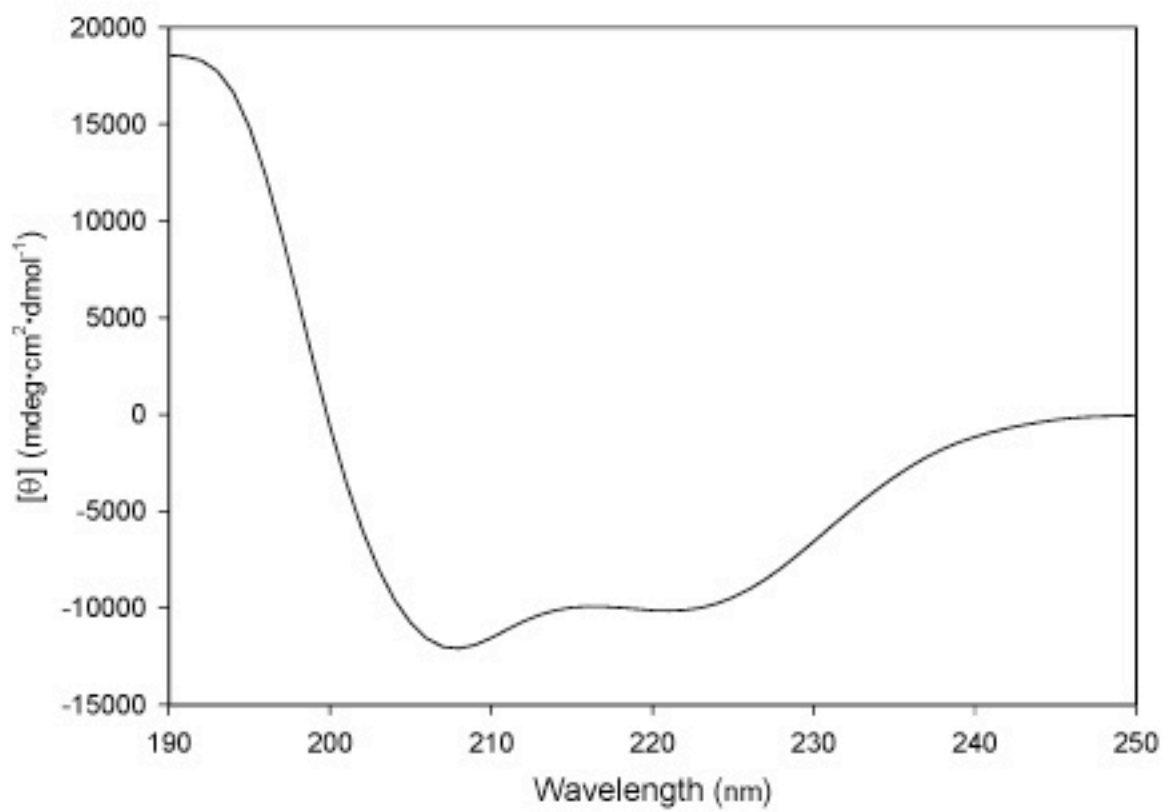


Figure 4

