

FUNCTIONAL LIPID CONSTRUCTS

TECHNICAL FIELD

The invention relates to methods for effecting qualitative and quantitative changes in the functional moieties expressed at the surface of cells and multi-cellular structures, and functional lipid constructs for use in such methods.

In particular, the invention relates to functional lipid constructs and their use in diagnostic and therapeutic applications, including serodiagnosis, where the functional moiety is a carbohydrate, peptide, chemically reactive group, conjugator or fluorophore.

BACKGROUND ART

The ability to effect qualitative and quantitative changes in the functional moieties expressed at the surface of liposomes, cells and multi-cellular structures provides for a range of diagnostic and therapeutic applications. A functional moiety may be a carbohydrate, peptide, chemically reactive group (e.g. maleimide), conjugator (e.g. biotin) or fluorophore (e.g. fluorescein).

The specification accompanying international application number PCT/NZ2005/000052 (publication number WO 2005/090368) describes the preparation of carbohydrate-lipid constructs for use in methods of effecting qualitative and quantitative changes in the level of carbohydrates expressed at the surface of cells and multi-cellular structures. The use of the constructs to prepare quality control cells for use in blood grouping and diagnostics is described.

The specification accompanying international application number PCT/NZ2006/000245 (publication number WO 2007/035116) describes another method for the preparation of carbohydrate-lipid constructs where the carbohydrate is the polymer hyaluronic acid. The use of the constructs to modify embryos to promote association with endometrial cells is described.

The specification accompanying international application number PCT/NZ2007/000256 (publication number WO 2008/030115) describes the preparation of fluorophore-lipid constructs. The use of the constructs in methods of fluorescently labeling cells is described.

Known methods of effecting changes in the peptides expressed at the surface of cells include gene manipulation, chemical modification of membrane peptides, and "cell surface painting" using lipid anchors such as GPI (Legler *et al.* (2004), McHugh *et al.* (1995), Medof *et al.* (1996), Metzner *et al.* (2008), Morandat *et al.* (2002), Premkumar *et al.* (2001), Ronzon *et al.* (2004), Skountzou *et al.* (2007)).

In addition to these methods of effecting changes of endogenously expressed peptides, exogenously prepared peptides may be coupled to lipids of the membrane utilising biotin-avidin conjugation. Biotin binds to the tetrameric protein avidin with a dissociation constant (K_D) of the order 10^{-15} mol/L. This strong binding is exploited in a number of laboratory applications.

In these laboratory applications biotin is linked to a molecule such as a carbohydrate or a peptide. The preferential binding of avidins to biotin is exploited in a

number of isolation or separation applications in addition to the coupling of peptides to the lipids of membranes.

The specification accompanying international application no. PCT/NZ02/00214 (publication no. WO 03/039074) describes a "two-step method" of localizing an antigen such as a peptide to the surface of cells. In the method the biotinylated glycoside (BioG) is contacted with a suspension of cells for a time and at a temperature sufficient to allow the BioG molecules to incorporate via their diacyl lipid tails into the cell membrane of the cells.

An exogenously prepared avidinylated peptide may then be localized to the surface of the BioG modified cells by contacting the peptide with the modified cells.

Alternatively, an exogenously prepared biotinylated peptide may be localized to the surface of the modified cells via a biotin-avidin bridge.

In either alternative of the "two-step method" the amount of peptide localized to the surface of the cells may be controlled by controlling the concentration, time and temperature at which the BioG molecules are contacted with the suspension of cells to provide the modified cells. However, the utility of the method is limited by the availability and dispersibility of BioG in biocompatible media such as a saline.

The specification accompanying international application no. PCT/NZ2005/000052 (publication no. WO 2005/090368) describes a "one-step method" of localizing carbohydrate antigen to the surface of cells. The "one-step method" utilizes carbohydrate-lipid constructs that are dispersible in biocompatible media and can therefore be used to prepare

modified cells without loss of vitality. However, a method of preparing peptide-lipid constructs with comparable dispersibility in biocompatible media and of general applicability to peptides has not been described.

Relatively little work has been performed on the coupling of peptides to phospholipids as individual components prior to their incorporation in self assembling lipid structures, such as liposomes. However, a variety of standard techniques have been described for the covalent coupling of peptides to liposome surfaces.

Martin *et al* (1990) has reviewed methods of attaching moieties including peptides, to the surface of liposomes.

Blume *et al* (1993) describes the coupling of the water soluble Glu-plasminogen to liposomes by the method described by Kung and Redemann (1986). The chemical ECDI (1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride) is used to activate the liposomes prior to incubation of the activated liposome suspension with Glu-plasminogen. Proteo-PEG-coated liposomes with Glu-plasminogen covalently attached to the ends of the distearylphosphatidylethanolamine (DSPE)-PEG-COOH are provided.

Haselgrübler *et al* (1995) describes a heterobifunctional crosslinker used to facilitate the preparation of immunoliposomes. The crosslinker is synthesised from a diamine derivative of poly(ethylene glycol) (PEG, average molecular weight 800 dalton (18mer)). The crosslinker has 2-(pyridylthio)propionyl (PDP) and N-hydroxysuccinimide ester (NHS) as functional groups.

Ishida *et al* (2001) describes the preparation of liposomes bearing polyethylene glycol-coupled transferrin. Transferrin was conjugated via the terminal carboxyl residue of DSPE-PEG-COOH. The liposomes were proposed as having utility in *in vivo* cytoplasmic targeting of chemotherapeutic agents or plasmid DNAs to target cells.

Massaguer *et al* (2001) describes the incorporation of a peptide sequence (GGRGRS) and hydrophobic derivatives to the surface of chemically activated liposomes. The incorporation was carried out through the carboxyl group of N-glutaryl dipalmitoyl phosphatidyl choline (NGPE).

Massaguer *et al* (2001) noted that considering potential *in vivo* applications, where sterility and simplicity would be some of the most important requirements, processes based on chemical reactions on the surface of liposomes involving extra steps would be more difficult to be scaled up at the industrial level. A hydrophobic derivative of the peptide sequence was identified as providing optimal properties for incorporation to the surface of liposomes.

Chung *et al* (2004) describe the antigenic determinant shielding effect of DOPE-PEG incorporated into the membranes of cells and speculated concerning the potential of lipid-PEG(n)(s) to regulate biological cell responses and the extension of this concept to the introduction of functional molecules at the end of the PEG chain.

Kato *et al* (2004) describe a method for anchoring of macromolecular proteins into the membranes of living mammalian cells. A dioleoylphosphatidylethanolamine (DOPE) derivative coupled with hydrophilic poly(ethylene glycol) (PEG80) was

used as the synthetic membrane anchor. Peptides were conjugated at the distal terminal of the PEG moiety via an amino-reactive N-hydroxysuccinimide derivative of the synthetic membrane anchor.

The PEG80 moiety facilitated solubilisation of the synthetic membrane anchor in water. As noted by Kato *et al* (2004) if the anchor is insoluble in water, undesirable and complicated processes such as liposome preparation and the fusion of liposomes with the cell membrane may be required to anchor the conjugates into the cell membrane.

An additional advantage noted by Kato *et al* (2004) was that synthetic membrane anchors with high hydrophile-lipophile balance values (attributable to PEG spacer with a high number of oxyethylene units) were concluded to have no cytolytic activity. However, difficulties arise in the use of synthetic membrane anchors including a PEG spacer with a high number of oxyethylene units.

Firstly, the expression of the conjugative peptide or other endogenous cell surface peptides may be masked by the PEG spacer. Secondly, a PEG spacer with a high number of oxyethylene units may elicit non-specific adherence of protein (including antibodies in certain individuals) and/or the non-specific activation of the complement cascade.

Winger *et al* (1996) describes the conjugation of bromoacetylated DSPE with a thiol terminated decapeptide comprising at its C-terminus the minimal human thrombin-receptor peptide agonist (HS---SerPheLeuLeuArgAsn).

Hashimoto et al (1986) describes the conjugation of iodoacetylated DSPE with thiolated compounds.

A need exists for a general method of preparing peptide-lipid constructs that may be incorporated as individual components in self assembling lipid structures, such as liposomes, by a "one-step method". The method should desirably provide peptide-lipid constructs that are readily dispersible in biocompatible media and spontaneously incorporate in to the membranes of cells and multi-cellular structures.

Peptide-lipid constructs with these characteristics are anticipated to have utility in a range of therapeutic and diagnostic applications, especially serodiagnosis, in addition to the preparation of functionalized liposomes.

It is an object of this invention to provide functional-lipid constructs that are dispersible in biocompatible media and spontaneously incorporate into the membranes of cells and multi-cellular structures.

It is an object of this invention to provide functional-lipid constructs for use in the preparation of peptide-lipid constructs that are dispersible in biocompatible media and spontaneously incorporate into the membranes of cells and multi-cellular structures.

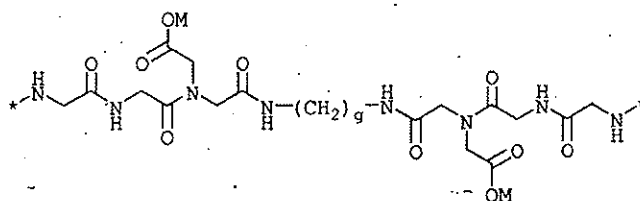
It is an object of this invention to provide peptide-lipid constructs that are dispersible in biocompatible media and spontaneously incorporate into the membranes of cells and multi-cellular structures.

These objects are to be read disjunctively with the object to

at least provide the public with a useful choice.

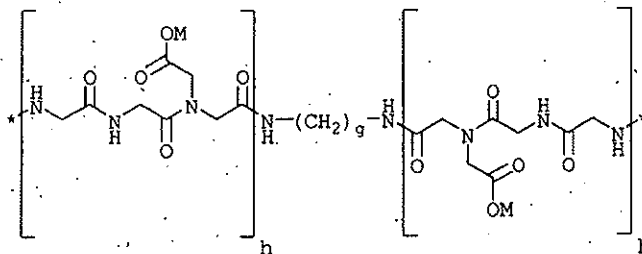
DISCLOSURE OF INVENTION

In a **first** aspect the invention provides a functional lipid construct of the structure F-S-L where F is a functional moiety, L is a diacyl or a dialkyl lipid,^o and S is a spacer covalently linking F to L including the substructure:



where g is the integer 1, 2 or 3, M is a monovalent cation or substituent, and * is other than H.

Preferably, the substructure is:



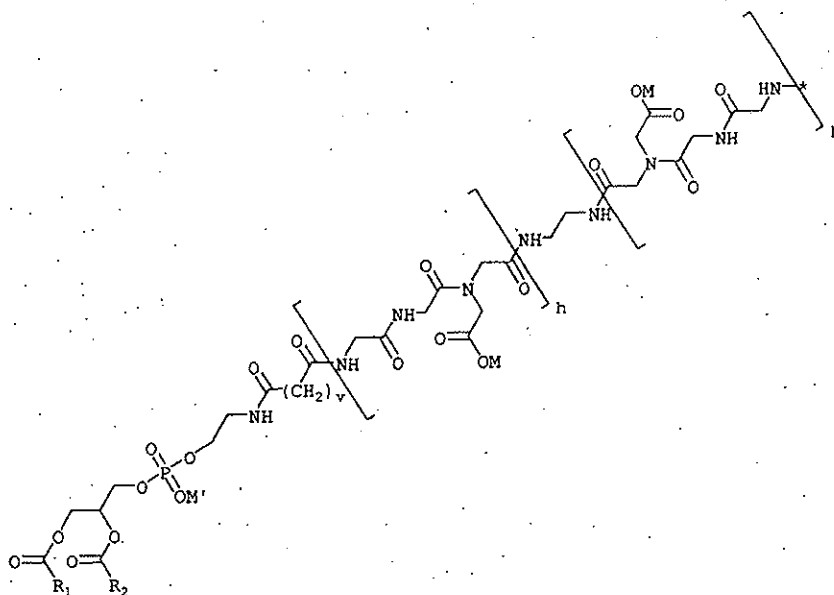
where h is the integer 1, 2, 3 or 4.

Preferably, g is the integer 2 and h is the integer 1, 2 or 4.

Preferably, M is H or CH₃.

Preferably, L is a diacylglycerophospholipid. More preferably, L is phosphatidylethanolamine.

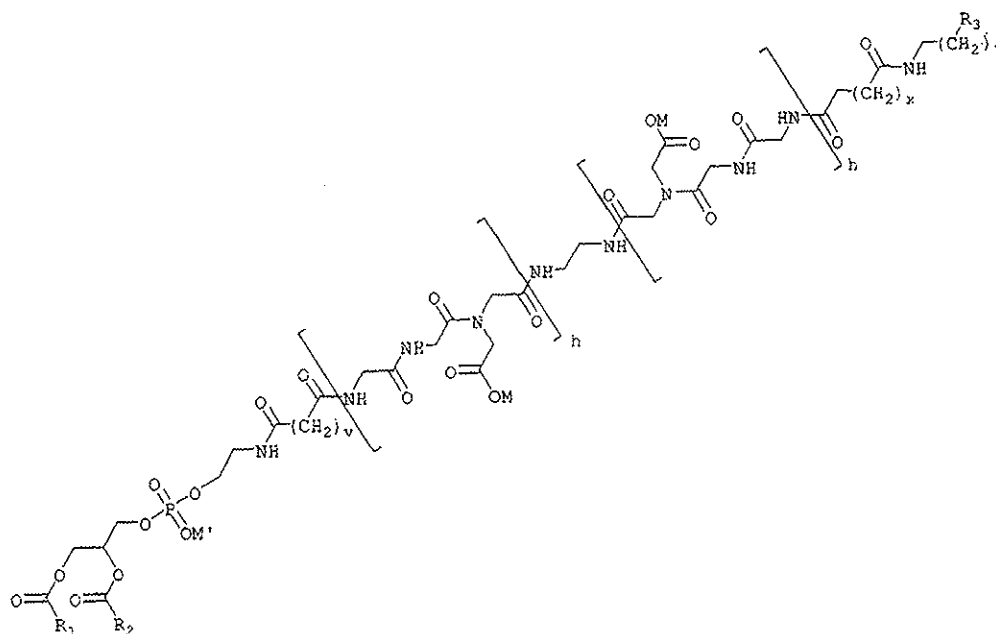
Preferably, the structure of the functional lipid construct includes the partial structure:



where v is the integer 3, 4 or 5, M' is a monovalent cation, and R_1 and R_2 are independently selected from the group consisting of: alkyl or alkenyl substituents of the fatty acids *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid.

Preferably, F is a functional moiety selected from the group consisting of: carbohydrate, peptide, chemically reactive group, conjugator or fluorophore.

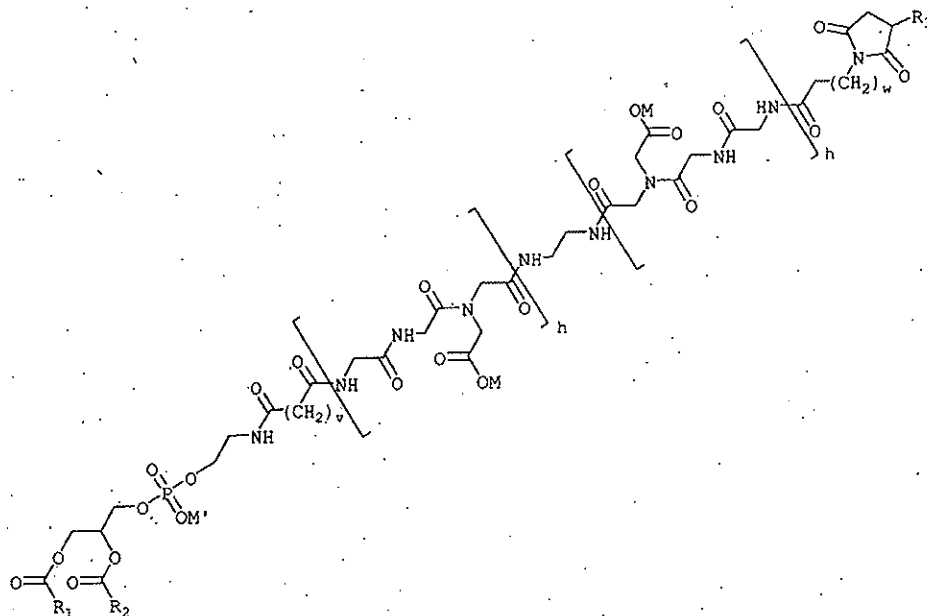
In a **first** alternative of the **first** aspect the invention provides a functional lipid construct of the structure:



where F is a carbohydrate, x is the integer 2, 3 or 4, y is the integer 1, 2 or 3, and R₃ is O of a substituted hydroxyl of the carbohydrate.

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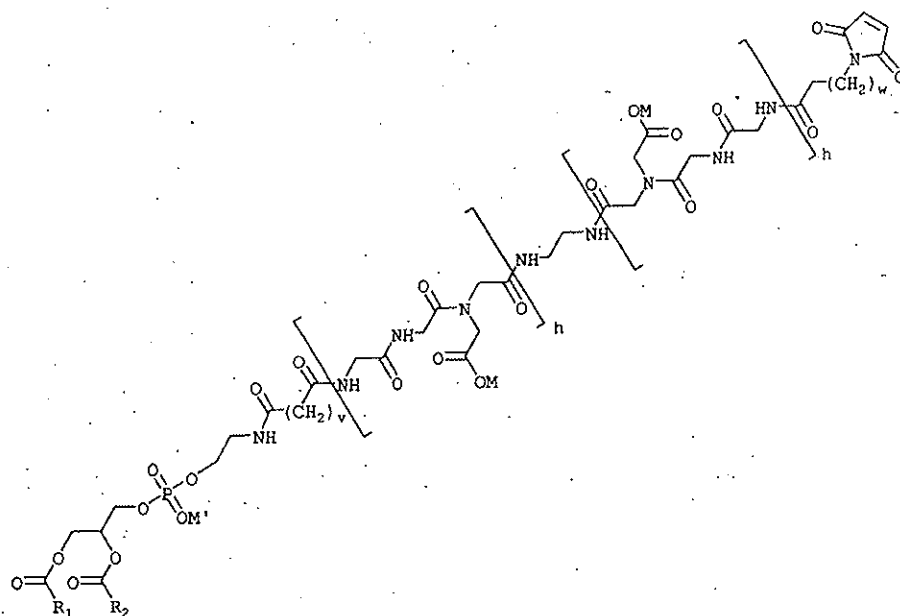
In a **second** alternative of the **first** aspect the invention provides a functional lipid construct of the structure:



where F is a peptide, w is the integer 1 or 2, and R₃ is S of a substituted sulfhydryl of a Cys residue of the peptide.

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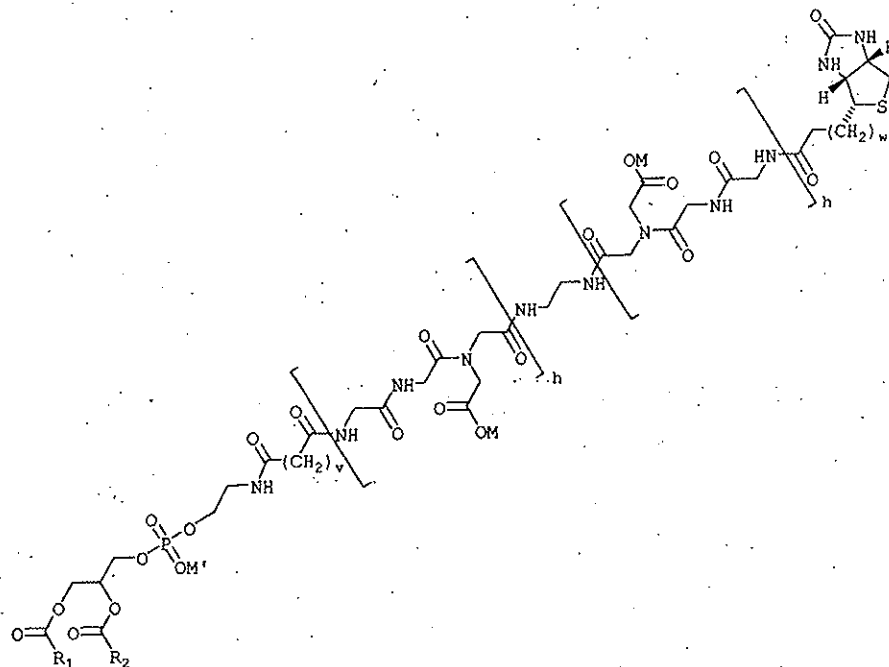
In a third alternative of the first aspect the invention provides a functional lipid construct of the structure:



where F is the chemically reactive group maleimide and w is the integer 1 or 2.

