SUMMARY

In a first aspect, there is provided a method for the measurement of at least two lipoprotein components in a blood sample from a subject, wherein said at least two lipoprotein components are selected from total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), said method comprising: combining at least a portion of said sample of blood with a first reagent to provide a first combined solution, wherein said first reagent comprises a lipoprotein solubilization agent, a lipoprotein interactant, and a buffer; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1 and wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measurable colored product at a substantially different rate than for conversion of LDL-cholesterol and VLDL-cholesterol to a measurable colored product; adding to said first combined solution a second reagent to provide a second combined solution, wherein said second reagent comprises a buffer, an amphiphilic agent, a cholesterol esterase, a cholesterol oxidase, and a colorant, wherein said time of addition of said second reagent to said first combined solution is termed the initial time; and measuring absorbance of light by said second combined solution within a first time period, within a second time period, and after a third time, wherein said time periods and said third time may be determined with respect to said initial time, and measuring the lipoprotein amounts of at least two lipoprotein components in the blood sample from said absorbance measurements, whereby at least two lipoprotein components in a blood sample from a subject are measured. In the second aspect, there is provided a method for the combined measurement of high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), very low density cholesterol (VLDL-C), total cholesterol (TC), and triglyceride level (TG) in no more than two samples of blood from a subject, said method comprising: combining at least a portion of a first sample or first portion of a sample of blood from said subject with a first reagent comprising a lipoprotein solubilization agent, a lipoprotein interactant, and a buffer to provide a first combined solution; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1 and wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measurable colored product at a substantially different rate than for conversion of LDL-cholesterol and VLDL-cholesterol to a measurable colored product; adding to said first combined solution a second reagent comprising a buffer, an amphiphilic agent, a cholesterol esterase, a cholesterol oxidase, and a colorant to provide a second combined solution, wherein said time of addition of said second reagent to said first combined solution is termed the initial time; and measuring absorbance of light by said second combined solution within a first time period, within a second time period, and at a third time, wherein said time periods and said third time may be determined with respect to said initial time, wherein HDL-C is measured by the difference in ΔA measured at said initial time and the ΔA measured at the end of said first time period, where ΔA is the difference between absorbance measured at two different wavelengths of light; wherein LDL-C is measured by the difference in ΔA measured at the beginning of said second time period and the ΔA measured at the end of said second time period; wherein TC is measured by ΔA measured at said third time; whereby measurement of HDL-C, LDL-C, and TC is performed on said first sample of blood or said first portion of said sample of blood; measuring TG in a second sample of blood or a second portion of a sample of blood from said subject; and calculating VLDL-C; whereby combined measurement of HDL-C, LDL-C, VLDL-C, TC, and TG in two samples of blood or two portions of a sample of blood is obtained. In the third aspect, there is provided a reagent for use in a cholesterol assay, said reagent comprising a lipoprotein solubilization agent, lipoprotein interactant, and a buffer, wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measureable colored product at a substantially different rate than for conversion of LDL-cholesterol to a measureable colored product and at a substantially different rate than for conversion of VLDL-cholesterol to a measureable colored product; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1. In the fourth aspect, there is provided a reagent for use in an assay for the simultaneous, rapid

measurement of at least two of total cholesterol, LDL-cholesterol and HDL-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, said reagent comprising a lipoprotein solubilization agent, lipoprotein interactant, and a buffer, wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measureable colored product at a substantially different rate than for conversion of LDL-cholesterol to a measureable colored product and at a substantially different rate than for conversion of VLDL-cholesterol to a measureable colored product; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1. In the fifth aspect, there is provided a reagent for use in an assay for the simultaneous, rapid measurement of at least two of total cholesterol, LDL-cholesterol and HDL-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, said reagent comprising α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene and a sodium phosphate buffer, wherein the ratio of dextran sulfate to magnesium ions is between about 0.002 to about 0.02. In the sixth aspect, there is provided a reagent for use in an assay for the measurement of at least two of total cholesterol, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, comprising a sodium phosphate buffer, Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, and cholesterol oxidase from *Pseudomonas sp.* In the seventh aspect, there is provided a kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a reagent as described herein. In the eighth aspect, there is provided a kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene and a sodium phosphate buffer, wherein the ratio of dextran sulfate to magnesium ions is between about 0.002 to about 0.02. In the ninth aspect, there is provided a kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a reagent as described herein. In the tenth aspect, there is provided a kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a sodium phosphate buffer, Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, and cholesterol oxidase from *Pseudomonas sp.*In the eleventh aspect, there is provided a kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises a reagent as described herein, and said second reagent comprises a reagent as described herein. In the twelfth aspect, there is provided a kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises the reagent as described herein, and said second reagent comprises the reagent as described herein. In the thirteenth aspect, there is provided a kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises the reagent as described herein, and said second reagent comprises the reagent as described herein.

