A rapid method for the synthesis of protein-lipid complexes using adsorption chromatography

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Summary A novel and rapid method for the detergent-mediated synthesis of protein-lipid complexes has been developed and has several advantages over detergent dialysis methods. This new method involves co-incubation of human apolipoprotein A-I (apoA-I), the major protein component of high density lipoproteins (HDL), and dipalmitoylphosphatidylcholine for 1 hr in the presence of cholate, after which removal of >99.7% of the detergent is achieved by a 2-hr batch adsorptive chromatography procedure. Complexes prepared by this method had a density of 1.10 g/ml, similar to plasma HDL. Chemical cross-linking of these products demonstrated that there was complete conversion of apoA-I to a protein-lipid complex that contained two molecules of apoA-I. One major band was resolved by gradient gel electrophoresis in the region of the gel expected for newly synthesized HDL. Results are described which show the application of this method to the study of lipid variation on the structure of model HDL, including the alteration of lipid-protein molar ratios and the addition of cholesterol. — **Bonomo, E. A., and J. B. Swaney.** A rapid method for the synthesis of protein-lipid complexes using adsorption chromatography. *J. Lipid Res.* 1988. **29:** 380-384.

Supplementary key words Bio-Beads SM-2 • detergent removal • detergent analysis • DPPC • lipoprotein

Several methods for the preparation of model high density lipoproteins (HDL) have been developed whereby protein-lipid complexes, similar in size and density to HDL, are synthesized through the recombination of apolipoproteins and individual lipid species (1). The components used most frequently for these methods have been apolipoprotein A-I(apoA-I), the major protein component of HDL, and pure synthetic phosphatidylcholines (2). Recently, a detergent-mediated synthesis of protein-lipid complexes has been reported whereby apoA-I and lipids are codispersed by the detergent action of the bile salt, sodium cholate (3). Subsequent removal of cholate by exhaustive dialysis results in the formation of models of discoidal, nascent HDL.

In attempting to employ the detergent dialysis method for a study of the effects of variation in lipid composition on HDL structure, several disadvantages of this method became apparent: *1*) multiple samples and minute volumes are difficult to handle, and *2*) this lengthy procedure requires a 12-18-hr incubation of the codispersion followed by a 48-hr dialysis at elevated temperatures to remove cholate. To avoid such hindrances, a simpler, alternative cholate-mediated method, based on the rapid, chromatographic removal of cholate by adsorption to Bio-Beads SM-2 (4), was developed. This report describes the details of this method which can be completed within 4 hr, accommodates multiple and minute samples, and is less likely to cause deterioration of the nascent HDL-like products such as through bacterial degradation and lipid oxidation.

MATERIALS AND METHODS

Materials

All experiments were performed in buffer A containing 10 mM Tris/HCl, pH 8.0, with 1 mM EDTA, 1 mM NaN_3, and 150 mM NaCl unless otherwise stated. Sodium cholate and lipids were purchased from Sigma Chemical Company (St. Louis, MO) and found to be 99% pure as assessed by thin-layer chromatography. Tritiated cholic acid was purchased from New England Nuclear Corporation (Boston, MA). Apolipoprotein A-I was isolated from the HDL fraction (d 1.063-1.21 g/ml) of human plasma

Abbreviations: HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; DPPC, dipalmitoylphosphatidylcholine; DMS, dimethylsuberimidate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

and labeled with [^{14}C]acetic anhydride as described previously (5). Quick-Sep micro-columns and caps were purchased from Isolab, Inc. (Akron, OH). Bio-Beads SM-2 (20–50 mesh) were obtained from Bio-Rad Laboratories (Richmond, CA) and washed before use in a manner similar to that previously described (6). Briefly, Bio-Beads SM-2 (20 g dry weight) were gently stirred in 200 ml of methanol for 15 min. During the wash procedure, precautions were taken never to allow the Bio-Beads to dry. The methanol was removed by vacuum filtration through a sintered glass filter. The methanol was then washed from the beads with 3 liters of distilled water. Finally, the beads were washed with 200 ml of buffer A and stored at 4°C.