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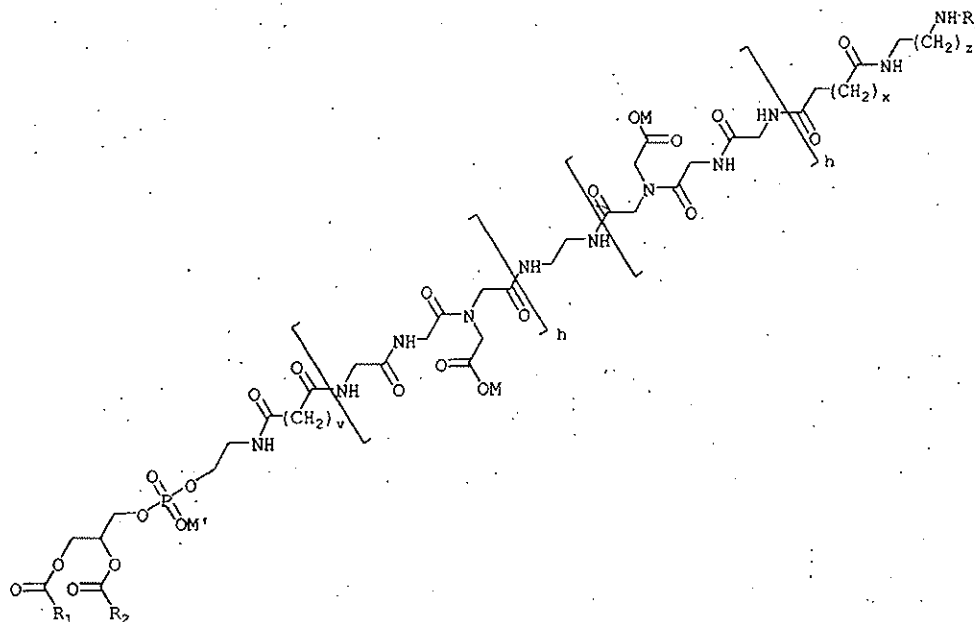
In a fourth alternative of the first aspect the invention provides a functional lipid construct of the structure:



where F is the conjugator biotin and k is the integer 2, 3 or 4.

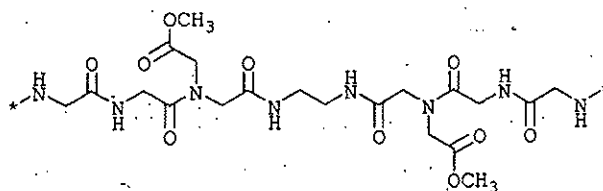
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In a fifth alternative of the first aspect the invention provides a functional lipid construct of the structure:

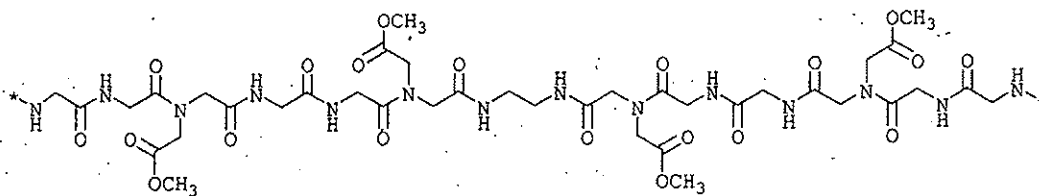


where F is the fluorophore of fluorescein (or one of its derivatives), z is the integer 3, 4 or 5, and R₃ is C of the thiocyanate substituent of the isothiocyanate derivative of fluorescein (or one of its derivatives).

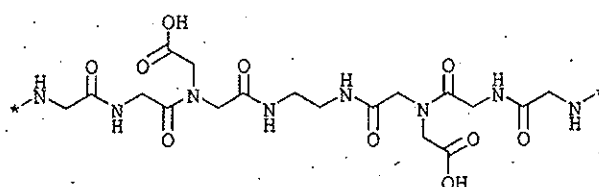
Preferably, the substructure is:



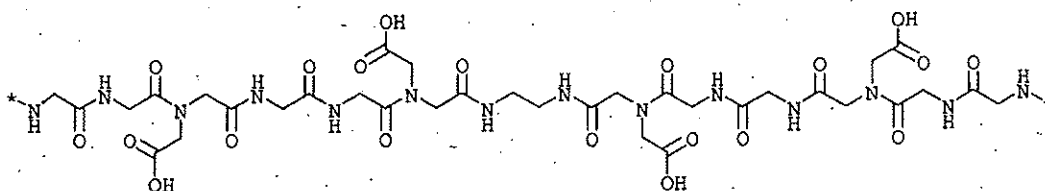
designated MCMG(1);



designated MCMG(2);

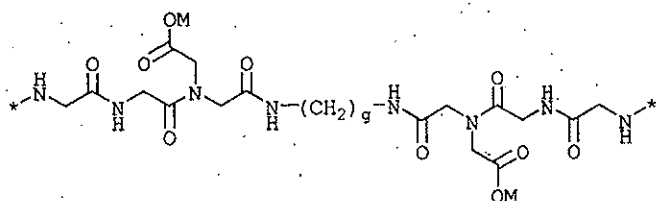


designated CMG(1); or



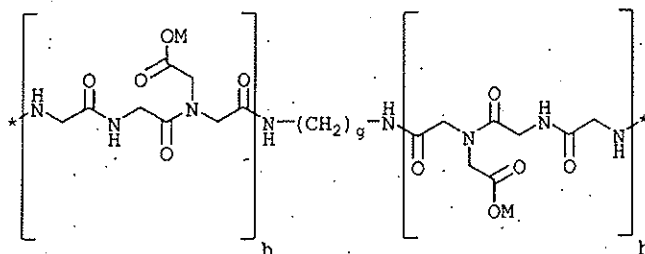
designated CMG(2).

In a second aspect the invention provides a water soluble peptide-lipid construct of the structure F-S-L where S is a spacer linked to F via a sulphide bond and includes the substructure:



where g is the integer 1, 2 or 3, M is a monovalent cation or substituent, and * is other than H.

Preferably, the substructure is:



where h is the integer 1, 2, 3 or 4.

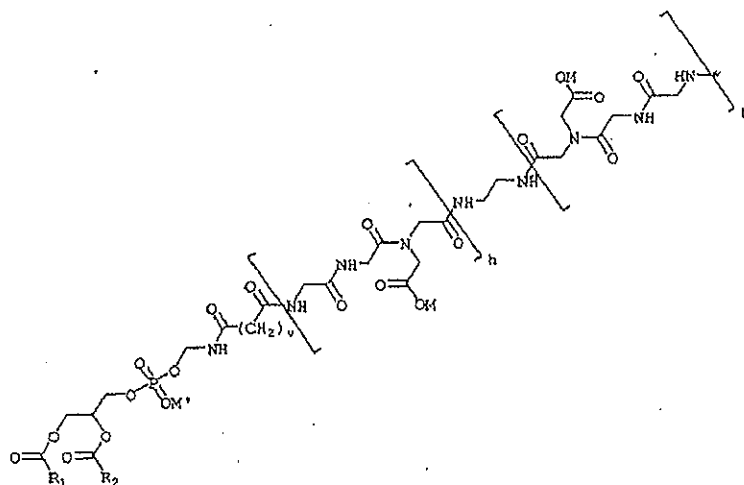
Preferably, g and h are the integer 2.

Preferably, M is H or CH₃.

Preferably, L is a diacylglycerophospholipid. More preferably, L is phosphatidylethanolamine.

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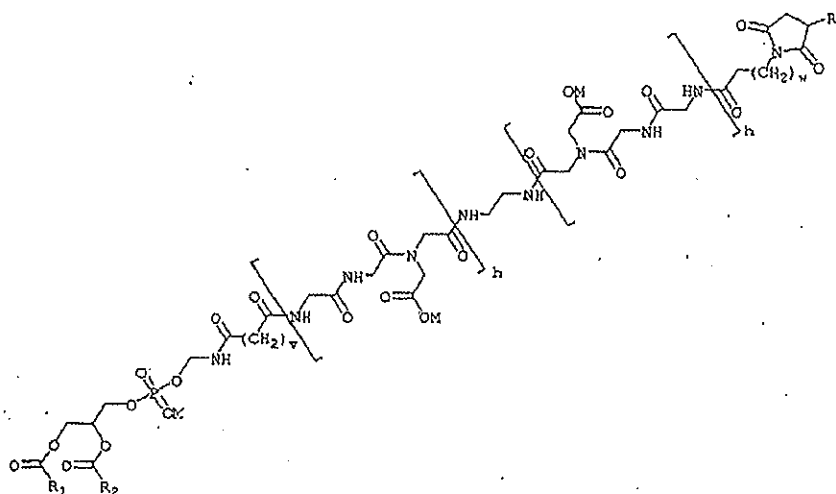
Préferably, the structure of the peptide-lipid construct includes the partial structure:



- 5 where v is the integer 3, 4 or 5, M' is a monovalent cation, and R_1 and R_2 are independently selected from the group consisting of: alkyl or alkenyl substituents of the fatty acids *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid.

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Preferably, the structure of the peptide-lipid construct includes the partial structure:



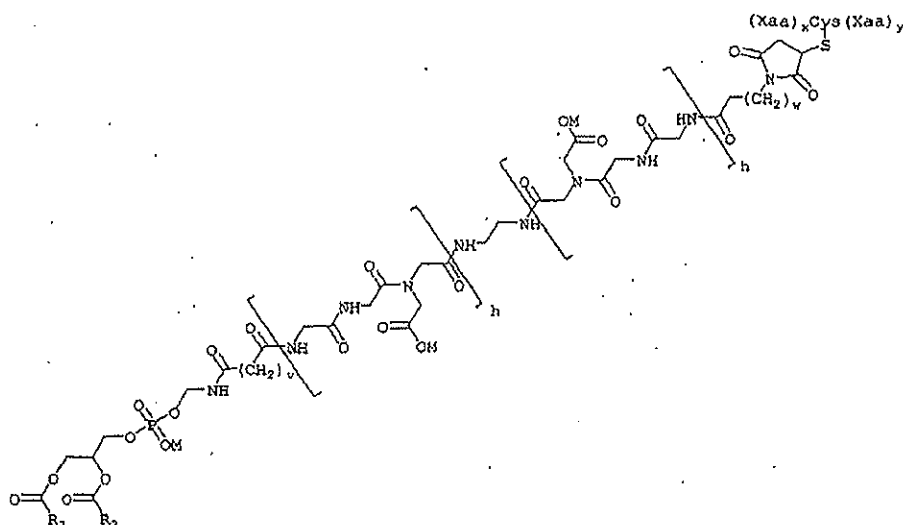
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where w is the integer 1 or 2, and R₃ is S of a substituted
sulfhydryl of a Cys residue of the peptide.

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In an embodiment of the second aspect the invention provides a peptide-lipid construct of the structure:



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where the sum of x and y is greater than 5.

Optionally, F is a peptide including a proximal terminal sequence (PTS) selected to promote solubility of the peptide.

- 10 In a preferment of this option, the PTS of the peptide is selected from the group consisting of:

SerLysLysLysLysGly

AlaAlaAlaAla

GlySerGlySerGly

- 15 Preferably, the Cys residue is a terminal Cys residue of the peptide (Cys).

Preferably, the terminal sequence of the peptide is selected from the group consisting of:

GlyLysLysLysLysSerCys

AlaAlaAlaAlaCys

GlySerGlySerGlyCys

CysSerLysLysLysLysGly

CysAlaAlaAlaAla

CysGlySerGlySerGly

Preferably, the Cys residue is a terminal Cys residue of the peptide at the carboxy-terminus of the peptide.

Preferably, F is a peptide comprising an epitope of antigens selected from the group consisting of: Glycophorin A, Glycophorin B, or mutations thereof (including the MNS blood group system). More preferably, F is a peptide selected from the *List of Peptides*. Most preferably, F is a peptide selected from the group consisting of:

GlnThrAsnAspLysHisLysArgAspThrTyrAlaAlaAlaAlaCys

GlnThrAsnAspLysHisLysArgAspThrTyrGlySerGlySerGlyCys

GlnThrAsnAspMetHisLysArgAspThrTyrGlySerGlySerGlyCys

SerSerGlnThrAsnAspLysHisLysArgAspThrTyrCys

ThrTyrProAlaHisThrAlaAsnGluValCys

ThrTyrProAlaHisThrAlaAsnGluCys

ProAlaHisThrAlaAsnGluValCys

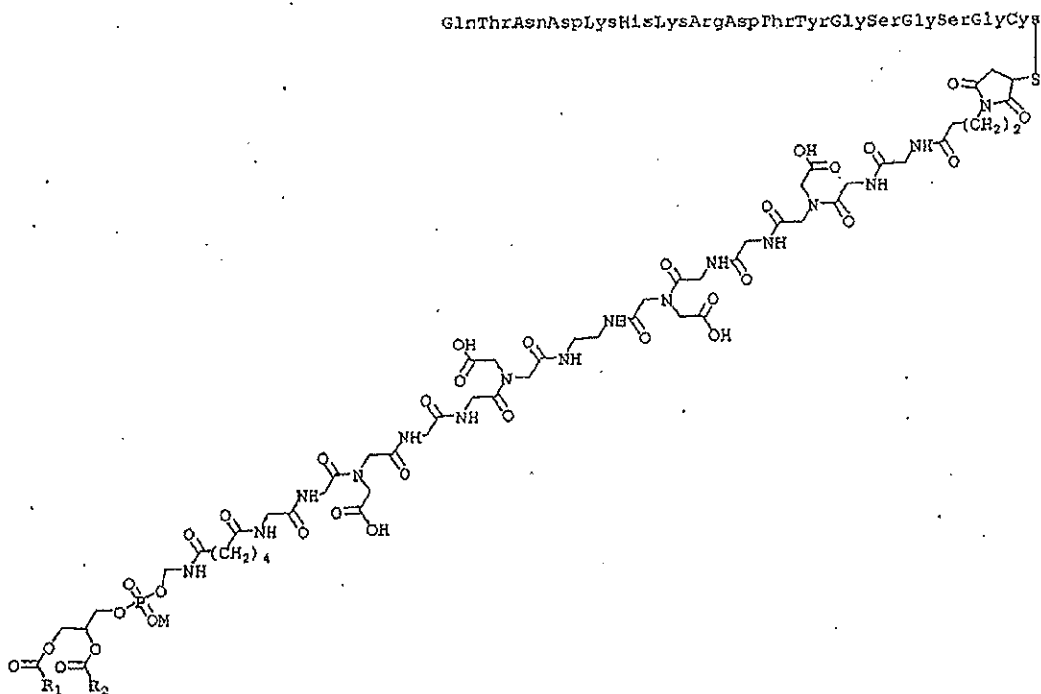
SerGlnThrAsnAspLysHisLysArgAspCys

CysThrTyrProAlaHisThrAlaAsnGlu

Preferably, L is a glycerophospholipid selected from the group consisting of: 1,2-O-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-O-distearyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE).

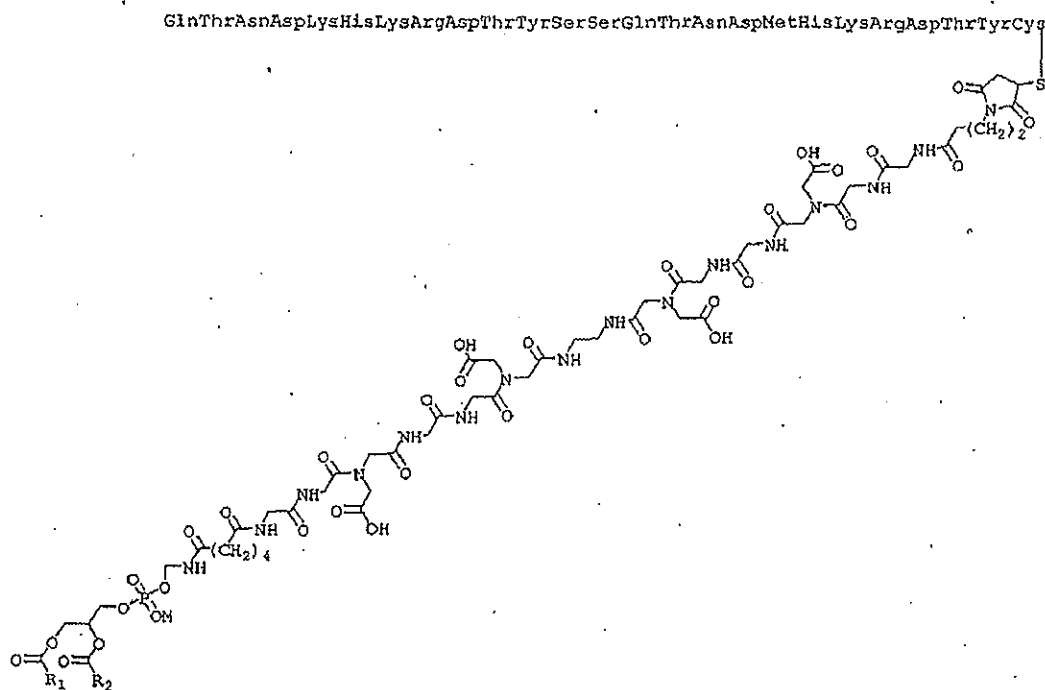
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In an exemplifying first embodiment of the second aspect the invention provides a peptide-lipid construct of the structure:



designated DOPE-Ad-CMG(2)- β Ala-Mal-PTS-Milt(K) (IX).

In an exemplifying **second** embodiment of the second aspect the invention provides a peptide-lipid construct of the structure:

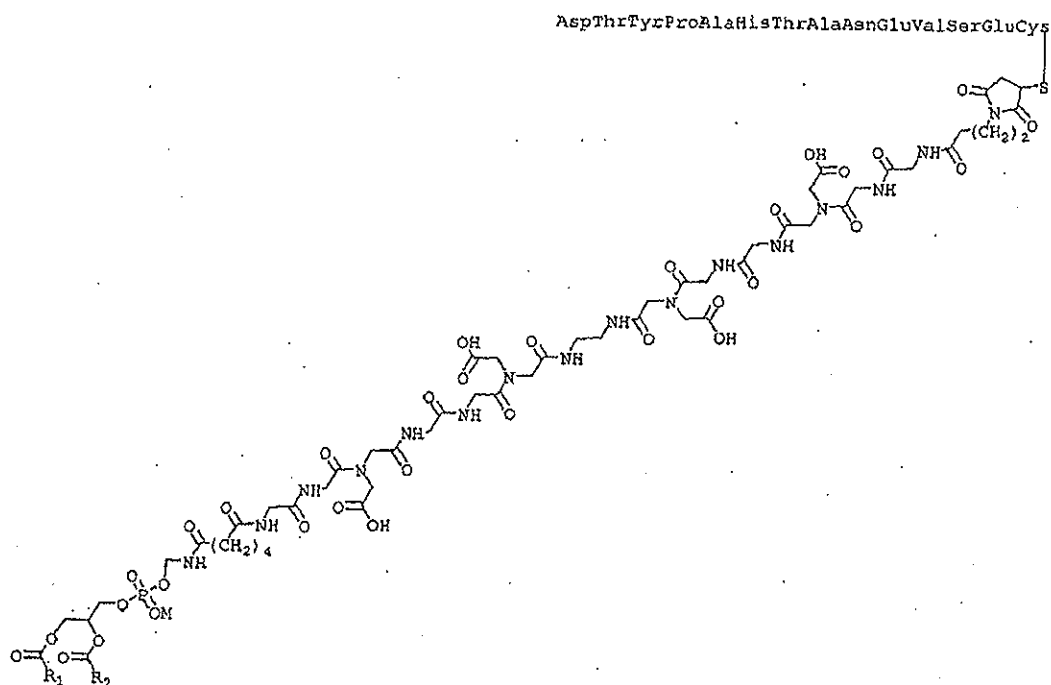


designated DOPE-Ad-CMG(2)- β Ala-Mal-Milt(K,M) (X).