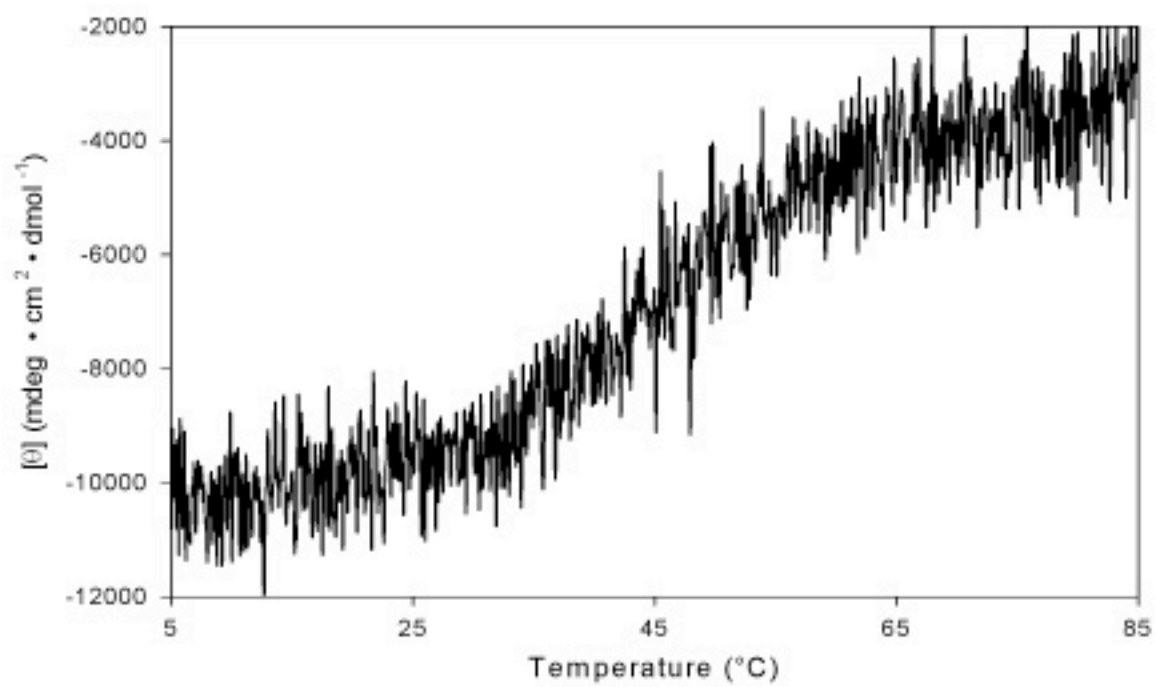


Figure 5

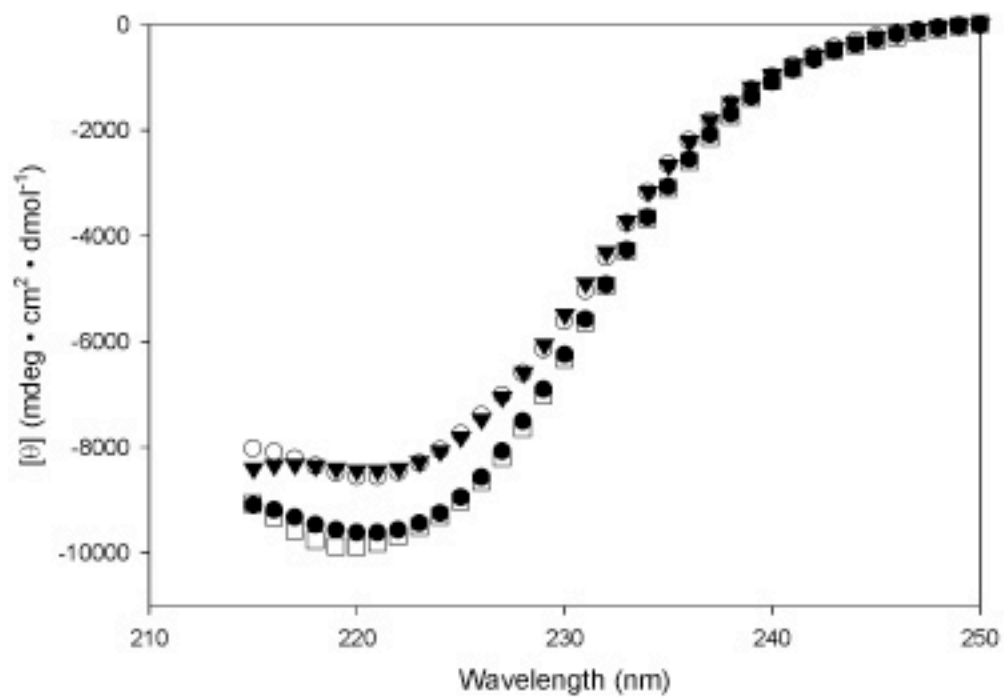