Preparation of DPPC–cholate–apoA-I codispersion 140:190:1 (mol/mol/mol)

Stock solutions of lipids in $CHCl_3$ (10 mg/ml) and [^{3}H]cholate in buffer A (30 mg/ml, 6740 dpm/μg) were prepared. An aliquot of the stock DPPC solution was pipetted into a conical glass vial (Pierce Chemical Co., Rockford, IL; Reactivial). A lipid film was formed on the walls of the vial by evaporating the solvent with nitrogen. Residual solvent was removed by placing the vial in a vacuum chamber overnight at room temperature. Buffer A was added to the vial which was then vortexed to disperse the lipid, yielding a turbid suspension of multilamellar vesicles at a final concentration of 20 mM DPPC. Mixed micelles were prepared by the addition of sodium cholate (30 mg/ml) to the DPPC multilamellar vesicles to yield a DPPC–cholate molar ratio of 0.737 which is within the range which yields optimal micelle formation (7) and has been employed for the detergent dialysis method (3). The mixture was vortexed (and warmed when necessary) until the suspension cleared (within less than 3 min). Finally, [^{14}C]apoA-I was added (final concentrations, 1.1 mg of apoA-I/ml, 3.2 mg of cholate/ml) and the vial was vortexed for 15 sec; the samples were then incubated for 1 hr. For initial experiments a temperature of 37°C was selected; however, in later work a temperature of 45°C was employed since this temperature, which is above the chain-melting temperature of DPPC (8), yielded a more homogeneous product. No differences in the nature of the products were observed when this incubation time was varied between 15 min and 18 hr. The final volume of the codispersion was 0.05–0.500 ml.

Adsorption of cholate to Bio-Beads SM-2

Immediately before using the Bio-Beads SM-2, excess buffer was removed by vacuum filtration to yield moist beads devoid of excess liquid, yet shiny and clumped due to wetness. (The adsorptive surface area of the beads decreases or is lost if the beads are too dry and a dilution problem arises if the beads remain too moist.) For codispersion volumes of less than 250 μl, the moist beads were weighed into Quik-Sep micro-column caps to which the codispersion was added (1 ml of codispersion/mg beads). The micro-columns were inverted and gently pushed down onto the caps. Each micro-column was then brought into a horizontal position and inserted into one of 30 clips on a rotator (Cole-Palmer Instrument Company, Chicago, IL, model no. 7637); the micro-column must remain horizontal and parallel to the axis of the rotator to prohibit the seeping of the codispersion out of the cap. The beads and codispersion were then gently mixed by low speed rotation for various lengths of time to determine optimum conditions, after which the product was eluted by placing the micro-column into a weighed collecting tube and centrifuging for 2 min in a table-top centrifuge at approximately 200 *g*. When the codispersion volume was greater than 250 μl, it was mixed with the appropriate amount of beads in a test tube. Then the contents of the tube were poured into a micro-column and the product was eluted as described above. The volume of the micro-column eluate was determined by calculating the difference in weight of the collecting tube before and after centrifugation.

Characterization of lipid–apoA-I complexes

To determine the extent of protein–lipid complex formation and the protein stoichiometry of the complexes, a cross-linking reagent, dimethylsuberimidate (DMS) (20 mg/ml in 1 M triethanolamine-HCl, pH 9.7), was added to aliquots of the micro-column eluates or density gradient fractions and allowed to react at room temperature for 2 hr (9). The cross-linked samples were subjected to 3–27% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (10) and the number of protein chains per particle in the protein–lipid complexes was determined by reference to cross-linked human apoA-I (11). The cross-linked, self-associated forms of lipid-free apoA-I range from monomers to pentamers. As a control, a codispersion with a cholate apoA-I molar ratio of 190:1 was incubated with Bio-Beads SM-2 for various periods of time up to 4 hr. The SDS-PAGE patterns of cross-linked aliquots from each time point were identical to the cross-linked pattern of apoA-I in the absence of cholate. In contrast, upon cholate-mediated complexation of apoA-I with lipids, the quaternary structure of the apoA-I undergoes changes that can be detected upon chemically cross-linking the protein moiety of the complexes and subjecting such samples to SDS-PAGE. The Stokes' diameter of the discoidal products was determined by pore limit gradient gel electrophoresis (Pharmacia PAA 4/30, Tris–borate, pH 8.4, 150 volts for 20 hr), correcting the observed diameters relative to spheroidal standards by adding 2 nm (12). To determine the product buoyant density and, when there was more than one product, isolate the products, the micro-column eluate was subjected to KBr density gradient ultracentrifugation (13). The tube fractionation was