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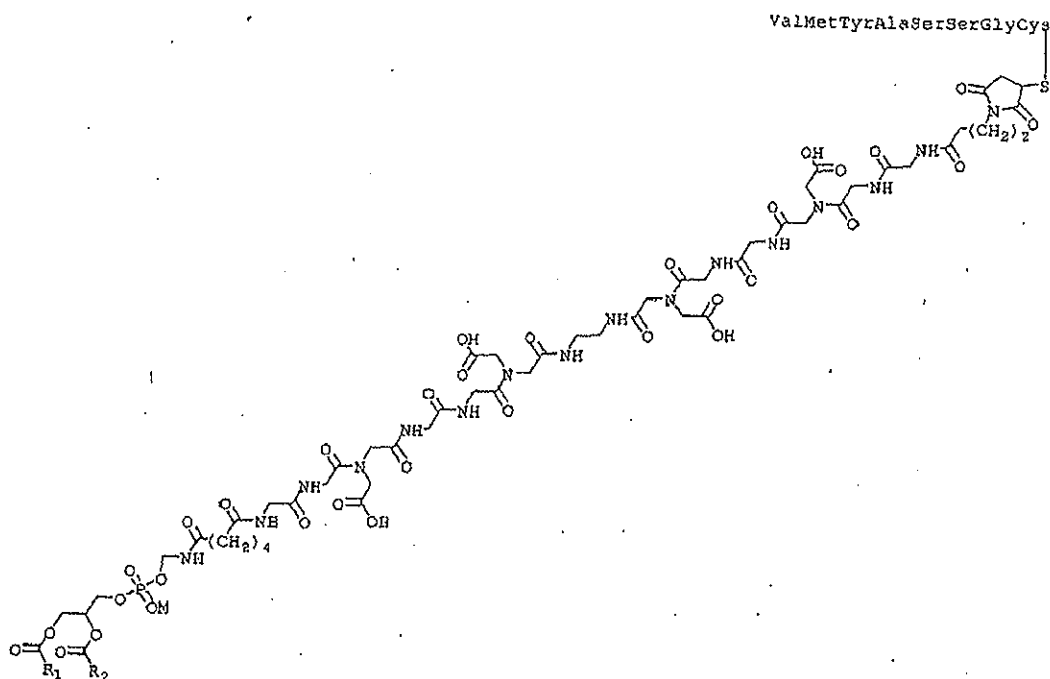
In an exemplifying third embodiment of the second aspect the invention provides a peptide-lipid construct of the structure:



where R_1 and R_2 are both $(CH_2)_7CHCH(CH_2)_7$ and designated DOPE-Ad-CMG(2)- β Ala-Mal-Mur(D14C)(XI).

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In an exemplifying fourth embodiment of the second aspect the invention provides a peptide-lipid construct of the structure:



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where R_1 and R_2 are both $(CH_2)_7CHCH(CH_2)_7$ and designated DOPE-Ad-CMG(2)- β Ala-Mal-Syph(V8C) (XII).

10 In a third aspect the invention provides a method of detecting reactive antibody in the serum of a subject including the steps of:

- 15 • Contacting a sample of the serum with a suspension of cells modified to incorporate a functional lipid construct (F-S-L) of the first or second alternatives of the first aspect of the invention or a peptide-lipid

- Incubating the mixture for a time and at a temperature sufficient to allow agglutination; and
- Determining the degree of agglutination of the cells in the mixture;

where:

F is a carbohydrate or peptide comprising an epitope for the reactive antibody.

Optionally, the method includes the intermediate step of:

- Adding an anti-subject globulin antibody to the mixture prior to determining the degree of agglutination of the cells of the mixture.

Preferably, the anti-subject globulin antibody is anti-human globulin (AHG) antibody.

Optionally, where F is a peptide, the method includes the preliminary step of:

- Adding an amount of the peptide to the sample of the serum;

where the amount of the peptide is sufficient to neutralize non-specific agglutination or confirm specificity of the reactive antibody.

Preferably, the reactive antibody is reactive to an antigen selected from the group consisting of: Glycophorin A, Glycophorin B, or mutations thereof (including the MNS blood group system).

Preferably, the subject is a human.

Preferably, the cells are red blood cells.

In a **third** aspect the invention provides a method of preparing a peptide-lipid construct of the second aspect of the invention including the step of:

- Reacting a peptide including a Cys residue with a functional lipid construct of the **third** alternative of the **first** aspect of the invention.

In a **fourth** aspect the invention provides a method of effecting qualitative and quantitative changes in the functional moieties expressed at the surface of a cell or a multi-cellular structure including the step of:

- contacting the cell or multi-cellular structure with a solution of a functional lipid construct of the **first** aspect of the invention for a time and at a temperature sufficient to allow the construct to incorporate into the cell or multi-cellular structure.

In a **fifth** aspect the invention provides a method of immobilizing one or more cells or multi-cellular structures including the steps of:

- Contacting the cells or multi-cellular structures with a solution of constructs of the **fourth** alternative of the **first** aspect of the invention for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells or multi-cellular structures to provide modified cells or multi-cellular structures; and
- Contacting the modified cells or multi-cellular structures with an avidin-coated substrate capable of being reversibly localized to a surface.

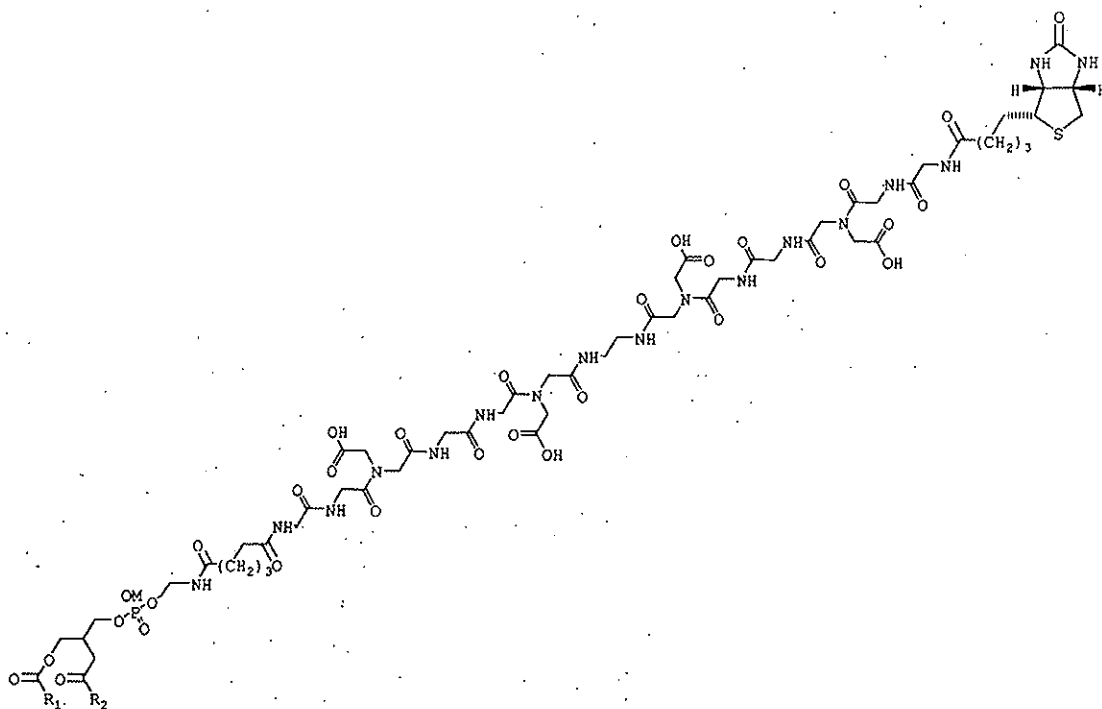
Preferably, the avidin-coated substrate is selected from the group consisting of: avidin-coated magnetic beads.

Preferably, the being reversibly localized to a surface is by application of a magnetic field.

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In an embodiment of the fifth aspect the invention provides a method of immobilizing one or more cells or multi-cellular structures including the steps of:

- Contacting the cells or multi-cellular structures with a dispersion of constructs of the structure:



for a time and at a temperature sufficient to allow an effective amount of the construct to incorporate into the cells or multi-cellular structures to provide modified cells or multi-cellular structures;

- Contacting the modified cells or multi-cellular structures with avidin-coated magnetic beads; and
- Applying a magnetic field to localize the beads to a surface;

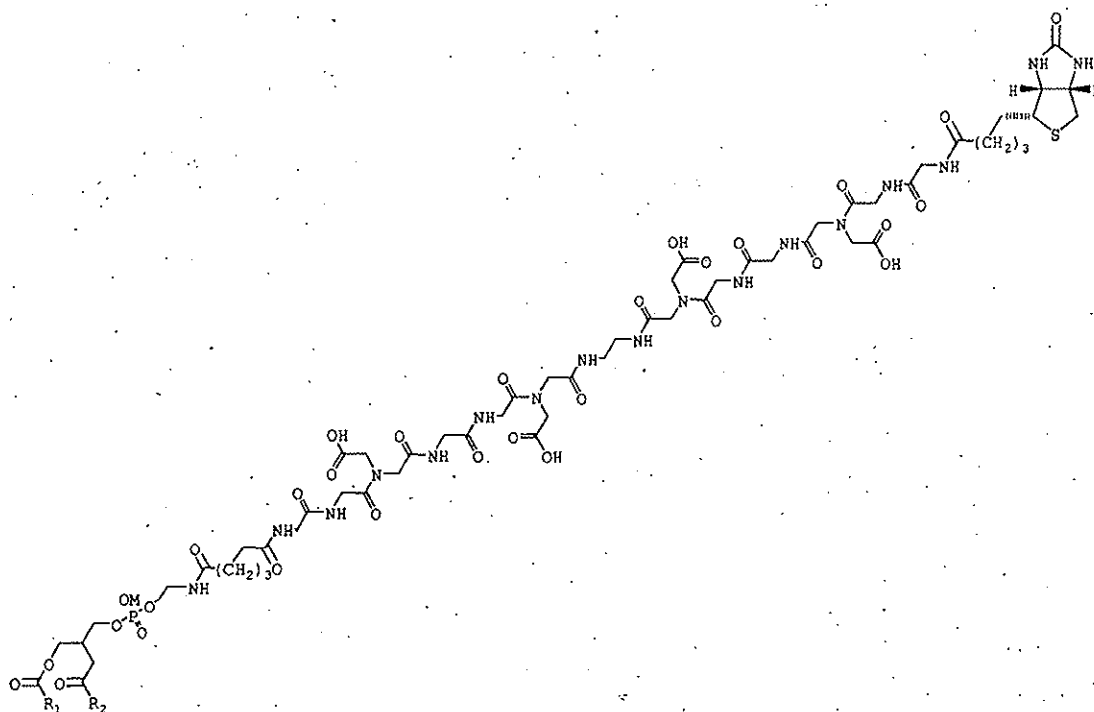
where R_1 and R_2 are both $(CH_2)_7CHCH(CH_2)_7$.

In a **sixth** aspect the invention provides a method of promoting the aggregation of a first and second population of cells including the steps of:

- Contacting the first population of cells with a solution of constructs of the **fourth** alternative of the **first** aspect of the invention for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;
- Contacting the second population of cells with a solution of constructs of the **fourth** alternative of the **first** aspect of the invention for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;
- Contacting the modified cells of one of the populations with an excess of avidin; and then
- Contacting the modified cells of the first and second populations...

In an embodiment of the sixth aspect the invention provides a method of promoting the aggregation of a first and second population of cells including the steps of:

- Contacting the first population of cells with a solution of constructs of the structure



for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;

- Contacting the second population of cells with a solution of the constructs for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;

- Contacting the modified cells of one of the populations with an excess of avidin; and then
- Contacting the modified cells of the first and second populations.

In all aspects of the invention M is typically H^+ , but may be replaced by another cation such as Na^+ , K^+ or NH_4^+ or monovalent substituent such as CH_3 . The notation M' excludes M being a monovalent substituent such as CH_3 .

For the most part amino acid residues of peptides are identified according to Table 3 of Appendix 2 of Annex C of the *Administrative Instructions under the Patent Cooperation Treaty* dated 7 February 2007 and in accordance with the convention:



In Tables the corresponding one-letter codes for amino acid residues may be employed to provide Tables of acceptable dimensions.

In the description and claims of the specification the following acronyms, terms and phrases have the meaning provided:

"Avidins" means the biotin-binding tetrameric protein produced in the oviducts of birds, reptiles and amphibians and deposited in the whites of their eggs, its biotin-binding homomers and biotin-binding modified forms thereof including EXTRAVIDIN™, NEUTRAVIDIN™ and NEUTRALITE™.

"Biotin-binding" means non-covalent binding to the biotin moiety with a dissociation constant (K_D) under biocompatible conditions of the order 10^{-15} M.

"Diagnostic marker" means a molecule, the presence of which in a body fluid of a subject is diagnostic of a phenotype or pathological condition of the subject.

"Dispersible in biocompatible media" means capable of forming a stable, single phase system in a medium at a concentration sufficient to effect qualitative and quantitative changes in the functional moieties expressed at the surface of a cell or a multi-cellular structure without loss of vitality.

"(or one of its derivatives)" means a chemical modification of the chemical structure to provide a fluorophore with substantially equivalent physico-chemical properties, but modified spectral characteristics.

"MNS blood group system " means blood group antigens or epitopes of those antigens and mutations which are present on either glycophorin A, glycophorin B or mutations which result in glycophorin A/B hybrids.

"pcv" means packed cell volume.

"Proximal terminal sequence" means that portion of the peptide sequence proximal to the amino- or carboxy- terminus of the peptide (F).

"Reactive antibody" means an immunoglobulin, the presence of which in a body fluid of a subject is diagnostic of a phenotype or pathological condition of the subject.

"RBC" means red blood cells.

"Water soluble" means a stable, single phase system is formed when the construct is contacted with water or saline (such as PBS) at a concentration of at least 100 µg/ml and in the absence of organic solvents or detergents. The terms "soluble" and "dispersible" are used synonymously.

Exemplifying embodiments of the invention will now be described in detail with reference to the Figures of the accompanying drawings pages.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. ¹H-NMR spectrum of the construct DOPE-Ad-CMG(I)amine (II) (5 mg/ml in D₂O/CD₃OD 2:1 δ ppm).

Figure 2. MALDI-TOF MS spectrum of DOPE-Ad-CMG(I)-βAla-Mal-Syph(V8C) (XII) (FLEX-PC (Bruker), DHB).

Figure 3. ¹H-NMR spectrum of the construct DOPE-Ad-CMG(I)-βAla-Mal-Syph(V8C) (XII) (7 mg/ml in D₂O/CD₃OD 4:1, pH c. 7.5; 600 MHz, 30 °C, δ ppm).

Figure 4. ¹H NMR spectrum of Biotin-CMG(2)-Ad-DOPE (I) (2.5 mg/ml in CD₃OD/D₂O 1:1, δ ppm, 600 MHz).

Figure 5. ESI-MS spectrum of Biotin-CMG(2)-Ad-DOPE (I) (ThermoFinnigan LCQDecaXP (negative mode, 30% MeOH)).

Figure 6. Fluorescence microscopy of avidin AF labelled red blood cells modified with the construct designated Biotin-CMG(2)-Ad-DOPE (I) (1 mg/mL) and stored for 14 days.

Figure 7. Fluorescence microscopy of avidin AF labelled zona-free D3.5pc murine embryos modified with the construct designated Biotin-CMG(2)-Ad-DOPE (I) (0.1 mg/mL) (400x magnification).

Figure 8. Fluorescence confocal microscopy of an avidin AF labelled Biotin-CMG(2)-Ad-DOPE (I) modified murine embryo.

Figure 9. Attachment of streptavidin beads to spermatozoa following modification of the spermatozoa by incubation with the construct designated Biotin-CMG(2)-Ad-DOPE (I).

Figure 10. Retention by streptavidin beads of following modification of the RBCs by incubation with the construct designated Biotin-CMG(2)-Ad-DOPE (I).

Figure 11. Structure of the construct designated Biotin-CMG(2)-Ad-DOPE.

Figure 12. RL95-2 monolayers modified with 20, 100 and 500 µg/mL biotin-CMG(2)-Ad-DOPE or media alone in serum-free media (A, C, E and G) and serum-containing media (B, D, F and H).

Figure 23. Addition of Avidin Alexa Fluor® 488 to RL95-2 monolayers modified with biotin-CMG(2)-Ad-DOPE. Fluorescence microscopy images of cells after addition of Avidin Alexa Fluor® 488 incubated at 37 °C show fluorescence starts to internalize within 4 hr (C) and is present in the cell interior in 24 hr (E). No internalization was observed when cells were incubated at 4°C (D, F).

DETAILED DESCRIPTION

The invention resides primarily in conjugating functional moieties to a diacyl or dialkyl lipid (L) via a spacer (S) to provide a construct (F-S-L) that is dispersible in biocompatible media, but will also spontaneously incorporate into the lipid bilayer of a cell membrane or multi-cellular structure.

The invention resides secondarily in the use of the selected structural motif (CMG) in the applications described and the advantages that accrue from using this structural motif and derivatives thereof.

Despite the advances in cell surface modification described in the specifications accompanying the international PCT applications referred to under the heading *Background Art*, the availability of constructs for use in the "one-step method", in particular peptide-lipid constructs, and the availability of BioG for use in the "two-step method", places a limitation on the broad application of these methods.

For example, a two-step method of localizing peptide antigen to the surface of cells or multi-cellular structures that avoids the use of BioG, or other conjugates obtained from biological sources, is desirable.

Although it was recognized that the biotinylation of the carbohydrate-lipid constructs described in the specification accompanying international application no. PCT/NZ2005/000052 provided a substitute for BioG in the "two-step method", it remains desirable to be able to use a biotin-lipid construct that has the favourable properties of these biotinylated carbohydrate-lipid constructs and could be used in the "one-step method".

In contrast with the preparation of constructs where the function (F) is a carbohydrate, the preparation of constructs where F is a peptide presents additional technical problems.

Firstly, it is desirable for the peptide (F) ligated to the L-S or S-L moiety to be dispersible in water such as a buffered

solution of solutes, e.g. PBS, or at least a biocompatible solvent.

Overcoming this difficulty may require the selection of a proximal terminal sequence (PTS) to promote solubility without modifying the desired biological properties of the construct.

Secondly, it is desirable for the peptide-lipid construct to be dispersible in water, or at least a biocompatible buffered solution or serum, according to the requirements of the proposed application (i.e. it is desirable for the construct to be "water soluble" as defined herein).