[0012] Applicants have found that very accurate and precise values for T-C, LDL-C and HDL-C can be obtained in a single assay for lipoprotein cholesterol using kinetic measurements, without need for precipitation of lipoproteins and without separation steps. Assays as disclosed herein use a lipase (e.g., a cholesterol esterase), an oxidase (e.g., a cholesterol oxidase) and a peroxidase to form a colored product. Assays as disclosed herein may use a lipase (e.g., a cholesterol esterase), a dehydrogenase (e.g., a cholesterol dehydrogenase) and nicotine adenine dinucleotide to form a light-absorbing product (e.g., NADH) or other detectable product. Such products may be measurable by spectrophotometry. In the assays disclosed herein, the amount of product formed from all the cholesterol-containing lipoprotein species is directly proportional to the quantity of cholesterol converted. The assays, methods, reagents, and kits disclosed herein are useful for rapid, efficient, and inexpensive assays of lipoprotein cholesterol and lipoprotein cholesterol sub-fractions, and advantageously provide for measurements of lipoprotein cholesterol and lipoprotein cholesterol sub-fractions in a single assay. Assays disclosed herein

advantageously provide for measurements of lipoprotein cholesterol and lipoprotein cholesterol sub-fractions without need for lipoprotein precipitation. Assays disclosed herein advantageously provide for measurements of lipoprotein cholesterol and lipoprotein cholesterol sub-fractions without the need for centrifugation or for electrophoresis.

[0013] Applicants have found that it is possible to provide assay conditions (e.g., reagents, protocol and temperature) so that the kinetics of reactions converting HDL-C, LDL-C and VLDL-C to colored product proceed at significantly different rates, while still allowing essentially complete reaction of all lipoprotein species (e.g., HDL, LDL, and VLDL) by the end of the assay. For example, in assays disclosed herein the time course for HDL-C has a half-time estimated at less than one minute whereas the LDL-C conversion has a lag phase and an overall sigmoid shape centered on about three minutes. In assays disclosed herein, the remaining lipoprotein cholesterol (chylomicrons and VLDL-C) react even more slowly (half-time of about five minutes or longer). These differences in half-time and reaction kinetics between different cholesterol species enable the de-convolution of an assay signal to that attributable to each species using a simple algorithm even though, for example, signal is being produced from more than one species during certain times during the assay. Accordingly, reagent formulations for use in the assays and methods disclosed herein are

CLAIMS

1. A method for the measurement of at least two lipoprotein components in a blood sample from a subject, wherein said at least two lipoprotein components are selected from total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), said method comprising:

combining at least a portion of said sample of blood with a first reagent to provide a first combined solution, wherein said first reagent comprises a lipoprotein solubilization agent, a lipoprotein interactant, and a buffer; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1 and wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measurable colored product at a substantially different rate than for conversion of LDL-cholesterol and VLDL-cholesterol to a measurable colored product;

adding to said first combined solution a second reagent to provide a second combined solution, wherein said second reagent comprises a buffer, an amphiphilic agent, a cholesterol esterase, a cholesterol oxidase, and a colorant, wherein said time of addition of said second reagent to said first combined solution is termed the initial time; and

measuring absorbance of light by said second combined solution within a first time period, within a second time period, and after a third time, wherein said time periods and said third time may be determined with respect to said initial time, and

measuring the lipoprotein amounts of at least two lipoprotein components in the blood sample from said absorbance measurements,

whereby at least two lipoprotein components in a blood sample from a subject are measured.

2. The method of claim 1 comprising measuring at least two lipoprotein components selected from TC, LDL-C, and HDL-C,

wherein HDL-C is measured by the difference in ΔA measured at said initial time and the ΔA measured at the end of said first time period, where ΔA is the difference between absorbance measured at two different wavelengths of light;

wherein LDL-C is measured by the difference in ΔA measured at the beginning of said second time period and the ΔA measured at the end of said second time period; and

wherein TC is measured by ΔA measured at said third time;

whereby two or more of HDL-C, LDL-C, and TC are measured in a sample of blood.

3. The method of claim 2, comprising the combined measurement of all of the lipoproteins high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) in the same sample of blood, or the same portion of a sample of blood.

4. The method of claim 2, wherein said lipoproteins are measured in a sample of blood without substantial precipitation of the lipoproteins.

5. The method of claim 2, where ΔA is the difference between absorbance measured at 560 nm and at 700 nm.

6. The method of claim 2, wherein said first time period comprises a time period of less than about 3 minutes after said initial time, and said third time is a time greater than about 5 minutes after said initial time.