monitored at 280 nm. The peak fractions were pooled and chemically analyzed for phospholipid phosphorus (14). The protein and cholate concentrations were determined by liquid scintillation counting. For comparison with the Bio-Beads technique, duplicate codispersions were subjected to cholate removal by dialysis over 2 days against three changes of buffer, 2 liters each as described by Matz and Jonas (3).

RESULTS AND DISCUSSION

One of the major drawbacks to existing procedures for detergent-mediated lipid apolipoprotein recombinant formation is the protracted time period required for detergent removal. To ascertain the time course of cholate removal by adsorption to Bio-Beads SM-2, aliquots of a DPPC-[^{3}H]cholate-A-I codispersion 140:190:1 (mol/mol/mol) were subjected to incubation with the beads for various time intervals in duplicate at room temperature or at 4°C. As determined by liquid scintillation counting, there was an exponential rate of cholate removal with >99.7% of the cholate removed within 2 hr (**Fig. 1**). Routinely, approximately 85% of the initial apoA-I and 70% of the DPPC were recovered in the micro-column eluate. These recoveries are comparable to those reported previously using the detergent dialysis technique (3).

DPPC-cholate-apoA-I codispersions with cholate-apoA-I molar ratios of 190:1 and a DPPC-apoA-I molar ratio of 34:1, 68:1, 100:1, or 135:1 were incubated at 37°C for 1 hr followed by a 2-hr incubation with Bio-Beads SM-2. As indicated by the KBr density gradient profiles of the products of these four different codispersions (**Fig. 2**), the extent of apoA-I complexation with DPPC neared completion as the DPPC-apoA-I molar ratio of

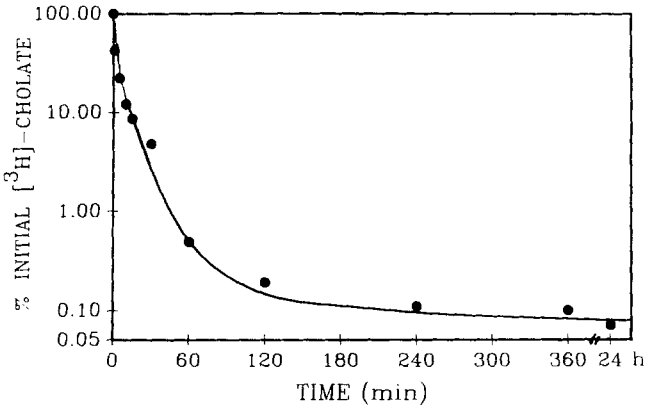

Fig. 1. The time-dependent removal of cholate from DPPC-cholate-A-I 140:190:1 (mol/mol/mol) codispersions by adsorption to Bio-Beads SM-2. The percentage of cholate in the eluate from a column of Bio-Beads SM-2 is shown as a function of the time of incubation of codispersions with the Bio-Beads prior to elution by centrifugation. The points plotted are averages of duplicate determinations.