Overcoming this difficulty requires the selection of a spacer (S) to promote solubility of the construct.

Thirdly, where the proposed application is the modification of cells such as red blood cells (RBCs) for use in diagnostic applications, or as quality controls in blood group typing, it is required for the construct to be dispersible in a biocompatible buffered solution without participating in antigen-antibody cross reactivity not specific to the diagnostic peptide or blood group type antigen.

Satisfying this requirement requires the identification of suitable structural motifs for the spacer (S) and/or proximal terminal sequence (PTS) when the latter is present.

Where the application is for use in the modification of the surface of cells or multi-cellular structures (e.g. an embryo) with a view to promoting the association of the modified cell or modified multi-cellular structure with a target surface (e.g. the endometrium) exposing the cell or multi-cellular

structure to solvents or buffered solutions that are not biocompatible must be avoided.

Fourthly, the presentation of the peptide of the peptide lipid construct at the surface of the modified cell or multi-cellular structure will have an influence on the extent of cross reactivity with diagnostic markers.

The ability to localise peptides to the surface of cells or multi-cellular structures via a residue proximal to either the N- or C- terminus of the peptide may allow the naturally occurring configuration of the peptide sequence relative to the cell surface to be approximated.

The presentation of the peptide sequence in the tertiary (or quaternary) structure of the parent polypeptide (or protein) may therefore be mimicked. It is contemplated that peptides may be localised to the surface of cells via multiple residues. For example, where both a residue proximal to the amino terminus and a residue proximal to the carboxyl terminus are used to localise the peptide a "looped" configuration of the peptide may be promoted at the surface.

The poly-ethylene glycol (PEG) spacer of known peptide-lipid constructs is selected to provide solubility. However, polymers of PEG may interfere with the expression and function of the peptide at the surface.

The as yet unpublished specification accompanying international application number PCT/NZ2008/000239 describes the preparation of peptide-lipid constructs for use in methods of effecting qualitative and quantitative changes in the level of peptides expressed at the surface of cells and multi-

cellular structures where an oligomer of ethylene glycol is used as a spacer covalently linking lipid of the construct to the peptide moiety. The use of the constructs to prepare cells for use in serodiagnosis is described.

In the peptide-lipid constructs of the present invention the structural motif designated CMG is used as a component (S_1) of the spacer (S) covalently linking the lipid (L) and peptide (F). Inclusion of this structural motif provides a degree of rigidity to the spacer, distancing the functional moiety (peptide) of the peptide-lipid construct from the surface of the modified cell or multi-cellular structure.

It will be recognized that this attribute of the invention may be favourably applied to the development of other functional lipid constructs as demonstrated here with reference to the use of constructs including this structural motif where the functional moiety is a carbohydrate, such as the glycotope of the antigens of the ABO blood grouping, a fluorophore such as fluorescein (or one of its derivatives), or a conjugator, such as biotin.

Biotin ([3aS-(3a α , 4 β , 6a α)]-hexahydro-2-oxo-1H-thieno[3,4-d]imidazole-4-pentanoic acid) is a water soluble vitamin of the B complex, also referred to as vitamin H. Biotin is a growth factor present in minute amounts in every living cell. The compound plays an indispensable role in numerous naturally occurring carboxylation reactions, including the production of fatty acids.

Biotin has a solubility (25°C) in water of approximately 22 mg/100 mL and approximately 80 mg/100 mL in 95% alcohol. The compound has increased solubility in hot water and in dilute

alkali, but is relatively insoluble in other common organic solvents. The ability to localize this functional moiety to the surface of cells and multi-cellular structures provides a number of applications as demonstrated.

Whilst not wishing to be bound by theory it is believed that the properties of the functional lipid constructs may be modified and refined to suit particular applications by selection of the cation (M^+) or derivation of the free carboxyl groups of the structural motif to provide modified structural motifs, e.g. by substitution with methyl (CH_3 ; MCMG).

The properties of the functional-lipid constructs for use in the claimed methods must be such that they can be readily dispersed in biologically compatible media in the absence of solvents or detergents, but incorporate into the lipid bilayer of a membrane when a solution of the construct is contacted with a suspension of cells or multi-cellular.

Peptide-lipid constructs with these potentially conflicting properties are prepared by selection of other components of the spacer (S) in addition to the inclusion of the unmodified (CMG) or modified (e.g. MCMG) structural motif and/or the inclusion of a proximal terminal sequence (PTS) in the peptide (F).

The preparation of the peptide-lipid constructs where S is linked to F via a sulphide bond formed with a terminal Cys (Cys) residue of the peptide at the carboxy-terminus of the peptide is preferred as the peptide is less prone to oxidation.

A range of peptides may therefore be prepared as peptide-lipid constructs for use in methods of effecting qualitative and quantitative changes in the levels of peptide expressed at the surface of cells and multi-cellular structures.

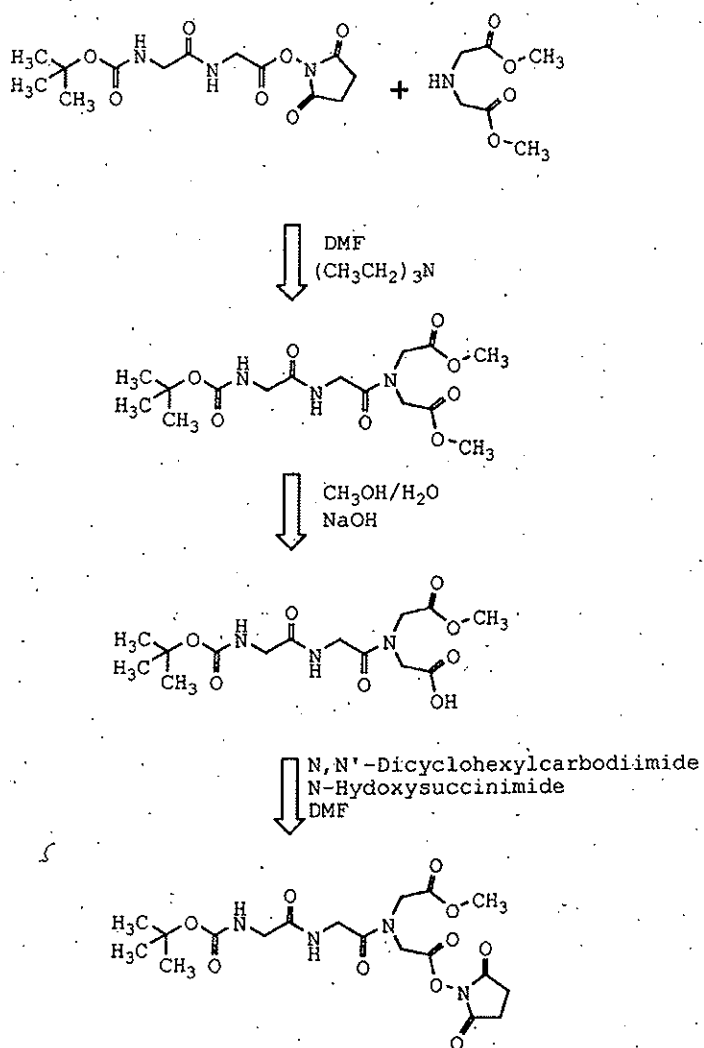
A particular advantage of the biotin-lipid constructs is that they permit cells or multi-cellular structures to be localized to surfaces with minimal detriment to the biological activity and viability of the cells or multi-cellular structure.

Examples of the localization of cells to a surface are provided. It will be noted that where the localization to a surface is achieved by means of avidin-coated magnetic beads the localization is reversible, thereby providing the opportunity to control the selection and positioning of cells on a surface.

The utility of the constructs in sub-cellular fractionation and localization of membrane bound organelles to surfaces is contemplated. The utility of the constructs in promoting the aggregation of populations of cells as may be required in the generation of hybridomas is also contemplated.

[followed by page 41]

SCHEME I



It will be understood that for a non-specific interaction, such as the interaction between diacyl- or dialkyl-glycerolipids or glycerophospholipids and a membrane, structural and stereo-isomers of naturally occurring lipids can be functionally equivalent.

For example, it is contemplated that diacylglycerol 2-phosphate could be substituted for phosphatidate (diacylglycerol 3-phosphate). Furthermore it is contemplated that the absolute configuration of phosphatidate can be either R or S.

The structural motif (CMG) may be prepared by the method summarized in Scheme I and Scheme II to provide the substructures designated MCMG(1) and CMG(2).

The preparation of the structural motif, the preparation of functional-lipid constructs utilizing this structural motif, and the use of these constructs in chemical and biological applications is described below

Preparation of the structural motif designated CMG

Materials and Methods

Acetone, benzene, chloroform, ethylacetate, methanol, toluene and o-xylene were from Chimmed (Russian Federation). Acetonitrile was from Cryochrom (Russian Federation). DMSO, DMF, CF₃COOH, Et₃N, N,N'-dicyclohexylcarbodiimide and N-hydroxysuccinimide were from Merck (Germany). Iminodiacetic acid dimethyl ester hydrochloride was from Reakhim (Russian Federation).

Dowex 50X4-400 and Sephadex LH-20 were from Amersham Biosciences AB (Sweden). Silica gel 60 was from Merck (Germany). Tetraamine $(\text{H}_2\text{N}-\text{CH}_2)_4\text{C} \times 2\text{H}_2\text{SO}_4$ was synthesized as described by Litherland et al. (1938). Thin-layer chromatography was performed using silica gel 60 F₂₅₄ aluminium sheets (Merck, 1.05554) with detection by charring after 7% H_3PO_4 soaking.

Preparation of {[2-(2-tert-butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (SCHEME I)

To a stirred solution of (methoxycarbonylmethyl-amino)-acetic acid methyl ester hydrochloride (988 mg, 5 mmol) in DMF (15 ml) were added Boc-GlyGlyNos (3293 mg, 10 mmol) and $(\text{CH}_3\text{CH}_2)_3\text{N}$ (3475 μL , 25 mmol) were added. The mixture was stirred overnight at room temperature and then diluted with o-xylene (70 ml) and evaporated.

Flash column chromatography on silica gel (packed in toluene, and eluted with ethyl acetate) resulted in a crude product. The crude product was dissolved in chloroform and washed sequentially with water, 0.5 M NaHCO_3 and saturated KCl.

The chloroform extract was evaporated and the product purified on a silica gel column (packed in chloroform and eluted with 15:1 (v/v) chloroform/methanol). Evaporation of the fractions and drying under vacuum of the residue provided a colourless thick syrup. Yield 1785 mg, (95%). TLC: $R_f=0.49$ (7:1 (v/v) chloroform/methanol).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C) δ , ppm: 7.826 (t, $J=5.1$ Hz, 1H; NHCO), 6.979 (t, $J=5.9$ Hz, 1H; NHCOO), 4.348 and 4.095 (s,

2H; NCH_2COO), 3.969 (d, $J=5.1$ Hz, 2H; COCH_2NH), 3.689 and 3.621 (s, 3H; OCH_3), 3.559 (d, $J=5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.380 (s, 9H; $\text{C}(\text{CH}_3)_3$).

Preparation of {[2-(2-tert-butoxycarbonylamino-acetyl-amino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid (SCHEME I)

To a stirred solution of {[2-(2-tert-butoxycarbonylamino-acetyl-amino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (1760 mg, 4.69 mmol) in methanol (25 ml) 0.2 M aqueous NaOH (23.5 ml) was added and the solution kept for 5 min at room temperature. The solution was then acidified with acetic acid (0.6 ml) and evaporated to dryness.

Column chromatography of the residue on silica gel (packed in ethyl acetate and eluted with 2:3:1 (v/v/v) *i*-PrOH/ethyl acetate/water) resulted in a recovered {[2-(2-tert-butoxycarbonylamino-acetyl-amino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (63 mg, 3.4%) and target compound (1320 mg). The intermediate product was then dissolved in methanol/water/pyridine mixture (20:10:1, 30 ml) and passed through an ion exchange column (Dowex 50X4-400, pyridine form, 5 ml) to remove residual sodium cations.

The column was then washed with the same solvent mixture, the eluant evaporated, the residue dissolved in chloroform/benzene mixture (1:1, 50 ml) and then evaporated and dried under vacuum. Yield of 10 was 1250 mg (74%), white solid. TLC: $R_f=0.47$ (4:3:1 (v/v/v) *i*-PrOH/ethyl acetate/water).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C), mixture of *cis*- and *trans*-conformers of *N*-carboxymethylglycine unit c.3:1. Major

conformer; δ , ppm: 7.717 (t, $J=5$ Hz, 1H; NHCO), 7.024 (t, $J=5.9$ Hz, 1H; NHCOO), 4.051 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 3.928 (d, $J=5$ Hz, 2H; COCH_2NH), 3.786 (s, 2H; NCH_2COOH), 3.616 (s, 3H; OCH_3), 3.563 (d, $J=5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; $\text{C}(\text{CH}_3)_3$) ppm; minor conformer, δ = 7.766 (t, $J=5$ Hz, 1H; NHCO), 7.015 (t, $J=5.9$ Hz, 1H; NHCOO), 4.288 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 3.928 (d, $J=5$ Hz, 2H; COCH_2NH), 3.858 (s, 2H; NCH_2COOH), 3.676 (s, 3H; OCH_3), 3.563 (d, $J=5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; $\text{C}(\text{CH}_3)_3$).

Preparation of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid N-hydroxysuccinimide ester (Boc-Gly₂(MCMGly)Nos) (SCHEME I)

To an ice-cooled stirred solution of {[2-(2-tert-butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid (1200 mg, 3.32 mmol) and *N*-hydroxysuccinimide (420 mg, 3.65 mmol) in DMF (10 ml) was added *N,N'*-dicyclohexylcarbodiimide (754 mg, 3.65 mmol). The mixture was stirred at 0°C for 30 min, then for 2 hours at room temperature.

The precipitate of *N,N'*-dicyclohexylurea was filtered off, washed with DMF (5 ml), and filtrates evaporated to a minimal volume. The residue was then agitated with $(\text{CH}_3\text{CH}_2)_2\text{O}$ (50 ml) for 1 hour and an ether extract removed by decantation. The residue was dried under vacuum providing the active ester (1400 mg, 92%) as a white foam. TLC: $R_f=0.71$ (40:1 (v/v) acetone/acetic acid).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C), mixture of *cis*- and *trans*-conformers of *N*-carboxymethylglycine unit c. 3:2.

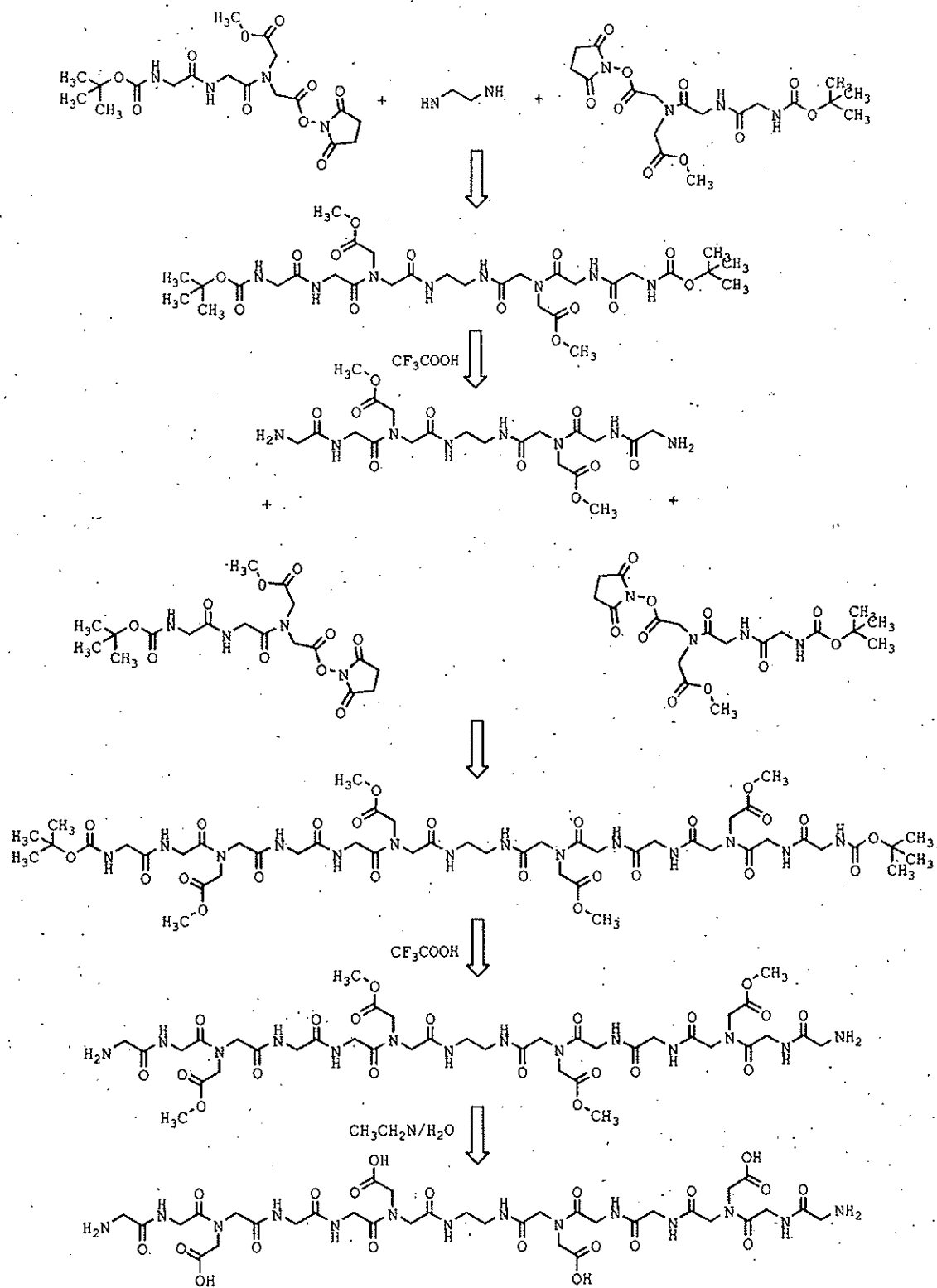
Major conformer; δ , ppm: 7.896 (t, $J=5.1$ Hz, 1H; NHCO), 6.972 (t, $J=5.9$ Hz, 1H; NHCOO), 4.533 (s, 2H; NCH_2COON), 4.399 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 3.997 (d, $J=5.1$ Hz, 2H; COCH_2NH), 3.695 (s, 3H; OCH_3), 3.566 (d, $J=5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.380 (s, 9H; $\text{C}(\text{CH}_3)_3$).

Minor conformer; δ , ppm: 7.882 (t, $J=5.1$ Hz, 1H; NHCO), 6.963 (t, $J=5.9$ Hz, 1H; NHCOO), 4.924 (s, 2H; NCH_2COON), 4.133 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 4.034 (d, $J=5.1$ Hz, 2H; COCH_2NH), 3.632 (s, 3H; OCH_3), 3.572 (d, $J=5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.380 (s, 9H; $\text{C}(\text{CH}_3)_3$).

The active ester (1380 mg) was dissolved in DMSO to provide a volume of 6 ml and used as a 0.5 M solution (stored at -18°C).

[followed by page 47]

SCHEME II



Preparation of ([2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino)-acetic acid methyl ester

To the stirred solution of (methoxycarbonylmethyl-amino)-acetic acid methyl ester hydrochloride (988 mg, 5 mmol) in DMF (15 ml) Boc-GlyGlyNos (3293 mg, 10 mmol) and Et₃N (3475 μ l, 25 mmol) were added.

The mixture was stirred overnight at room temperature (r.t.), then diluted with o-xylene (70 ml) and evaporated. Flash column chromatography on silica gel (packed in toluene and eluted with ethyl acetate) resulted in crude product.