7. The method of claim 2, wherein said first time period comprises a time period of between about 0 minutes to about 2 minutes after said initial time, said second time period comprises a time period of between about 2 minutes to about 6 minutes after said initial time, and said third time is a time between about 6 minutes to about 10 minutes after said initial time.

8. The method of claim 2, wherein said amphiphilic agent comprises a surfactant selected from the group consisting of nonionic surfactants; anionic surfactants; cationic surfactants; zwitterionic surfactants; and derivatives, analogues, and combinations thereof.

9. The method of claim 2, wherein said colorant comprises one or more of a peroxidase, a peroxidase substrate, an aminoantipyrene compound, and an aniline-containing compound.

10. The method of claim 2, wherein said first reagent comprises α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene, and a sodium phosphate buffer, and said second reagent comprises Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, cholesterol oxidase from *Pseudomonas sp.*, and a sodium phosphate buffer.

11. A method for the combined measurement of high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), very low density cholesterol (VLDL-C), total cholesterol (TC), and triglyceride level (TG) in no more than two samples of blood from a subject, said method comprising:

combining at least a portion of a first sample or first portion of a sample of blood from said subject with a first reagent comprising a lipoprotein solubilization agent, a lipoprotein interactant, and a buffer to provide a first combined solution; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1 and wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measurable colored product at a substantially different rate than for conversion of LDL-cholesterol and VLDL-cholesterol to a measurable colored product;

adding to said first combined solution a second reagent comprising a buffer, an amphiphilic agent, a cholesterol esterase, a cholesterol oxidase, and a colorant to provide a second combined solution, wherein said time of addition of said second reagent to said first combined solution is termed the initial time; and

measuring absorbance of light by said second combined solution within a first time period, within a second time period, and at a third time, wherein said time periods and said third time may be determined with respect to said initial time,

wherein HDL-C is measured by the difference in ΔA measured at said initial time and the ΔA measured at the end of said first time period, where ΔA is the difference between absorbance measured at two different wavelengths of light;

wherein LDL-C is measured by the difference in ΔA measured at the beginning of said second time period and the ΔA measured at the end of said second time period;

wherein TC is measured by ΔA measured at said third time;

whereby measurement of HDL-C, LDL-C, and TC is performed on said first sample of blood or said first portion of said sample of blood;

measuring TG in a second sample of blood or a second portion of a sample of blood from said subject; and

calculating VLDL-C;

whereby combined measurement of HDL-C, LDL-C, VLDL-C, TC, and TG in two samples of blood or two portions of a sample of blood is obtained.

12. The method of claim 11, wherein said first time period comprises a time period of less than about 3 minutes after said initial time, and said third time is a time greater than about 5 minutes after said initial time.

13. The method of claim 11, wherein said first time period comprises a time period of between about 0 minutes to about 2 minutes after said initial time, said second time period comprises a time period of between about 2 minutes to about 6 minutes after said initial time, and said third time is a time between about 6 minutes to about 10 minutes after said initial time.

14. The method of claim 11, where ΔA is the difference between absorbance measured at 560 nm and at 700 nm.

15. The method of claim 11, wherein combined measurement of HDL-C, LDL-C, VLDL-C, TC, and TG in two samples of blood or two portions of a sample of blood is obtained without substantial precipitation of said lipoproteins..

16. The method of claim 11, wherein calculating VLDL-C comprises calculating VLDL-C by the formula:

$$\text{VLDL-C} = \alpha\text{TC} + \beta\text{HDL-C} + \delta\text{TG} + a_1(\text{TG}+\varepsilon)(\text{TC}+\kappa) + \lambda$$

where λ has a value of between about 10 and about -10; α has a value of between about 0.05 and about 0.5; β has a value of between about 0 and about -5; δ has a value of between about 0.05 and about 0.5; a_1 has a value of between about 0.0005 and about 0.005; ε and κ have a value of between about -50 and about -500.

17. The method of claim 16, where λ has a value of between about 0 and about -5; α has a value of between about 0.1 and about 0.3; β has a value of between about 0 and about -2; δ has a value of between about 0.1 and about 0.3; a_1 has a value of between about 0.001 and about 0.003; ε and κ each have a value of between about -100 and about -300.

18. The method of claim 16 wherein very low density cholesterol (VLDL-C) is calculated by the formula:

$$\text{VLDL-C} = \alpha\text{TC} + \beta\text{HDL-C} + \gamma\text{LDL-C} + \delta\text{TG} + \lambda + a_1(\text{TG}+\varepsilon)(\text{TC}+\kappa) + a_2(\text{TG}+\varepsilon)(\text{HDL-C}+\mu) + a_3(\text{TG}+\varepsilon)(\text{LDL-C}+\nu) + a_4(\text{TC}+\kappa)(\text{HDL-C}+\mu) + a_5(\text{TC}+\kappa)(\text{LDL-C}+\nu) + a_6(\text{LDL-C}+\nu)(\text{HDL-C}+\mu)$$

wherein the multiplicative coefficients α, β, γ, δ, a_1, a_2, a_3, a_4, a_5, and a_6, may take any value, whether positive, negative, or zero; and

wherein the additive constants λ, ε, κ, μ, and ν may take any value, whether positive, negative, or zero.