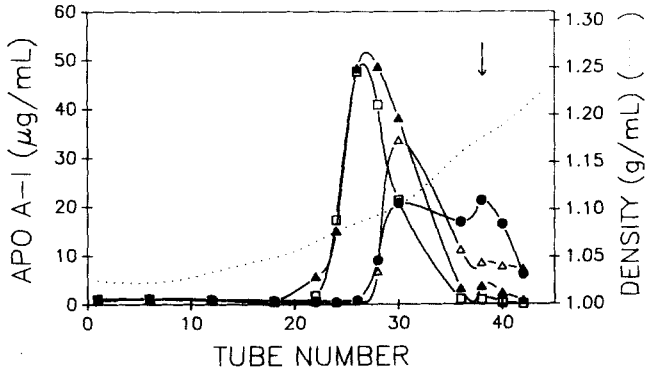

Fig. 2. Effect of varying the DPPC-apoA-I molar ratio on the density of resultant complexes and the extent of complex formation. The KBr density gradient ultracentrifugation profiles of Bio-Beads SM-2 micro-column eluates obtained from DPPC-cholate-apoA-I codispersions at DPPC-apoA-I molar ratios of 34:1 (●—●), 68:1 (△—△), 100:1 (▲—▲), or 135:1 (□—□) is shown. The arrow indicates where lipid-free apoA-I eluted from a control density gradient.

the initial codispersion increased to 100:1 and was essentially complete at a ratio of 135:1. The stoichiometry of the DPPC-apoA-I complex and the extent of complex formation as a function of the DPPC-apoA-I molar ratio of the codispersions were studied by cross-linking each of the four micro-column eluates with DMS and performing SDS-PAGE (9). As the DPPC-apoA-I molar ratio increased, the intensity of the dimer band increased and that of the other oligomeric bands decreased (data not shown).

Pore limit gradient gel electrophoresis of products of the codispersions that contained a DPPC-apoA-I molar ratio of 34:1 or 68:1 demonstrated a single band which correlated to a particle Stokes' diameter of 11.3 nm (data not shown). The calculated DPPC-apoA-I molar ratio of these products is 204:2, based upon two apoA-I molecules per particle. At higher DPPC-apoA-I ratios, the electrophoretic pattern showed two major bands, the smaller of which migrated as a larger particle than that observed for the single product obtained at DPPC-A-I ratios of 34:1 or 68:1. When the DPPC-apoA-I ratio was 100:1, these bands correlated to particle Stokes' diameters of 11.5 nm and 12.2 nm with calculated DPPC-apoA-I molar ratios of 220:2 and 280:2, respectively. When the DPPC-apoA-I molar ratio was 135:1, the Stokes' diameters of the products were 11.7 nm and 12.4 nm with calculated DPPC-apoA-I molar ratios of 237:2 and 298:2, respectively.

In order to directly compare the complexes prepared by the Bio-Beads method with those generated by the detergent dialysis approach, aliquots of the same codispersion with a DPPC-cholate-apoA-I molar ratio of 140:190:1 were incubated for 1 hr at 45°C and were subjected in duplicate to exhaustive dialysis or to Bio-Beads treatment. When the pore limit electrophoretic patterns of these products were compared (**Fig. 3**), it was shown that the major product generated by the Bio-Beads method was

similar to that obtained by the dialysis method; however, there was a difference in the distribution of the minor components. SDS-PAGE of cross-linked samples showed that dialysis and Bio-Beads products possessed two apoA-I molecules per complex (data not shown). In parallel experiments we found that the DPPC–apoA-I complex size and stoichiometry were independent of the time of incubation before cholate removal; however, the temperature of incubation did affect the size of the product such that incubation at 45°C before cholate removal yielded a particle size that was slightly larger and more homogeneous than that found for the Bio-Beads product when incubation prior to cholate removal was at 37°C.

The buoyant density of the complexes eluted from the Bio-Beads micro-columns and of the products prepared by dialysis was 1.10 g/ml, as determined by KBr density gradient ultracentrifugation and pycnometry. The complexes prepared by the Bio-Beads treatment had a DPPC–apoA-I molar ratio of 240:2, and less than 1 molecule of cholate, and were similar in composition to the product prepared by detergent dialysis method from the same codispersion.

Based upon these results our standard protocol for forming model HDL by the cholate–Bio-Beads method involves a 1-hr pre-incubation of the reaction mixtures at 45°C followed by a 2-hr incubation of these mixtures with Bio-Beads SM-2 at 4°C, and a 2-min centrifugation to liberate a cholate-free eluate containing model lipoproteins.