The crude product was dissolved in chloroform and washed sequentially with water, 0.5 M NaHCO₃ and saturated KCl. The chloroform extract was evaporated, and the product was purified on a silica gel column (packed in chloroform and eluted with chloroform/methanol 15:1).

Evaporation of fractions and vacuum drying of residue resulted in a colorless thick syrup of (3) (1785 mg, 95%).

TLC: R_f = 0.49 (chloroform/methanol 7:1).

¹H NMR (500 MHz, [D₆]DMSO, 30 °C) δ = 7.826 (t, J = 5.1 Hz, 1H; NHCO), 6.979 (t, J = 5.9 Hz, 1H; NHCOO), 4.348 and 4.095 (s, 2H; NCH₂COO), 3.969 (d, J = 5.1 Hz, 2H; COCH₂NH), 3.689 and 3.621 (s, 3H; OCH₃), 3.559 (d, J = 5.9 Hz, 2H; COCH₂NHCOO), 1.380 (s, 9H; CMe₃) ppm.

Preparation of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid

To the stirred solution of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (1760 mg, 4.69 mmol) in methanol (25 ml) 0.2 M aqueous NaOH (23.5 ml) was added. The solution was kept for 5 min at r.t., then acidified with acetic acid (0.6 ml) and evaporated to dryness.

Column chromatography of the residue on silica gel (packed in ethyl acetate and eluted with iPrOH/ethyl acetate/water (2:3:1)) resulted in recovered (3) (63 mg, 3.4%) and crude target compound (1320 mg).

The crude target compound was dissolved in methanol/water/pyridine mixture (20:10:1, 30 ml) and passed through an ion-exchange column (Dowex 50X4-400, pyridine form, 5 ml) to remove residual Na cations.

The column was washed with the same mixture, eluant evaporated, dissolved in chloroform/benzene mixture (1:1, 50 ml) then evaporated and dried in vacuum to provide a yield of pure (10) was 1250 mg (74%), white solid.

TLC: $R_f = 0.47$ (iPrOH/ethyl acetate/water (4:3:1)).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C) of mixture of *cis*- and *trans*- conformers of N-carboxymethyl-glycine unit c.3:1.

Major conformer: $\delta = 7.717$ (t, $J = 5$ Hz, 1H; NHCO), 7.024 (t, $J = 5.9$ Hz, 1H; NHCOO), 4.051 (s, 2H; NCH_2COOMe), 3.928 (d, $J = 5$ Hz, 2H; COCH_2NH), 3.786 (s, 2H; NCH_2COOH), 3.616 (s, 3H;

OCH_3), 3.563 (d, $J = 5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; CMe_3) ppm.

Minor conformer: $\delta = 7.766$ (t, $J = 5$ Hz, 1H; NHCO), 7.015 (t, $J = 5.9$ Hz, 1H; NHCOO), 4.288 (s, 2H; NCH_2COOMe), 3.928 (d, $J = 5$ Hz, 2H; COCH_2NH), 3.858 (s, 2H; NCH_2COOH), 3.676 (s, 3H; OCH_3), 3.563 (d, $J = 5.9$ Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; CMe_3) ppm.

Preparation of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid N-oxy succinimide ester Boc-Gly2(MCMGly)Nos

To an ice-cooled stirred solution of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid (1200 mg, 3.32 mmol) and N-hydroxysuccinimide (420 mg, 3.65 mmol) in DMF (10 ml) N,N'-dicyclohexylcarbodiimide (754 mg, 3.65 mmol) was added. The mixture was stirred at 0 °C for 30 min, then for 2 h at r.t..

The precipitate of N,N'-dicyclohexylurea was filtered off, washed with DMF (5 ml) and the filtrates evaporated to a minimal volume.

The residue was agitated with Et_2O (50 ml) for 1 h. An ether extract was removed by decantation, and the residue dried in vacuum to yield the target compound (1400 mg, 92%) as a white foam.

TLC: $R_f = 0.71$ (acetone/acetic acid 40:1).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C), mixture of *cis*- and *trans*- conformers of N-carboxymethyl-glycine unit c. 3:2.

Major conformer: δ = 7.896 (t, J = 5.1 Hz, 1H; NHCO), 6.972 (t, J = 5.9 Hz, 1H; NHCOO), 4.533 (s, 2H; NCH_2COON), 4.399 (s, 2H; NCH_2COOMe), 3.997 (d, J = 5.1 Hz, 2H; COCH_2NH), 3.695 (s, 3H; OCH_3), 3.566 (d, J = 5.9 Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.380 (s, 9H; CMe_3) ppm.

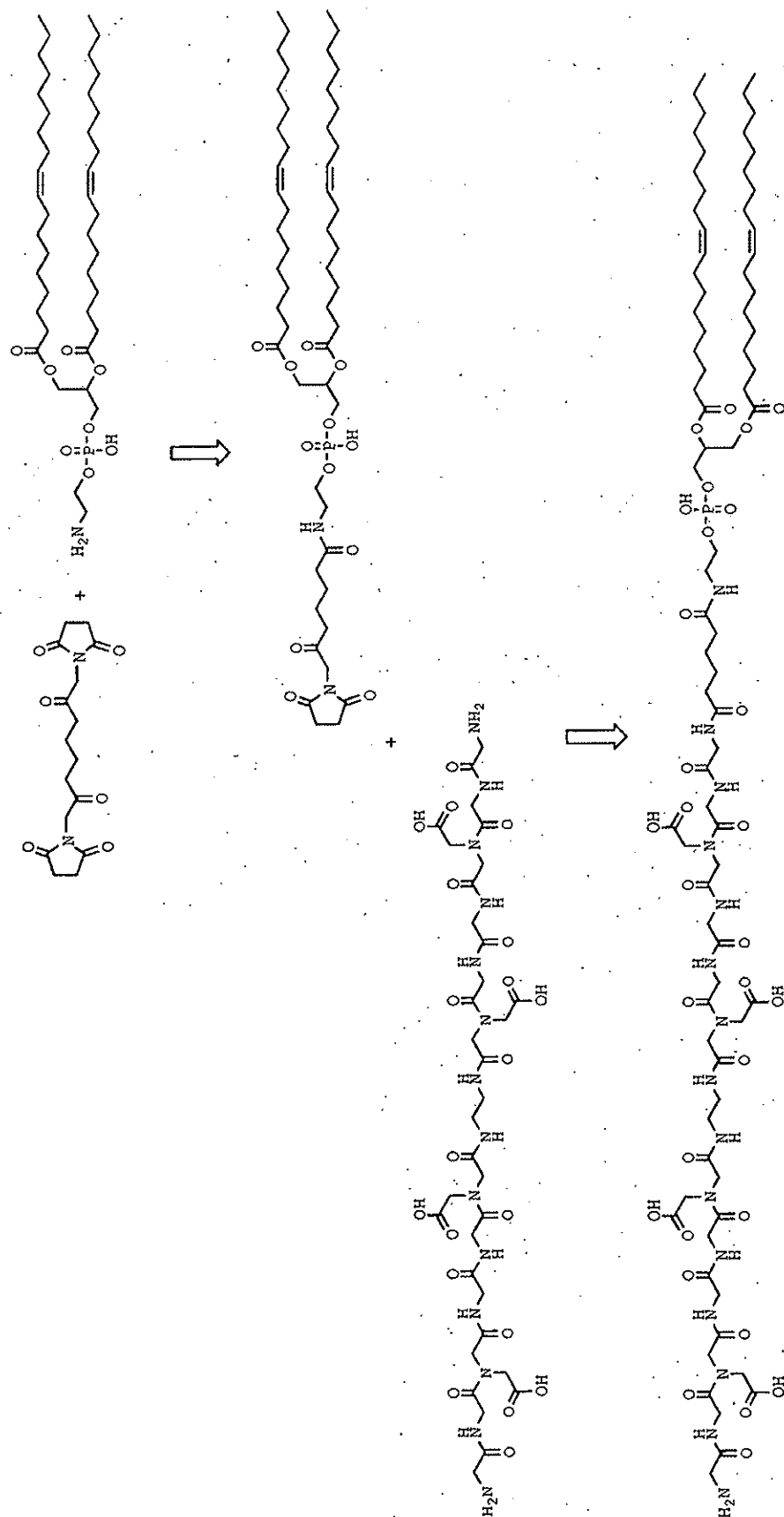
Minor conformer: δ = 7.882 (t, J = 5.1 Hz, 1H; NHCO), 6.963 (t, J = 5.9 Hz, 1H; NHCOO), 4.924 (s, 2H; NCH_2COON), 4.133 (s, 2H; NCH_2COOMe), 4.034 (d, J = 5.1 Hz, 2H; COCH_2NH), 3.632 (s, 3H; OCH_3), 3.572 (d, J = 5.9 Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.380 (s, 9H; CMe_3) ppm.

Preparation of DOPE-Ad-CMG(I)amine (SCHEME III)

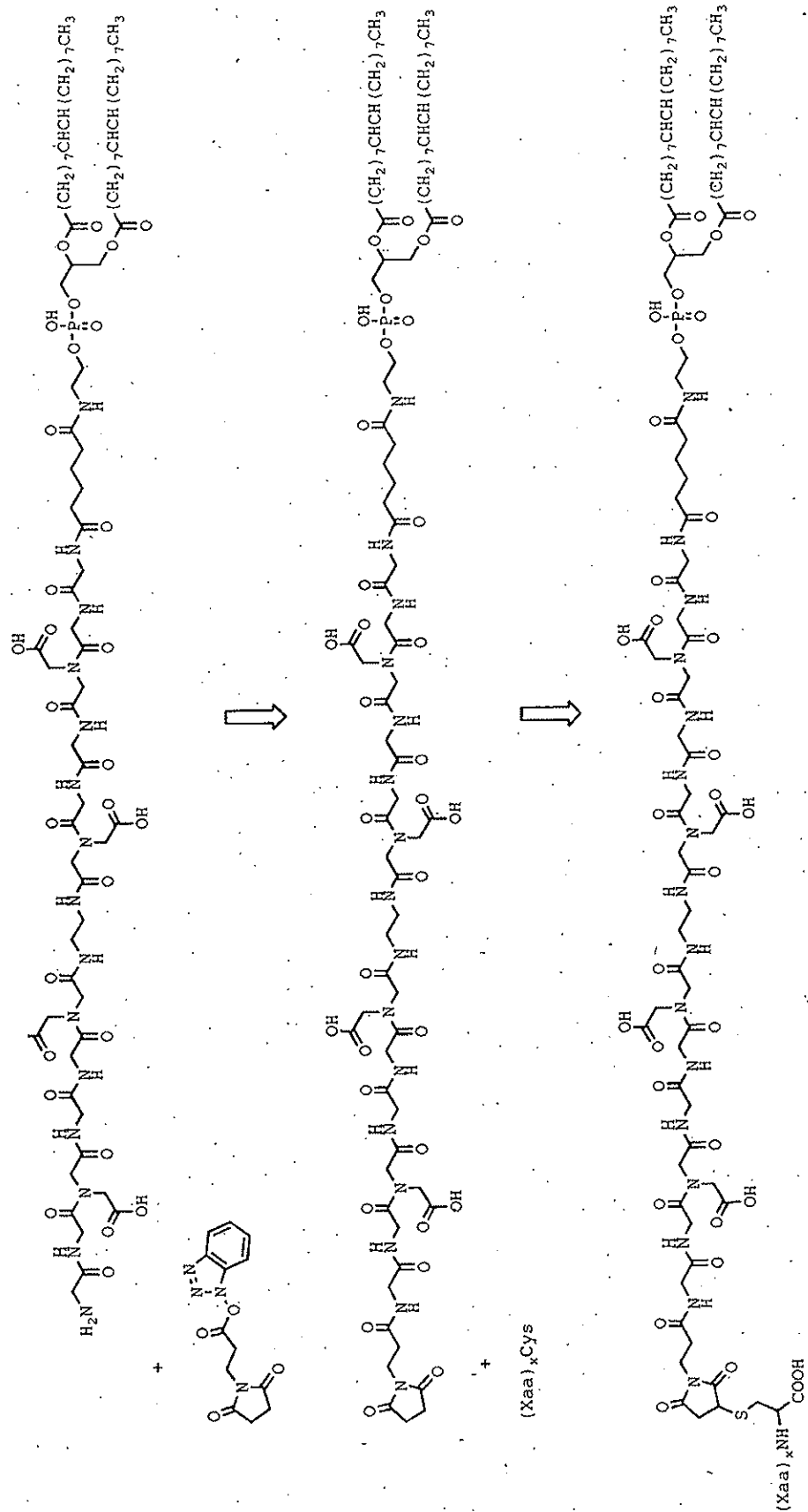
DOPE-Ad-CMG(2)amine was prepared from {[2-(2-tert-butoxycarbonylamino-acetyl-amino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid N-oxysuccinimide ester Boc-Gly₂(MCMGly)Nos according to Scheme III.

[followed by page 52]

SCHEME III



SCHEME IV



Preparation of functional lipid constructs (F-S-L) (SCHEME IV)

Preparation of DOPE-Ad-CMG(2)- β Ala-Mal-Milt(K,M) (X)

The construct DOPE-Ad-CMG(2)- β Ala-Mal-Milt(K,M) (X) was prepared according to Scheme IV.

DOPE-Ad-CMG(2)amine was treated with 5-fold excess of 3-maleimidopropionic acid oxybenztriazol ester (12) in i-PrOH-water.

Conversion of DOPE-Ad-CMG(2) into was somewhat low maleimido-derivative (about 70%), presumably due to fast hydrolysis of the intermediate promoted by the amount of organic base, diisopropylethylamine; required to be added to keep DOPE-Ad-CMG(2) in solution.

The maleimido-derivative was isolated in 40% yield after gel-permeation chromatography on Sephadex LH-20 (i-PrOH-water, 1:2).

Initially, the conjugation of the maleimido-derivative with peptide was attempted using i-PrOH-TRIS buffer, pH 8 (1:2), but the intermediate appeared to be almost insoluble in this medium. However, addition of pyridin (1 μ l/mg of intermediate) resulted in immediate dissolution of reactants and a surprisingly clean and substantially complete conversion.

Notably, although no reducing agent was used to prevent oxidative deactivation of the peptide, MS analysis of the whole reaction mixture revealed no traces of S-S dimer.

The desired construct (X) was purified on a Sephadex LH-20 column. A solubility problem was again encountered as fractions containing (X) were slightly opaque.

This would appear to indicate that the amount of base added to the eluent was insufficient to keep compounds properly charged and soluble in the concentration range of 1-5 mg/ml.

The structure of purified construct (X) was unambiguously established by NMR and MS spectra.

NMR spectrum revealed the expected peptide: DOPE ratio as deduced from the signal ratio for the most characteristic aromatic and olefin protons.

According to MS data, almost half of the final product (X) spontaneously formed pyroglutamyl derivative ($[M-17]^+$ ion).

In MALDI MS spectra of (X) peaks corresponding to unmodified peptide are present while the related peaks are absent in ESI-MS spectrum of the same substance. This is ascribed to facile fragmentation at the thiosuccinimide bond (retro-Michael reaction) under MALDI ionization conditions (destructive technique).

The general method of preparing peptide-lipid constructs was applied with minor modification to the preparation of constructs including peptides (F) selected from the following *List of Peptides*:

List of Peptides	
<u>Cys</u> (Xaa) ₂ TrpThrProProArgAlaGlnIleThrGlyTyrLeuThrValGlyLeuThrArgArg	
<u>Cys</u> (Xaa) ₂ TrpThrProProArgAlaGlnIleThrGlyTyrArgLeuThrValGlyLeuThrArgArg	
<u>Cys</u> (Xaa) ₂ ValMetTyrAlaSerSerGly	
<u>Cys</u> (Xaa) ₂ TyrProAlaHisThrAlaAsnGlu	ValMetTyrAlaSerSerGly(Xaa) ₂ <u>Cys</u>
	AspTyrHisArgValMetTyrAlaSerSerGly(Xaa) ₂ <u>Cys</u>
	ThrAsnGlyGluThrGlyGlnLeuValHisArgPhe(Xaa) ₂ <u>Cys</u>
	ThrAsnGlyGluMetGlyGlnLeuValHisArgPhe(Xaa) ₂ <u>Cys</u>
	AspThrTyrProAlaHisThrAlaAsnGluValSerGlu(Xaa) ₂ <u>Cys</u>
	ThrTyrProAlaHisThrAlaAsnGluVal(Xaa) ₂ <u>Cys</u>
	ProAlaHisThrAlaAsnGluVal(Xaa) ₂ <u>Cys</u>
	TyrProAlaHisThrAlaAsnGlu(Xaa) ₂ <u>Cys</u>
	ThrTyrProAlaHisThrAlaAsn(Xaa) ₂ <u>Cys</u>
	ThrTyrProAlaHisThrAlaAsnGlu(Xaa) ₂ <u>Cys</u>
	TyrProAlaHisThrAlaAsnGluVal(Xaa) ₂ <u>Cys</u>

List of Peptides	
	<u>TyrProAlaHisThrAlaAsnGlu(Xaa)₂Cys</u>
	<u>ProAlaHisThrAlaAsnGluValSer(Xaa)₂Cys</u>
	<u>AspThrTyrProAlaHisThrAlaAsnGlu(Xaa)₂Cys</u>
	<u>TyrProAlaHisThrAlaAsnGluValSer(Xaa)₂Cys</u>
	<u>SerGlnThrAsnAspLysHisLysArgAsp(Xaa)₂Cys</u>
	<u>GlnThrAsnAspLysHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>GlnThrAsnAspLysHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>GlnThrAsnAspMetHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>SerSerGlnThrAsnAspLysHisLysArg(Xaa)₂Cys</u>
	<u>SerSerGlnThrAsnAspLysHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>SerSerGlnThrAsnAspMetHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>SerSerGlnThrAsnAspMetHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>SerSerGlnThrAsnAspLysHisLysArgAspThrTyr(Xaa)₂Cys</u>
	<u>GlnThrAsnAspLysHisLysArgAspThr(Xaa)₂Cys</u>
	<u>SerGlnThrAsnAspLysHisLysArgAspThr(Xaa)₂Cys</u>
	<u>ThrAsnAspLysHisLysArgAspThrTyrPro(Xaa)₂Cys</u>
	<u>GluGluThrGlyGluThrGlyGlnLeuVal(Xaa)₂Cys</u>
	<u>GluGluGluThrGlyGluThrGlyGlnLeu(Xaa)₂Cys</u>

List of Peptides	
	<u>GluThrGlyGluThrGlyGlnLeuValHis (Xaa)₂Cys</u>
	<u>SerProProArgArgAlaArgValThr (Xaa)₂Cys</u>
	<u>TyrArgTyrArgTyrThrProLysGluLysThrGlyProMetLysGlu (Xaa)₂Cys</u>
	<u>TrpGlnProProArgAlaArgIle (Xaa)₂Cys</u>
	<u>ThrIleThrGlyLeuGluProGlyThrGlu (Xaa)₂Cys</u>

[followed by page 59]

The use of the peptide-lipid constructs in methods for effecting qualitative and quantitative changes in the levels of peptide expressed at the surface of cells and multicellular structures was illustrated with reference to serodiagnosis.

In the following table cross-reactivity of polyclonal sera and monoclonal antibodies of known specificities and red blood cells (RBCs) modified with the construct DOPE-Ad-CMG(I)- β Ala-Mal-Mur(D14C) (XI) (2 hours, 37 °C) is summarized.