19. The method of claim 16, wherein λ has a value of between about 10 and about -10; α has a value of between about 0.05 and about 0.5; β has a value of between about 0 and

about -5; δ has a value of between about 0.05 and about 0.5; a_1, a_2, a_3, a_4, a_5, and a_6 each have a value of between about 0.0005 and about 0.005; and ε, κ, μ, and ν each have a value of between about -50 and about -500.

20. The method of claim 11, wherein said first reagent comprises α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene, and a sodium phosphate buffer, and said second reagent comprises Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, cholesterol oxidase from *Pseudomonas sp.*, and a sodium phosphate buffer.

21. A reagent for use in a cholesterol assay, said reagent comprising a lipoprotein solubilization agent, lipoprotein interactant, and a buffer, wherein said lipoprotein interactant is present at a concentration level that provides for conversion of HDL-cholesterol to a measureable colored product at a substantially different rate than for conversion of LDL-cholesterol to a measureable colored product and at a substantially different rate than for conversion of VLDL-cholesterol to a measureable colored product; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1.

22. The reagent of claim 21, comprising α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene and a sodium phosphate buffer.

23. The reagent of claim 21, said reagent comprising magnesium and dextran sulfate in a ratio of dextran sulfate to magnesium ions effective that there is no substantial precipitation of low-density lipoprotein (LDL), or that there is no substantial precipitation of very low-density lipoprotein (VLDL), or both.

24. The reagent of claim 23, wherein said ratio of dextran sulfate to magnesium ions is between about 0.002 to about 0.02.

25. The reagent of claim 21, wherein said lipoprotein solubilization agent is selected from a surfactant, and a negatively charged α-cyclodextrin derivative; and said lipoprotein interactant comprises a low molecular weight negatively charged dextran derivative.

26. A reagent for use in an assay for the simultaneous, rapid measurement of at least two of total cholesterol, LDL-cholesterol and HDL-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, said reagent comprising a lipoprotein solubilization agent, lipoprotein interactant, and a buffer, wherein said lipoprotein interactant is present at a concentration level that provides for conversion of

HDL-cholesterol to a measureable colored product at a substantially different rate than for conversion of LDL-cholesterol to a measureable colored product and at a substantially different rate than for conversion of VLDL-cholesterol to a measureable colored product; wherein the lipoprotein interactant comprises a negatively charged polysaccharide and a divalent cation, or a salt thereof, in a ratio of between 0.001 to 0.1.

27. A reagent for use in an assay for the simultaneous, rapid measurement of at least two of total cholesterol, LDL-cholesterol and HDL-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, said reagent comprising α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene and a sodium phosphate buffer, wherein the ratio of dextran sulfate to magnesium ions is between about 0.002 to about 0.02.

28. A reagent for use in an assay for the measurement of at least two of total cholesterol, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol in lipoproteins in a sample of blood from a subject without substantial precipitation of said lipoproteins, said reagent comprising a sodium phosphate buffer, Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, and cholesterol oxidase from *Pseudomonas sp.*

29. A kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a reagent of any of claims 21 to 27.

30. A kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising α-cyclodextrin sulfate, dextran sulfate, magnesium chloride, 4-aminoantipyrene and a sodium phosphate buffer, wherein the ratio of dextran sulfate to magnesium ions is between about 0.002 to about 0.02.

31. A kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a reagent of claim 28.

32. A kit comprising a reagent and instructions for the use of said reagent in a cholesterol assay, the reagent comprising a sodium phosphate buffer, Triton X-100, pluronic L64, N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (ALPS), cholesterol esterase from *Pseudomonas sp.*, and cholesterol oxidase from *Pseudomonas sp.*

33. A kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises a reagent of any of claims 21 to 27, and said second reagent comprises a reagent of claim 28.

34. A kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises the reagent of claim 25, and said second reagent comprises the reagent of claim 28.

35. A kit comprising at least a first reagent and a second reagent, and instructions for the use of said reagents in a cholesterol assay, wherein said first reagent comprises the reagent of claim 26 or claim 27, and said second reagent comprises either a) a buffer, an amphiphilic agent, a lipase, an oxidase, and a colorant or b) a buffer, an amphiphilic agent, a cholesterol esterase, a cholesterol oxidase, and a peroxidase.