To test the feasibility of applying the cholate–Bio-Beads method to studies of the effect of lipid variation on model

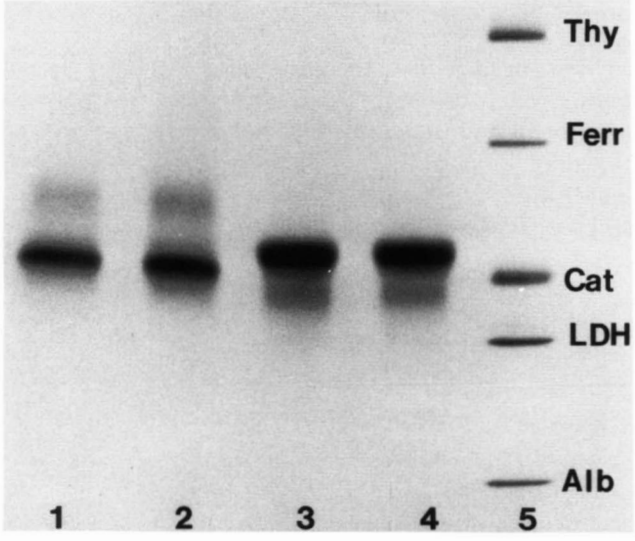

Fig. 3. A comparison of the extent of formation and size of DPPC–A-I complexes prepared in duplicate from the same DPPC–cholate–A-I codispersion by the removal of cholate using the Bio-Beads method (120 min) or detergent dialysis (48 hr). Pore limit gradient gel electrophoresis of aliquots of duplicate Bio-Beads eluates (lanes 1 and 2) and dialysis products (lanes 3 and 4); protein standards: thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine serum albumin (lane 5).

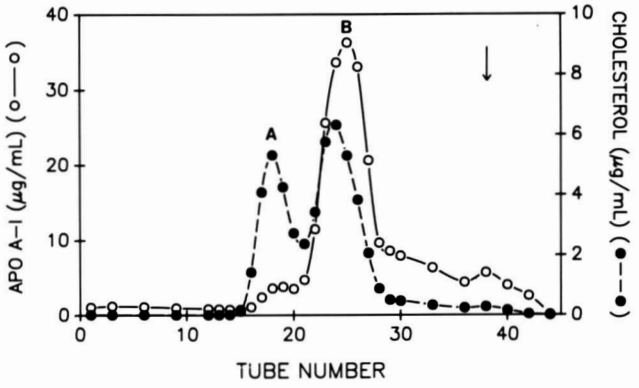

Fig. 4. Effect of cholesterol on the density of protein–lipid complexes and on the extent of complex formation. The KBr density gradient ultracentrifugation profile of the Bio-Beads SM-2 micro-column eluate obtained from a DPPC–cholate–cholesterol–apoA-I codispersion with a molar ratio of 140:190:14:1, respectively. The arrow indicates where lipid-free apoA-I eluted from a control density gradient.

HDL formation, a study which involved the addition of cholesterol to yield a codispersion with a DPPC–cholesterol molar ratio of 10 was performed. Complexes were prepared by the cholate–Bio-Beads method from an incubation of a DPPC–cholate–cholesterol–apoA-I codispersion with a molar ratio of 140:190:14:1, respectively. The pore limit gradient gel electrophoretic pattern of the Bio-Beads micro-column eluate showed two bands with Stokes' diameters of 11.3 nm and 12.4 nm. **Fig. 4** shows the KBr density gradient isolation of these products. Peak A (d 1.06 g/ml) and peak B (d 1.09 g/ml) correspond to DPPC–cholesterol–apoA-I complexes with a molar ratio of 107:220:2 and 140:28:2, respectively.

In this work a cholate–Bio-Beads procedure has been developed for the preparation of protein–lipid complexes within less than 4 hr, compared with 2–4 days required by previous methods. The method has been designed to accommodate the handling of multiple samples of minute volume with relatively high recoveries of protein and phospholipid and negligible dilution of sample. This method also avoids days of dialysis at 37°C as is required in the dialysis procedures.

Plasma HDL is known to originate in intestinal mucosal cells and hepatocytes, but factors influencing the structure of the newly assembled HDL are poorly understood. Because of the simplicity and rapidity of the cholate–Bio-Beads method, we are currently employing this method for investigating the effect of variation in lipid composition on HDL structure.

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