[followed by page 60]

Reagent.	ID	Type	EIA/Miltenberger Specificity
2	T217	Human group AB serum	Reactive with MUT-T peptides by EIA
3	T165	Human group O serum	Reactive with MUR peptides by EIA
4	T7202	Human group B serum	Reactive with MUT-M peptides by EIA
6	T6025	Human group A serum	Reactive with MUT-T peptides by EIA
7	T8445	Human group O serum	Uncertain
8	T5896	Human group O serum	Uncertain
9	MIII	Monoclonal antibody	Reactive with Mi III red cells
10	Mia	Monoclonal antibody	Reactive with Mi III red cells
11	Mur	Monoclonal antibody	Reactive with Mur positive red cells
12	Gam	IgG monoclonal antibody	Reactive with Mi III red cells
13	BoxH	Human serum	Uncertain
14	TAP1	Human group O serum	Presumed MUT-K specificity
15	TAP2	Human serum	Presumed MUR specificity

	Antibody ID		Specificities	Transformed cells	Untransformed cells
Expected positives	T165	serum	Mur	10	0
	T6025	serum	K+Mur	5	0
	T8445	serum	Mur+Hil+Tsen	5	0
	T5896	serum	M+K+ (Mur)	0	0
Expected negatives	Japan	MoAb	Mur	0	0
	T4130	serum	Hil+Tsen	0	0
	T217	serum	T	0	0
	T7202	serum	M	0	0
	T8012	serum	M+K+T	0	0
	Japan	MoAb	Mi III (1:10)	0	0
	Box Hill	serum	?	0	0
	Japan	MoAb	Mi ^a (1:50)	0	0
	E119 KBL 7201	MoAb	Mia GAMMA(1:100)	0	0

Modification of red blood cells with peptide-lipid constructs

Red blood cells are modified by mixing 1 part by volume of washed packed red blood cells with 1 part by volume of peptide-lipid construct dispersed at a concentration of 10 to 1000 µg/ml in cell media (Celpresol™).

The suspensions are either:

1. incubated for 2 hours at 37 °C before being washed and suspended in a cell medium for serological analysis at a concentration of 0.8 to 3% (Method 1); or
2. incubated for 3 to 4 hours at room temperature (circa 25 °C) followed by 18 hours at 4 °C before being washed and suspended in a cell medium for serological analysis at a concentration of 0.8 to 3% (Method 2).

Tube serology testing of modified red blood cells

Serological reactions are graded or scored by either of two established systems (0 or '-' = no agglutination, 1+ or 3 = very weak agglutination, 2+ or 5 = weak agglutination, 3+ or 8 = moderate strong agglutination, 4+ or 10/12 = strong agglutination)

Serological platforms used are Tube (addition of reagents and reactants into plastic or glass serology tubes and after appropriate incubations, washing and centrifugation observing reactions macroscopically by eye and a 10X magnification eyepiece and scoring) and BioVue™ (addition of reactants into cassettes containing beads (including some reactants) and

after appropriate incubations and centrifugation observing the reaction patterns trapped within the Gel matrix). BioVue is the serological column agglutination platform of Ortho-Clinical Diagnostics. Diamed is the serological column agglutination platform of Diamed AG.

Serum samples were available from 47 blood donors of negative antibody screen status. These samples were designated "negative samples", but not determined not to have anti-Miltenberger antibodies).

Three serum samples known to have Miltenberger related antibodies T217, T6025, T5896. These samples were designated "positive samples", but not determined to have anti-antibodies against the peptide of the peptide of the construct designated DOPE-PEG₆- β Ala-Mal-Milt(K) (M00).

A suspension of 3 % modified RBCs was prepared in PBS and 30 μ l of the suspension mixed with 30 μ l serum sample. The mixtures were then incubated for 45 min at 37 °C. Following incubation the RBCs were centrifuged for 10 s in an Immufuge™ (setting: "high") and observed for agglutination before being washed 3 times with PBS.

After washing one drop of Epiclone™ anti-human globulin (AHG) was added and the tubes then centrifuged for 10 s in an Immufuge™ (setting: "high"). Tubes were then read and serology scores recorded.

Comments on the observed serology scores are provided in the legends to the following tables.

Age of modified RBCs (days)	Serum	Concentration of DOPE-PEG ₆ - β Ala-Mal-Milt(K) (M00) (mg/ml)					
		1.0 (n = 47)		0.5 (n = 21)		0.25 (n = 21)	
3	Negative samples	AHG+	AHG-	AHG+	AHG-	AHG+	AHG-
		1	46	0	21	0	21

Table 1. Summary of reactivity of samples of serum from 47 blood donors not expected to have anti-Miltenger activity ("negative samples"). AHG+ means sample reacted by the anti-human globulin test. AHG- means sample is unreactive. RBCs were modified with the peptide-lipid construct designated DOPE-PEG₆- β Ala-Mal-Milt(K) at the concentrations indicated. Sera were tested against modified RBCs following 3 days storage.

Age of modified RBCs (days)	Serum	Concentration of DOPE-PEG ₆ - β Ala-Mal-Milt(K) (M00) (mg/ml)		
		1.0	0.5	0.25
3	T217	2+	1+	-
3	T6025	4+	4+	4+
3	T5896	-	-	-
24	T217	-	-	n.t.
24	T6025	2+	2+	n.t.
24	T5896	-	-	n.t.

Table 2. Results by tube serology of 3 serums known to contain antibodies against antigens of the Miltenger complex. Score results show sample reactivity by the anti-human globulin test, 1+ = weak, 2+ = medium, 3+ = medium/strong, 4+ = strong, - means sample is unreactive. RBCs were modified with the peptide-lipid construct at the concentrations indicated. Sera were tested against modified RBCs following 3 days and 24 days storage. (n.t. - not tested).

Age of modified RBCs (days)		Concentration of DOPE-PEG ₆ - β Ala-Mal-Milt(K) (M00) (mg/ml)		
		1.0	0.5	0.25
	Serum			
3	T217	-	-	1+
3	T6025	1+	2+	1+
3	T5896	-	-	-
24	T217	-	-	-
24	T6025	2+	2+	1+
24	T5896	-	-	-

Table 3. Results by Diamed column serology of 3 serums known to contain antibodies against the Miltenberger complex. Score results show sample reactivity by the anti-human globulin test, 1+ = weak, 2+ = medium, 3+ = medium/strong, 4+ = strong, - means sample is unreactive. RBCs were modified with the peptide-lipid construct at the concentrations indicated. Sera were tested against modified RBCs following 3 days and 24 days storage.

Cell ID	Antigen	Polyclonal sera								Monoclonal antibodies			
		2	3	4	6	7	8	14	15	9	10	11	12
		T217	T165	T7202	T6025	T8445	T5896	TAP1	TAP2	MIII	Mia	Mur	Gam
9422184	Vw	8	5	3	0	8	0	5	0	0	10	0	12
11297161	MiIII	12	10	12	12	10	10	10		10	10	12	12
4131850	MiIV	12			12				10	0	10	12	12
1523	MiVI	12			12				8	0	10	12	10
T1569	MiVII	0	0	0	0	10	0	0		0	0	0	0
C.BR	Mi?X	12	10	12	12	8	12	12	8	0	10	10	10

Table 6. Identification of naturally occurring Miltenberger antigen positive (Milt⁺) human red cells as determined in BioVue AHG cards.

		Peptide sequence													Leader	Spacer
13	MUTK	Ser	Ser	Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	c	PEG6
34	MUTK	Ser	Ser	Gln	Thr	Asn	Asp	Lys	His	Lys	Arg				c	CMG(2)
21	MUTK	Ser	Gln	Thr	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp			c	CMG(2)
22	MUTK	Ser	Gln	Thr	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp			c	CMG(2)
36	MUTK	Ser	Gln	Thr	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr		c	CMG(2)
35	MUTK			Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr		c	CMG(2)
1	MUTK			Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	AAAAA	PEG6
2	MUTK			Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	GSerGSerGc	PEG6
3	MUTM			Gln	Thr	Asn	Asp	Met	His	Lys	Arg	Asp	Thr	Tyr	GSerGSerGc	PEG6
9	MUTK			Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	GSerGSerGc	CMG(2)
33	MUTK			Gln	Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	c	CMG(2)
37	MUTK				Thr	Asn	Asp	Lys	His	Lys	Arg	Asp	Thr	Tyr	Pro	CMG(2)
14	Mur	Asp	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val	Ser	Glu	c	CMG(2)
14	Mur	Asp	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val	Ser	Glu	c	CMG(2)
14	Mur	Asp	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val	Ser	Glu	c	CMG(2)
30	Mur	Asp	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu				c	CMG(2)
16	Mur	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val				c	PEG
17	Mur	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val				c	CMG(2)
28	Mur	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu					c	CMG(2)

		Peptide sequence										Leader	Spacer
27	Mur	Thr	Tyr	Pro	Ala	His	Thr	Ala	Asn			c	CMG(2)
25	Mur		Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu		c	CMG(2)
26	Mur		Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val	c	CMG(2)
31	Mur		Tyr	Pro	Ala	His	Thr	Ala	Asn	Glu	Val	ser	CMG(2)
18	Mur			Pro	Ala	His	Thr	Ala	Asn	Glu	Val	c	CMG(2)
19	Mur			Pro	Ala	His	Thr	Ala	Asn	Glu	Val	c	CMG(2)
29	Mur			Pro	Ala	His	Thr	Ala	Asn	Glu	Val	ser	CMG(2)
40	Hil	Glu	Glu	Glu	Thr	Gly	Glu	Thr	Gly	Gln	Leu	c	CMG(2)
23	Hil		Glu	Glu	Thr	Gly	Glu	Thr	Gly	Gln	Leu	Val	CMG(2)
24	Hil		Glu	Glu	Thr	Gly	Glu	Thr	Gly	Gln	Leu	Val	CMG(2)
41	Hil		Glu	Thr	Gly	Glu	Thr	Gly	Gln	Leu	Val	His	CMG(2)

Table 7. Identification of peptide-lipid constructs. Lowercase 'c' denotes a cysteine residue (Cys). All peptide-lipid constructs (F-S-L or L-S-F) were prepared as the DOPE (L) variant. M refers to a shorthand name for the molecule construct and is used in the following tables. The terminal peptide sequence is as indicated with "little c" representing Cys via which S is linked to L. Spacer refers to the structural motif of the spacer (S). CMG denotes the peptide-lipid constructs described in this specification. PEG denotes peptide-lipid constructs of the structure described as the second aspect of the invention in the specification accompanying the international PCT application filed on 11 September 2008 at the Intellectual Property Office of New Zealand as receiving Office (RO/NZ). All constructs were prepared as the DOPE variant.

Miltnerberger Antibody Positive Panel														
	4	8	2	6	3	14	7	9	10	11	12	13	15	
M	µg/ml	T7202	T5896	T217	T6025	T165	TAP1	T8445	M111	M1a	Mur	Gam	BoxH	TAP2
13	250	8	3	8	8	0		0	0	0	0	0	8	
34	50	0	0	0		0	3							0
21	200	0	0	0	8	8		0	0	0	0	3		5
22	200	0	0	0	10	0		0	0	0	0	3		0
36	50	0	0	0		0	8							0
35	50	0	0	0		0	5							0
1	500	5	0	3	8	0		0	0	5	0	8		
2	500	8	8	8	8	5		0	0	5	0	8		
9	300	8	10	8	8	8		3	0	0	0	8	10	
33	50	0	0	0		0	8							0
37	50	8	0	5		0	8							0
3	1000	8	10	0		5		0	0	0	0		5	

Table 8. Analysis of sorted data for the reactivity against the Miltnerberger Antibody Positive Panel of RBCs modified to incorporate the MUT peptide-lipid constructs identified at the concentration indicated. Constructs were able to show reactivity with one or more polyclonal serums indicating specificity to one or more peptide variations.

		Miltenberger Antibody Positive Panel											
M	µg/ml	3	6	7	4	8	2	15	9	10	11	12	13
		T165	T6025	T8445	T7202	T5896	T217	TAP2	MIII	Mia	Mur	Gam	BoxH
14	10	10	8	5	0	0	0		0	0	0		0
14	50	10	5	8	3	0	0		0	0	0		
14	100	10	10	5	5	0	3		0	0	0		0
30	50			8	10	0	0	8					
16	100	10	5	12	5	0	0		0	0	0	0	0
17	100	10	10	10	8	0	0	0	0	0	0	0	
28	50			8	10	0	0	8					
27	50			0	10	0	0	0					
25	50	3	0	3	0	0	0	8	0	0	0	0	
26	50	10	8	8	0	0	0	3	0	0	0	0	
31	50			8	10	0	0	0					
18	100	10	10	8	0	0	0		0	0	0	0	
19	100	10	8	10	0	3	0		0	0	0	0	
29	50				10	0	0	8					

Table 9. Analysis of sorted data for the reactivity against the Miltenberger Antibody Positive Panel of RBCs modified to incorporate the MUR peptide-lipid constructs identified at the concentration indicated. Constructs were able to show reactivity with one or more polyclonal serums indicating specificity to one or more peptide variations.

Country	Laboratory	Number Tested			Number Positive			% Positive		
		BioVue	DiaMed	Tube	BioVue	DiaMed	Tube	BioVue	DiaMed	Tube
Australia	CSL	100			3			3.0		
	Melb Path	45			2			4.5		
	RNSH	500	500		2	2		0.4	0.4	
Malaysia	UMMC	749			19			2.5		
Philippines	Metrop Hosp			60			0			0

Table 10. Negative serum reactivity. Miltenberger negative red cells were modified with the peptide-lipid construct M22 at a transformation concentration of 50 µg/ml and tested against antibody negative serums in the field to determine rates of false positivity. Studies were undertaken in clinical laboratories in Australia, Malaysia and Philippines using three different serological platforms; Column agglutination platforms BioVue and DiaMed as well as the simple technique of tube reactivity. Equal volumes of packed RBCs and a solution containing 50 µg/ml of the construct were contacted for 3 hours at room temperature and then 18 hours at 4°C. This field trial found that clinical antibody negative serums reacted with M22 transformed cells at rates 0.4 to 4.5 % in the BioVue™ platform and at a rate of 0.4 % in the DiaMed™ platform. No reactivity was observed in the tube platform. These results can be considered as false positive reactions.

Sample No.	22 (50 µg/ml)	17 (200 µg/ml)	24 (200 µg/ml)	Interpretation
488-6	10			K
9327986660	8	3	0	K
9325490091	5	5	0	K
9328791834	5	3	0	K
621-3	5			K
922390844-5	0	12	0	Mur
9322338631	0	10	0	Mur
914146821-8	0	10	0	Mur
932809044-1	0	8	0	Mur
942433813-3	0	8	0	Mur
942404708-4	0	5	0	Mur
942421413-0	0	5	0	Mur
942223755-1	0	5	0	Mur
942442720-2	0	5	0	Mur
927619701-8	0	3	0	Mur
912485657-9	0	3	0	Mur
926190919-0	0	3	0	Mur
9328154853	0	10	3	Mur + Hil
9328118428	0	10	5	Mur + Hil
9425256505	0	8	8	Mur + Hil
942433855-3	0	8	8	Mur + Hil
942753165-4	0	8	8	Mur + Hil
9424292604	0	8	5	Mur + Hil
9427455417	0	5	5	Mur + Hil
S-3	0	3	5	Mur + Hil
942448627-8	0	0	5	Hil
942423002-4	0	0	3	Hil
942762589-1	0	0	3	Hil

Sample No.	22 (50 µg/ml)	17 (200 µg/ml)	24 (200 µg/ml)	Interpretation
9424248012	0	0	0	other
9427615156	0	0	0	other
9424396133	0	0	0	other
9427613497	0	0	0	other
927175131-4	0	0	0	other
932467774-5	0	0	0	other
927299700-1	0	0	0	other
926555294-1	0	0	0	other
932360876-4	0	0	0	other
927516053-2	0	0	0	other
942404708-4	0	0	0	other
589-6	0			other

Table 11. Positive serum reactivity. Miltenberger negative red cells were modified with the peptide-lipid construct **M22** at a transformation concentration of 50 µg/ml, **M17** at a transformation concentration of 200 µg/ml, **M24** at a transformation concentration of 200 µg/ml, and tested against natural Mi III antibody reactive human serums in the field to determine rates of reactivity. Equal volumes of packed RBCs and a solution containing 50 µg/ml of the construct were contacted for 3 hours at room temperature and then 18 hours at 4°C. The three different constructs of MUT, MUR and HIL were able to discriminate most natural Mi III reactive polyclonal antibodies into specific reactivity profiles. Twelve serums were unreactive with the modified cells, suggesting they may have specificity against another Mi III antigen.

[followed by page 73]

RBCs		TAP 1 Serum	
		Saline	AHG
Natural Mi III positive cells			
R2R2, 11297161		8	10
R1Rz 11291347		8	10
Abtectcell III 8245009			10
	Peptide-lipid Construct (µg/ml)		
Modified cells			
M22 (MUT)	200	0	10
M22 (MUT)	100	0	8
M22 (MUT)	50	0	8
M22 (MUT)	20	0	8
M22 (MUT)	10	0	5
M22 (MUT)	0	0	0
M17 (Mür)		0	0
M24 (Hil)		0	0
Antibody Screen/ID panel Abtectcell III Batch 2223005 Cells I - III			0
Phenocell B Batch 2653046 Cells 1-11			0

Table 12. Anti-MUT serum reactivity. Miltenberger negative RBCs were modified with the peptide-lipid constructs M22 at concentrations ranging from 10 to 200 $\mu\text{g/ml}$, M17 at a concentration of 50 $\mu\text{g/ml}$ and M24 at a concentration of 50 $\mu\text{g/ml}$. The modified cells were tested against a natural Mi III antibody reactive Taiwan human serum (TAP1) detected in the field to determine reactivity profile. Reactivity was compared against natural Mi III antigen positive cells. TAP1 (Taiwan Miltenberger antibody

positive sample). TAP1 serum was shown to contain both IgG and IgM antibodies (the latter being saline reactive) directed against natural M1111 positive cells. The lack of reactivity against Abtectcell™ and Phenocell™ antibody screening and identification panels concludes no other antibodies against red cells are present. Reactivity with M22 modified cells over transformation concentrations of 10 to 200 µg/ml (and not with untransformed cells - 0 µg/ml) concludes the presence of an antibody directed against MUT. The failure of the M22 modified cells to detect a saline reaction, suggests the assay is sensitive to the IgG class of antibody and not IgM - a clinically favourable result. The lack of reactivity with the M17 and M24 transformed cells concludes an absence of antibodies to the MUR and HIL mutations.

[followed by page 75]

RED CELLS		TAP 2 Serum
		BioVue AHG
Natural Mi III positive cells		
Mi III PDN No. 54		5
Mi IV No;4131850		10
Mi VI 1523A		8
Mi ?x ID: CBR		8
Vw No: 9422184		0
	Peptide-lipid construct ($\mu\text{g/ml}$)	
M28 (MUR)	100	10
M22 (MUT)	100	0
Antibody Screen panel Abtectcell III Batch 2223009		
Cells I - III		0

Table 13. Anti-MUT serum reactivity. Miltenberger negative red cells were transformed with the peptide-lipid constructs M28 at a concentration of 100 $\mu\text{g/ml}$ and M22 at a concentration of 100 $\mu\text{g/ml}$ and tested against a natural Mi III antibody reactive Taiwan human serum (TAP2) detected in the field to determine reactivity profile. Reactivity was compared against natural Mi III antigen positive cells. TAP 2 (Taiwan Miltenberger antibody positive sample). TAP2 serum was shown to contain IgG antibodies directed against natural Mi III positive cells. The lack of reactivity against Abtectcell[™] antibody screening and identification panels concludes no other antibodies against red cells are present. Reactivity with M28 modified cells and not with M22 modified cells concludes the presence of an antibody directed against the MUR peptide.