Figure 6

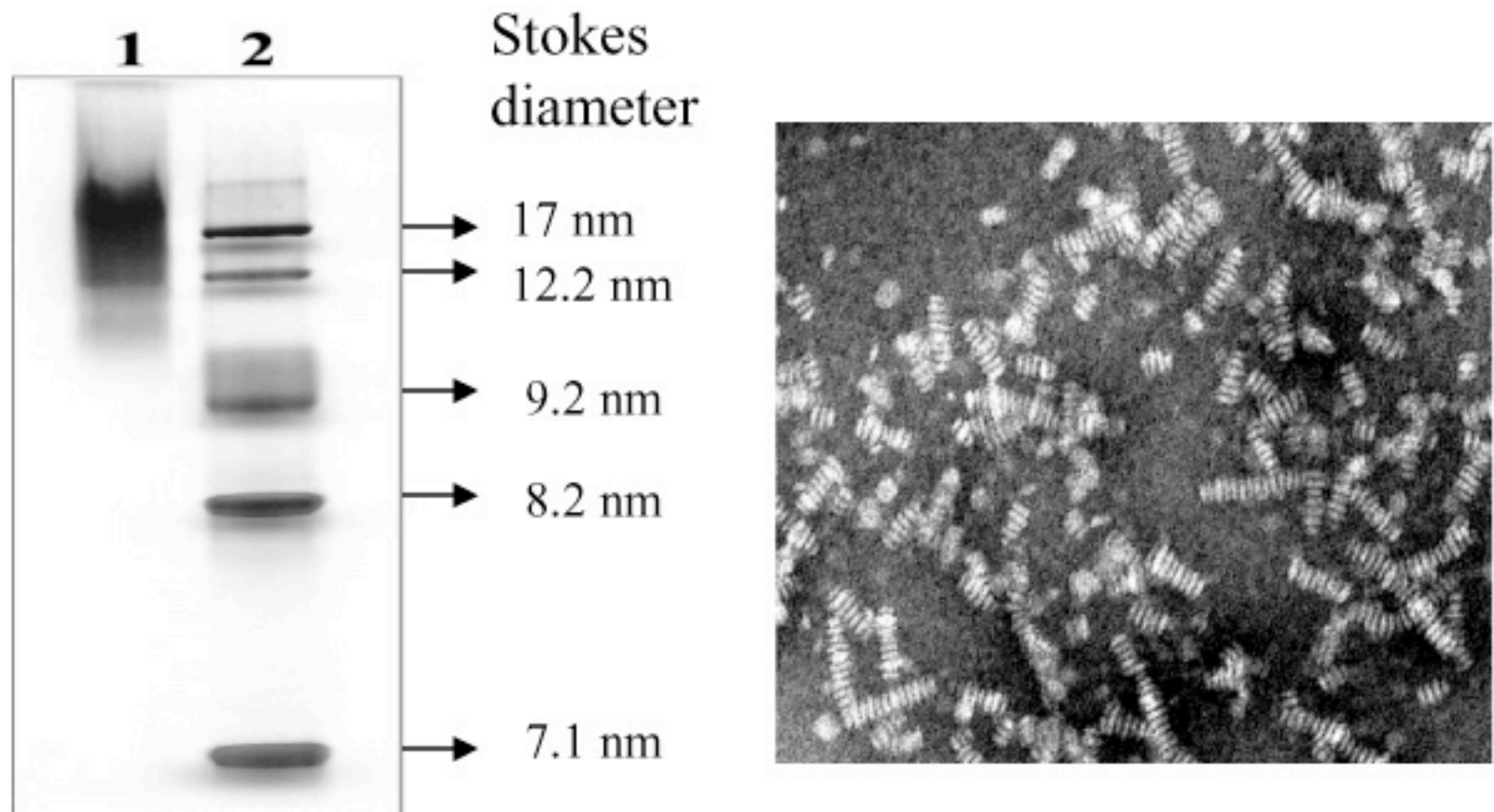


Figure 7