Positive Sample	ID	Specificities	BioVue									
			M22	M33	M34	M35	M36	M37	M40	M41	Unmodified	
3	T165	Mur	0	0	0	0	0	0	0	0	0	0
8	T5896	M+K+Mur	0	0	0	0	0	0	0	0	0	0
2	T217	T	0	0	0	0	0	5	0	0	0	0
4	T7202	M	0	0	0	0	0	8	0	0	0	0
	TAP1		8	3	5	5	8	8	0	0	0	0

		MUT peptides												MUT		MUT/MUR	MUR	
		S	S	Q	T	N	D	K	H	K	R			#2	#4			TAP1
34	MUTK4	S	S	Q	T	N	D	K	H	K	R			-	-	++	-	-
22	MUTK3		S	Q	T	N	D	K	H	K	R	D		-	-	+++	-	-
36	MUTK6		S	Q	T	N	D	K	H	K	R	D	T	-	-	+++	-	-
35	MUTK5			Q	T	N	D	K	H	K	R	D	T	-	-	++	-	-
33	MUTK1			Q	T	N	D	K	H	K	R	D	T	-	-	+	-	-
37	MUTK7				T	N	D	K	H	K	R	D	T	++	+++	+++	-	-
		HIL peptides												MUT		MUT/MUR	MUR	
		#2	#4	TAP1	#2	#4	TAP1	#2	#4	TAP1	#2	#4	TAP1	#2	#4			TAP1
40	Hil 2	E	E	E	T	G	E	T	G	Q	L			-	-	-	-	-
41	Hil 3			E	T	G	E	T	G	Q	L	V	H	-	-	-	-	-

Table 14. Identification of the M37 sequence as a candidate for the detection of anti-MUT. Miltenberger negative cells were modified with the peptide-lipid constructs M22, M33, M34, M35, M36, M37, M40 and M41 a concentration of 50 µg/ml. Modified cells were tested against serums 2, 3, 4 and 8 of the Miltenberger Antibody Positive Panel and Taiwan Mi IIX antibody positive serum TAP1 to determine its MUT reactivity profile. TAP 1 (Taiwan Miltenberger antibody positive sample). Cells modified with the peptide-lipid construct M37 were able to detect the anti-MUT activity of Miltenberger Antibody Positive Panel samples 2 and 4. Sample 3 containing MUR activity was expected negative. Sample 8 containing multiple antibodies was unexpectedly negative, but may have lost specificity. TAP1 serum was able to detect all MUT variations, indicating some polyclonal serums may have less defined anti-MUT activity than others.

[followed by page 78]

		MUT Peptides												Reactivity
34	MUTK4	S	S	Q	T	N	D	K	H	K	R			5
22	MUTK3		S	Q	T	N	D	K	H	K	R	D		8
36	MUTK6		S	Q	T	N	D	K	H	K	R	D	T	8
35	MUTK5			Q	T	N	D	K	H	K	R	D	T	5
33	MUTK1			Q	T	N	D	K	H	K	R	D	T	3
37	MUTK7				T	N	D	K	H	K	R	D	T	8
		HIL peptides												
40	Hil 2	E	E	E	T	G	E	T	G	Q	L			-
41	Hil 3			E	T	G	E	T	G	Q	L	V	H	-

Table 15. Anti-MUT serum (TAP1) reactivity. Miltenberger negative cells were modified with the peptide-lipid constructs M22, M33, M34, M35, M36, M37, M40 and M41 at a concentration of 50 µg/ml (2 hours, 37 °C). Modified cells were tested against Taiwan Mi III antibody positive serum TAP1 to determine its MUT reactivity profile. TAP 1 (Taiwan Miltenberger antibody positive sample). The TAP1 serum is able to recognize some, but not all, peptide variations of MUT. The lack of reactivity with M40 and M41 (HIL peptide) modified cells and the untransformed cells is expected.

														Panel Serum #7 (T8445)
30	Mur 9	D	T	Y	P	A	H	T	A	N	E			8
17	Mur 2		T	Y	P	A	H	T	A	N	E	V		10
28	Mur 6		T	Y	P	A	H	T	A	N	E			8
27	Mur 5		T	Y	P	A	H	T	A	N				-
25	Mur 4			Y	P	A	H	T	A	N	E			3
26	Mur 7			Y	P	A	H	T	A	N	E	V		10
31	Mur 10			Y	P	A	H	T	A	N	E	V	S	8
19	Mur 3				P	A	H	T	A	N	E	V		8
29	Mur 8				P	A	H	T	A	N	E	V	S	8

Table 16. Anti-MUR serum (TAP1) reactivity. Miltenberger negative cells were modified with the peptide-lipid constructs M17, M19, M25, M26, M27, M28, M29, M30 and M31 at a concentration of 50 µg/ml (2 hours, 37 °C). Modified cells were tested against antibody positive serum #7 from the Miltenberger Antibody Positive Panel to determine their MUR reactivity profile.

	False positive reactions with M17			
Score (n=102)	12-10	8-5	3	0
	17	18	3	65
	17%	18%	3%	64%

Table 18. False positive MUR M17 construct reactions with 102 negative serums. Cells modified with the peptide-lipid construct M17 were tested against 102 negative serum samples. Cells modified with the peptide-lipid construct M17 give the most "false positive" reactive construct showing up to 36% false positive rate with negative serums.

T series reactives (n=58)

	2	4	31	44	61	18	21	28	55	42	63	62	7	20	48	22	39	23	30	% positive
M17	10	8	8	10	10	12	12	12	12	10	10	8	8	8	8	8	5	3	3	33%
M28	10	8	8	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9%
M30	10	8	8	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9%

39 T negative samples were negative with all 3 constructs. Larger series
= 102

																T18	T21	T28	T55	T78	T92
30	Mur 9	D	T	Y	P	A	H	T	A	N	E					-	-	-	-	-	-
17	Mur 2		T	Y	P	A	H	T	A	N	E	V				12	12	12	12	12	12
28	Mur 6		T	Y	P	A	H	T	A	N	E					-	-	-	-	-	-
27	Mur 5		T	Y	P	A	H	T	A	N						-	-	-	-	-	-
25	Mur 4			Y	P	A	H	T	A	N	E					-	-	-	-	5	-
26	Mur 7			Y	P	A	H	T	A	N	E	V				5	10	12	10	10	12
31	Mur 10			Y	P	A	H	T	A	N	E	V	S			-	10	12	-	8	12
19	Mur 3				P	A	H	T	A	N	E	V				-	-	-	12	-	-
29	Mur 8				P	A	H	T	A	N	E	V	S			-	-	5	-	-	8

5 samples reacted with all 3 constructs

															Panel Serum #7 (T8445)	False Positivity
30	Mur 9	D	T	Y	P	A	H	T	A	N	E				8	-
17	Mur 2		T	Y	P	A	H	T	A	N	E	V			10	+++
28	Mur 6		T	Y	P	A	H	T	A	N	E				8	-
27	Mur 5		T	Y	P	A	H	T	A	N					-	-
25	Mur 4			Y	P	A	H	T	A	N	E				3	
26	Mur 7			Y	P	A	H	T	A	N	E	V			10	+++
31	Mur 10			Y	P	A	H	T	A	N	E	V	S		8	++
19	Mur 3				P	A	H	T	A	N	E	V			8	+
29	Mur 8				P	A	H	T	A	N	E	V	S		8	+

Table 19. "M17-false-positive" negative serum reactivity against other Mur constructs. The 6 most false positive negative serums reactive against cells modified with the peptide-lipid construct M17 were tested against cells modified by contacting with the peptide-lipid constructs M19, M25, M26, M27, M28, M29, M30 and M31 at a concentration of 50 µg/ml (2 hour 37°C). The modified cells were tested in BioVue™ AHG cards. Cell modified with the peptide-lipid construct M17 provided the most "false positive" reactions with negative serums. The reactivity of the 6 most false positive samples when tested against other modified cells shows that some are unreactive (M28, M27), some are poorly reactive or show a single discrete reactivity (M5, M29, M19) while others are more reactive (M31, M26). Minor changes in amino acid sequence can influence the rate of false positive reactivity. Cells modified with the constructs M30 and M28 show both specificity and low non-specificity.

	M1 modified cells			M2 cells vs serum		
Identity of sera	#4	#5	#6	#2	#6	#8
Serum alone	5	5	10	8	8	8
Serum + peptide	0	0	0	0	2	0

Table 20. Sera reactive with RBCs modified to incorporate the M1 peptide-lipid construct or M2 peptide-lipid construct constructs by contacting the cells with a 500 µg/ml dispersion of the construct (Method 1) were "neutralised" with the peptide QTNDKHKRDTY and retested against the modified cells. Sera were neutralized by adding 10 µL of 1 mg/ml solution of peptide to a 50 µL volume of sera and incubating for 30 minutes at 37 °C. Testing was performed using BioVue™ cards.

Identity of sera	M13 modified cells			
	#3	#42	#37	#34
Serum alone	8	8	8	8
Serum + peptide	0	0	0	0

Table 21. Sera reactive with RBCs modified to incorporate the M13 peptide-lipid construct by contacting the cells with a 500 µg/ml dispersion of the construct (Method 1) were "neutralised" with the peptide SSQTNDKHKRDTY and retested against the modified cells. Sera were neutralized by adding 10 µL of 1 mg/ml solution of peptide to a 50 µL volume of sera and incubating for 30 minutes at 37 °C. Testing was performed using BioVue™ cards.

			PAC74	TAP1
Pre-neutralisation serology (cells modified with the peptide-lipid construct M22)			8	8
Neutraliser	F	Linker	Post-neutralisation serology (cells modified with the peptide-lipid construct M22)	
nil	nil	nil	8	8
M22 peptide	SQTNDKHKRDC	nil	8	-
M28 peptide	TYPANTANEC	nil	8	8
M22 molecule	SQTNDKHKRDC	CMG(2)	-	-
Cys-CMG-DE	Ac-C	CMG(2)	-	8
	VMYASSG?		8	8

Table 22. Cells were modified by the peptide-lipid construct M22 (2 hours, 37°C) and two positive reactions were identified. Neutralisation experiments were then performed. A volume of 40 µl of plasma was incubated with 10 µl of peptide or Ac-C at a concentration of 1.0 mg/ml for 30 minutes at 37 °C. The standard AHG test in BioVue™ was then performed. The false positive reaction for PAC74 was confirmed as a reaction not neutralised by addition of peptide. The true positive reaction for TAP1 confirmed as reaction neutralised by peptide and the whole construct, but not the construct bearing only acetylated cysteine.

			PAC70	PAC71	PAC72	TAP2
Pre-neutralisation serology (cells modified with the peptide-lipid construct M28)			8	5	5	8
Neutraliser	F group	Linker	Post-neutralisation serology (cells modified with the peptide-lipid construct M22)			
saline	nil	nil	8	5	5	8
M22 peptide	SQTNDKHKRDC	nil	8	-	-	3
M28 peptide	TYPAHTANEC	nil	-	-	-	8
Cys-CMG-DE	Ac-C	CMG(2)	8	3	3	5
Atri-CMG-DE	GalNAc α 3[Fuc α 2]Gal β	CMG(2)	8	3	5	8
Atri-adipate-DE	GalNAc α 3[Fuc α 2]Gal β	adipate	8	3	5	8
	VMYASSG?		8	5	5	8

Table 23. Cells were modified by the peptide-lipid construct **M28** (2 hours, 37°C) and four positive reactions were identified. Neutralisation experiments were then performed. A volume of 40 μ l of plasma was incubated with 10 μ l of peptide or Ac-C at a concentration of 1.0 mg/ml for 30 minutes at 37 °C. The standard AHG test in BioVue™ was then performed. The neutralisations of PAC70, 71 and 72 with the **M28** peptide suggests specificity. The fact that the unrelated peptide **M22** was also able to cause neutralisation of serums PAC71 and PAC72, together with reductions in score with other unrelated structures, revises the results for these two sera as being false positive reactivity. The fact that PAC70 does not react with Miltenberger positive cells suggests that although an antibody appears to be present to the peptide sequence it is not blood group specific. In contrast although TAP2 was not fully inhibited by peptide, the substantial reduction in score suggests specificity, although it is possible that specificity may be present with a low level of non-specificity as suggested by the reaction score reduction against Cys-CMG-DE.

Consideration of the MUT peptide reactivities presented in the foregoing in Tables shows that peptides **M22**, **M36** and **M37** all showed superior sensitivity and specificity towards a human polyclonal antibody panel when compared with sequence 1, the sequence identified in the prior art (Reid and Lomas-Francis (2004)).

Modification of red blood cells with peptide-lipid constructs with peptide in alternative configurations

Peptide-lipid constructs comprising CMG(2) and the following peptides were prepared:

ThrTyrProAlaHisThrAlaAsnGluCys (**M44**) and
CysThrTyrProAlaHisThrAlaAsnGlu (**M45**)

The termini of the peptides were formylated and amidated to provide "capped" peptides. The reactivity of random Miltenberger antibody positive samples and three false positive antibody negative samples were tested against RBCs modified to incorporate these "capped" peptide-lipid constructs (50 µg/mL, 2 hours at 37 °C).

The reactivity was assessed and recorded in Table 24. The capping of the peptide did not affect reactivity with positive samples, nor did it appear to affect reactivity with false-positive antibody-negative samples. However, linkage of the peptide via a Cys residue located at the amino terminus (as opposed to the carboxy terminus) appeared to reduce the likelihood of reactivity with the false-positive antibody negative samples and improve reactivity with the known antibody positive samples.

Whilst not wishing to be bound by theory it is speculated that the presentation of the peptide by cells modified to incorporate M45 may be more analogous to the presentation of the corresponding peptide sequence expressed by the naturally occurring antigen.

	Sample	M44	M45	Not modified	Natural MiIII cells
Antibody positive serums	71	10	10	0	8
	67	8	10	0	5
	65	10	8	0	12
	55	8	8	0	10
	61	8	5	0	8
	942-433813-3	0	5	0	12
	68	0	0	0	10
False-positive antibody negative serums	PAC302	5	0	0	0
	PAC332	12	0	0	0
	PAC340	5	0	0	0

Table 24.

Modification of cells and multi-cellular structures with biotin-lipid constructs

The modification of red blood cells (RBCs) and murine embryos by the construct designated Biotin-CMG(2)-Ad-DOPE was demonstrated using Avidin-Alexafluor (Avidin AF).

Materials

A stock solution of the construct designated Biotin-CMG(2)-Ad-DOPE (100 μ L) was prepared in water at a concentration of 10 mg/mL. A stock solution of Avidin-Alexafluor (Avidin-AF) was prepared in sterile phosphate buffered saline (PBS) at a concentration of 2 mg/mL. A stock solution of biotinylated

gangliocide (BioG) was prepared in sterile PBS at a concentration of 5 mg/mL.

Red Blood Cells

Dilution series of the construct designated Biotin-CMG(2)-Ad-DOPE and BioG (positive control) were prepared at concentrations of 0.001, 0.1, 0.1 and 1 mg/mL with Celpresol™. O group red blood cells (RBCs) were modified by incubation of 15 μ L of packed RBCs and 5 μ L of a dilution of the construct designated biotin-CMG(2)-Ad-DOPE or BioG. Incubations were performed in a plastic ependorf tube of nominal volume 1.5 mL for 2 hours at 37°C in a water bath. O group RBCs were incubated with a solution of BioG at a concentration of 0.33 mg/mL as a positive control.

Incubated RBCs were washed 3 times with PBS in a mini centrifuge. Fluorescent labeling of the washed, modified RBCs was performed by adding 10 μ L of a solution of Avidin-AF at a concentration of 0.1 mg/mL. The RBCs were then incubated in the dark for 1 hour at 37 °C in a water bath and washed 3 times with PBS.

85% solution of the washed, fluorescent labeled RBCs were viewed on a slide with cover slip under a fluorescent microscope at 488 nm and photographic exposure of 1.903 (Figure 6). The intensity of the fluorescent signal was recorded using a scoring system of 0 (no fluorescence observed) to 4 (maximum fluorescence observed).

An aliquot of the washed, modified RBCs obtained from incubation with the construct designated biotin-CMG(2)-Ad-DOPE at a concentration of 1 mg/mL was retained and stored at 14 °C

for 14 days. Retention of the construct by the RBCs was assessed by incubation with Avidin-AF as before.

The assessment of the fluorescence is recorded in Table 25.

Dilution (mg/ml)	1	0.1	0.01	0.001	0
Biotin-CMG(2)-Ad-DOPE Day 0	4	2	1	0	0
Biotin-CMG(2)-Ad-DOPE Day 14	4	-	-	-	-
BioG Day 0	3	2	0	0	0

Table 25.

Murine Embryos

Murine embryos (morula/early blastocyst stage, 3.5 day) were incubated in 50 microlitre microdrops in blastassist™ culture media. Embryos were incubated with construct designated biotin-CMG(2)-Ad-DOPE at a concentration of 0.1, 1 or 2 mg/mL or BioG at a concentration of 0.5 mg/mL (positive control).

The zona pellucida of the embryos was removed by treatment with 0.5% pronase (4 minute incubation) and washing 3 times in embryo handling media prior to introduction into the microdrops. Each microdrop was equilibrated 5% CO₂ at 37 °C overnight prior to introduction of the embryos.

Microdrops containing embryos and the construct designated biotin-CMG(2)-Ad-DOPE were incubated for 2 hours in 5% CO₂ at 37 °C. Microdrops containing embryos and BioG were incubated for 40 minutes in 5% CO₂ at 37 °C.

Following incubation each group of embryos was washed 3 times in handling media and transferred to 50 microlitre microdrops containing 2 mg/mL Avidin-AF microdrops for fluorescent labelling. The microdrops containing transferred embryos were incubated at 37 °C for 30 minutes in the dark.

Each group of embryos was then washed 3 times in handling media and mounted on a glass microscope slide for viewing. Embryos were viewed under a fluorescent microscope at 488 nm. The intensity of the fluorescence was recorded using a scoring system of 0 (no fluorescence) to 4 (maximum fluorescence).

Biotin-CMG(2)-Ad-DOPE 0.1 mg/mL	Biotin-CMG(2)-Ad-DOPE 1 mg/mL	Biotin-CMG(2)-Ad-DOPE 2 mg/mL	BioG 0.5 mg/mL	Media alone
2	1	1	3	1
n=21	n=19	n=19	n=19	n=19

Table 26.

Immobilization of spermatozoa and cells

The immobilization of spermatozoa and red blood cells (RBCs) was demonstrated by use of the construct designated Biotin-CMG(2)-Ad-DOPE and streptavidin beads (Dynabeads® M-280).

Materials

A stock solution of the construct designated Biotin-CMG(2)-Ad-DOPE (100 μ L) was prepared in water at a concentration of 10 mg/mL and diluted in culture media (Medicult 10310060A) to provide a test dilution at 0.1 mg/mL.

The spermatozoa in fresh semen (less than one day old) were assessed for motility (80%, grade 3 (fast, forward progression) by 10-fold dilution in culture medium (Medicult 10310060A; pre-incubated for a minimum of 2 hours at 37 °C in a 5% CO₂ atmosphere). Spermatozoa counts (91.5×10^6 /mL) were performed by 10-fold dilution in deionised water.

Spermatozoa were washed and isolated by layering 1.1 mL of fresh semen over a gradient of SpermGrad 125 (Vitrolife 10099; 2 mL of 40% solution over 2 mL of 80% solution in a 15 mL round bottom tube) and centrifuging at 500 x g for 20 min.

The bottom layer of the gradient (c. 0.7 mL) was transferred to 4 mL round bottom tubes and c. 2 mL flushing (handling) media (Medicult 10840125A) added. The tube was centrifuged at 300 x g for 10 min and the spermatozoa washed two more times (mixing by tube inversion).

Samples of washed spermatozoa were incubated overnight at 37 °C in a 5% CO₂ atmosphere. Spermatozoa counts (c. 25×10^6 /mL) were performed post overnight incubation by 10-fold dilution in deionised water.

Spermatozoa

A volume of 100 μ L of the test dilution of the construct designated Biotin-CMG(2)-Ad-DOPE (I) was added to each of four 0.6 mL ependorf tubes (A-D) and 100 μ L of culture media added to one 0.6 mL ependorf tube (E).

Open tubes were incubated at 37 °C in a 5% CO₂ atmosphere prior to addition of c. 70 μ L spermatozoa (c. 25×10^6 /mL) to each tube and incubation for 120 min (A), 60 min (B), 30 min (C), 10 min (B) and 120 min (E).

Following incubation a couple of drops of flushing media were added and the tubes centrifuged at 300 x g for 5 min. The spermatozoa were washed two more times with flushing media and before being re-suspended in culture media to a final volume of 100 μ L.

Streptavidin beads at a concentration of c. 6.25×10^6 /100 μ L were diluted 35 times in BSA plus flushing media to provide a ratio of 0.1 beads/spermatozoa when mixed in equal volume with a diluted suspension of the modified spermatozoa.

A volume of 5 μ L of a diluted suspension of the modified spermatozoa was mixed on a slide with 5 μ L of a diluted suspension of streptavidin beads and covered with a coverslip.

The mixture was observed under a microscope at 400x magnification. Figure 9 provide a photomicrograph of the mixture provided following incubation with 0.1 mg/mL of the construct designated Biotin-CMG(2)-Ad-DOPE for 60 min (B).

The assessment of the attachment of streptavidin beads to modified spermatozoa is recorded in Table 27.

Biotin-CMG(2)-Ad-DOPE (I) treatment	Incubation time (min)	Number of beads attached to spermatozoa	
		Immediate	30 min
A	120	10-12	10-15 (with cross-linking)
B	460	2, 4, 8	8
C	30	1-3	3
D	10	1	1
E (Control)	120	0	0

Table 27.

Spermatozoa were observed to retain motile capacity despite attachment of beads (no acrosome reaction was evident) with a preference of attachment to motile spermatozoa.

Red Blood Cells.

A dilution of the construct designated Biotin-CMG(2)-Ad-DOPE was prepared at a concentration of 1 mg/mL with Celpresol™. A volume of 60 µL washed A group red blood cells (RBCs) was modified by incubation with 20 µL of the dilution of the construct at 37 °C for 2 hours.

The modified RBCs were washed twice in PBS and once in Celpresol™ as described above. A 2% cell suspension of washed cells (modified or control) was prepared in Celpresol™ and cell concentration ($150 \times 10^6/\text{mL}$) determined using a haemocytometer. Similarly the concentration of a suspension streptavidin beads ($134 \times 10^6/\text{mL}$) was determined.

A volume of 50 μ L of the suspension of streptavidin beads was added to the wells of a 96-well plat with a Neodymium (Rare-Earth) Super magnet (Magnets NZ Limited) affixed to the base. A volume of 50 μ L of a suspension of RBCs was added to provide a bead to RBC ratio of c. 1:1 and incubated at room temperature for 1 hour to allow RBCs to settle.

The wells were washed 3 x with PBS, aspirating the washing solution with a pipette. The washed wells were observed under a microscope and the RBCs determined to be retained (Figure 10).

Separation of populations of cells

A 0.5 mg/ml solution of the construct designated Biotin-CMG(2)-Ad-DOPE was prepared in Celpresol™ and a volume of 10 μ l used to modify 30 μ l packed cell volume of group O RBCs in a 1 ml eppendorf tube to provide a first population of cells. Unmodified group A RBCs were used as a second population of cells.

Both populations of cells were incubated for 37 °C for 2 hrs in a water bath and then washed 2x in PBS and 1x in Celpresol using an Immufuge II (low, 1 min). The concentration of cells in each suspension was made up to 2% by adding 1.5 mL.

The suspensions of RBCs were mixed with avidinylated magnetic Dynabeads at an approximate ratio of RBC:bead of 1 and incubated for 10 min at room temperature on a gyrator. Samples of the first and second populations of RBCs were then mixed in equal volumes (35 μ l each) in an eppendorf tube for two minutes.

The contents of the ependorf were transferred to the well of a 96-well plate and a magnet was applied to the underside of the well for 1 minute. The supernatant was carefully removed with the magnet applied and without disruption of the beads. The blood grouping of the cells of the supernatants were then assessed by applying 30 μ l of supernatant and 30 μ l anti-A antibody to a Dynamed™ gel card. Cards were spun for 10 min in a centrifuge. Retention of the O group RBCs by the magnet was demonstrated by the absence of a pellet of group O cells.

Modification of cell layers with biotin-lipid constructs

The modification of monolayers of the cell line RL95-2 (established from a human endometrial adenocarcinoma (ATCC HTB CRL 1671)) in serum-free and serum-containing media was evaluated.

D-MEM/F12 (Gibco 11320-033, Invitrogen NZ) containing 1% penicillin/streptomycin (Gibco 15140-122, Invitrogen NZ) was used as a serum-free medium. D-MEM/F12 10% FBS (Gibco 10091-130, Invitrogen NZ) containing 1% penicillin/streptomycin and 5 μ g/mL insulin (Gibco 12585-014, Invitrogen NZ) was used as a serum-containing medium.

A suspension of the cell line RL95-2 was diluted in pre-warmed serum-containing media to the required concentration e.g. 4×10^5 cells/mL. A 25 μ L volume of the suspension was used to seed the required wells in a Terasaki tray so that each treatment was performed in duplicate. The plates were incubated overnight in a 5% CO₂, 37 °C incubator until the monolayer was approximately 60% confluent.

Dilutions of Biotin-CMG(2)-Ad-DOPE were prepared and 12 μ l volumes added to wells containing washed cell layers to provide final concentrations of 20, 100 or 500 μ g/mL. Trays were incubated at 37 °C, 5% CO₂ for 120 min.

The cells were then washed and a 12 μ l volume of a 0.1 mg/mL solution of Avidin Alexa Fluor® 488 added. The cells were then incubated at room temperature for a further 30 minutes in the dark.

The monolayers were finally washed 3 times with PBS, the trays inverted and photographed using an Olympus BX51 fluorescent microscope at 200x magnification, exposure time 475 ms (Figure 12).

When the construct is inserted in serum-free media at 20, 100 and 500 μ g/mL, a homogenous intense fluorescent signal is observed in the cell membrane that intensifies with increasing concentration of the construct (Figure 12A, C and E). When the construct is inserted in serum-containing media, weakened fluorescence is observed at the same concentrations (Figure 12B, D and F). These results imply that optimal insertion of construct into the cell membranes requires serum-free media.

When the construct is inserted in serum-free media at 20, 100 and 500 μ g/mL, an homogenous intense fluorescent signal is observed in the cell membrane that intensifies with increasing concentration of construct. The intensity of the fluorescence also increased with increasing insertion time (Table 28). These results imply that optimal insertion of construct into cell membranes occurs with increased concentration of construct and/or increased insertion time.

	Mean Fluorescence*			
	Insertion time			
Concentration of biotin-CMG(2)-Ad-DOPE ($\mu\text{g/mL}$)	10	30	60	120
20	1+	1-2+	2-3+	3-4+
100	2+	3	3+	4+
500	3+	3-4+	na	na
Media alone	0	0	0	0

Table. 28. Optimal insertion of construct into cell membranes occurs with increased concentration of construct and/or increased insertion time. 'na' denotes "not assessed".

When avidin Alexa Fluor® 488 was added to construct modified RL95-2 cells and incubated at 37 °C for 4 and 24 hr the fluorescence gradually shifted from the cell surface to the cell interior (Figure 13). No internalization was observed when cells were incubated at 4°C.

Fluorescence was detected in construct modified RL95-2 cells 24 hr post-insertion when cultured in serum-free media, albeit with reduced fluorescence from T=0 (Table 29). However, when cells were cultured in serum-containing media a fluorescence score of 1+ was detected at the highest concentration of construct (500 mg/mL), but not at lower. These results imply that the construct is optimally retained in cell membranes 24 hr post-insertion when cultured in serum-free media, but not in serum-containing media.

Concentration of biotin-CMG(2)-Ad-DOPE ($\mu\text{g/mL}$)	Mean Fluorescence*		
	T=0	T=24 hr	
		Serum-free	Serum-containing
20	3-4+	1-2+	0
100	4+	2-3+	0
500		4+	1+
Media alone	0.	0	0

Table 29. Retention of construct 24 hours post-insertion. The construct was detected in cells cultured for 24 hr post-insertion in serum-free media, but was only detected at the highest concentration in serum-containing media. 'na' denotes "not assessed".

Modification of antigen presentation

The amount of construct used in the manufacture of quality controls cells as described in the specification accompanying international application no. PCT/NZ2005/000052 (publication no. WO 2005/090368) is a determinant of the cost of manufacture.

It was anticipated that presentation of antigen at a distance from the immediate milieu of the cell surface may promote recognition by cross-reactive antibody and subsequent agglutination. The estimated distances from the cell surface for an antigen (F) of a functional lipid construct (F-S-L) where the spacer (S) includes the structural motif of the present invention are 7.2 nm (CMG(2)) and 11.5 nm (CMG(4)). These distances compare with 1.9 nm for the antigen of a construct (F-S-L) where the spacer is one described in the specification accompanying international application no.

PCT/NZ2005/000052 (publication no. WO 2005/090368), i.e. A_{tri}-sp-Ad-DOPE (I).

To test the hypothesis solutions of four different trisaccharide (A_{tri})-lipid constructs were prepared as 50 μ M, 10 μ M and 5 μ M solutions in Celpresol™. Modified red blood cells were then prepared by contacting 0.6 mls (pcv) of washed RBCs with 0.6 mls of the relevant solution. The mixtures were incubated at 37 °C for 2 hours and then washed 3 times to provide a modified cell suspension.

The modified cell suspensions were prepared in 0.8% in Celpresol LISS™ suitable for BioVue serology cards. A volume of 0.05 mls of CSL monoclonal anti-A (026129801) followed by 0.05 mls of modified cell suspension was applied to each card. Reactions were recorded following centrifugation in a BioVue cassette centrifuge (Table 29).

A comparison of the recorded reaction for molar equivalents of the A_{tri}-lipid constructs demonstrates that less CMG(2) or CMG(4) construct is required to provide an equivalent serological result. There was no significant gain with CMG(2) compared with CMG(4). There was a minor, but not significant change for MCMG (2).

Although the invention has been described by way of examples it should be appreciated that variations and modifications may be made to the claimed methods without departing from the scope of the invention. As noted it will be understood that for a non-specific interaction, such as the interaction between the diacyl- or dialkyl- glycerolipid portion of the functional-lipid constructs and a membrane, structural and

stereo-isomers of naturally occurring lipids can be functionally equivalent.

Where known equivalents exist to specific features, such equivalents are incorporated as if specifically referred to in this specification. For example, it is contemplated that diacylglycerol 2-phosphate could be substituted for phosphatidate (diacylglycerol 3-phosphate) and that the absolute configuration of phosphatidate could be either R or S.

[followed by page 99]

A ₄₄ -lipid construct		Serological result at reciprocal of dilution of anti-A										
Structural motif of spacer	Conc (μM)	Neat	2	4	8	16	32	64	128	256	512	1024
Adipate	50	12	12	12	12	12	12	10	8	8	3	-
	10	10	10	10	8	8	8	8	3	-	-	-
	5	8	8	5	3	-	-	-	-	-	-	-
CMG(2)	50	12	12	12	12	12	12	10	10	8	5	3
	10	12	12	12	10	10	10	10	8	5	3	-
	5	10	10	10	10	8	8	8	5	3	-	-
mCMG(2)	50	12	12	12	12	12	12	12	10	10	8	5
	10	12	12	12	12	12	10	10	8	8	5	-
	5	10	10	10	10	10	8	8	5	3	-	-
CMG(4)	50	12	12	12	12	12	12	10	10	8	5	3
	10	12	12	12	10	10	10	10	8	8	3	-
	5	10	10	10	10	8	8	8	5	3	-	-

Table 29.

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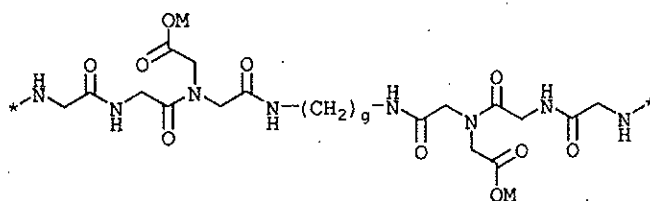
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[followed by page 103 - Claims]

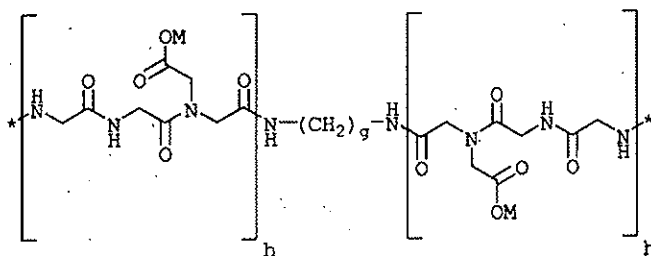
CLAIMS

- 1) A functional lipid construct of the structure F-S-L where F is a functional moiety, L is a diacyl or a dialkyl lipid, and S is a spacer covalently linking F to L including the substructure:



where g is the integer 1, 2 or 3, M is a monovalent cation or substituent, and * is other than H.

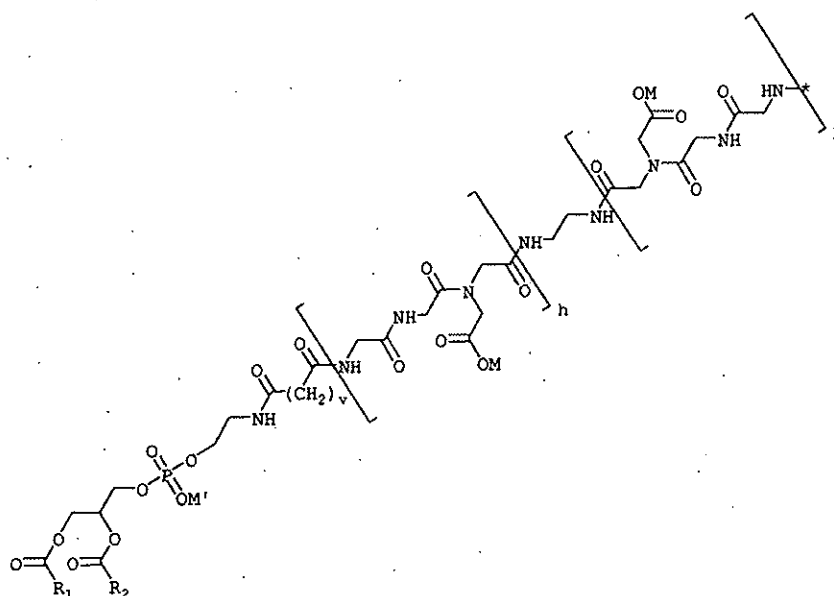
- 2) The functional lipid construct of claim 1 where the substructure is:



where h is the integer 1, 2, 3 or 4.

- 3) The functional lipid construct of claim 2 where g is the integer 2 and h is the integer 1, 2 or 4.
- 4) The functional lipid construct of claim 3 where M is H or CH₃.

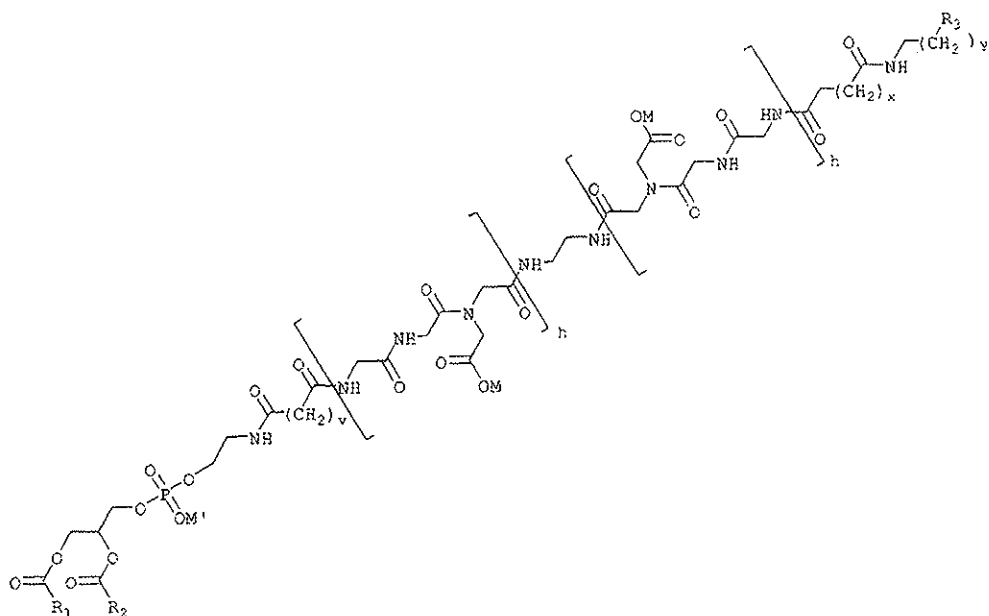
- 5) The functional lipid construct of claim 1 where L is a diacylglycerophospholipid.
- 6) The functional lipid construct of claim 1 where L is phosphatidylethanolamine.
- 7) The functional lipid construct of claim 6 where the structure of the functional lipid construct includes the partial structure:



where v is the integer 3, 4 or 5, M' is a monovalent cation, and R_1 and R_2 are independently selected from the group consisting of: alkyl or alkenyl substituents of the fatty acids *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid.

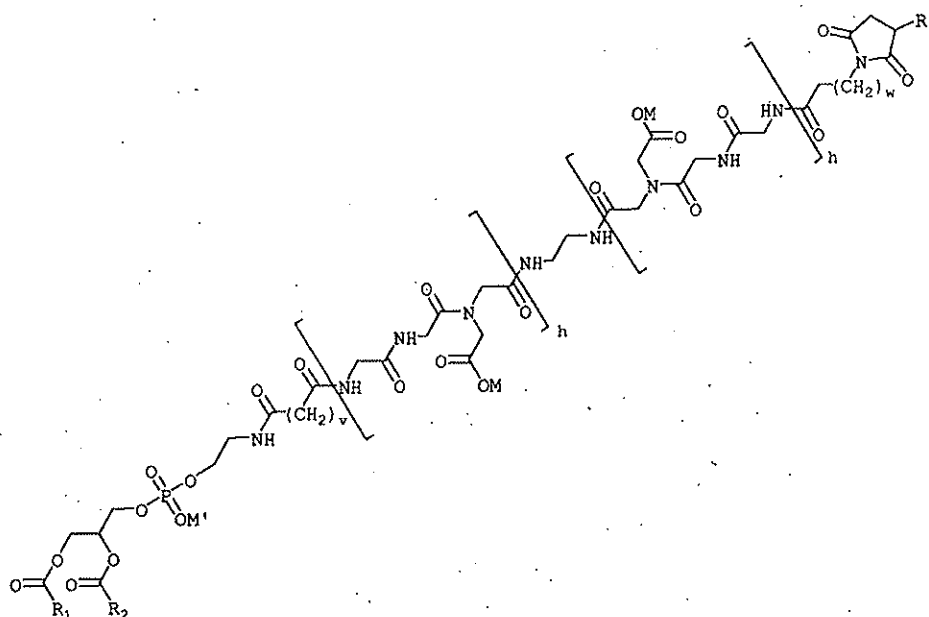
8) The functional lipid construct of claim 7 where F is a functional moiety selected from the group consisting of: carbohydrate, peptide, chemically reactive group, conjugator or fluorophore.

9) The functional lipid construct of claim 8 of the structure:



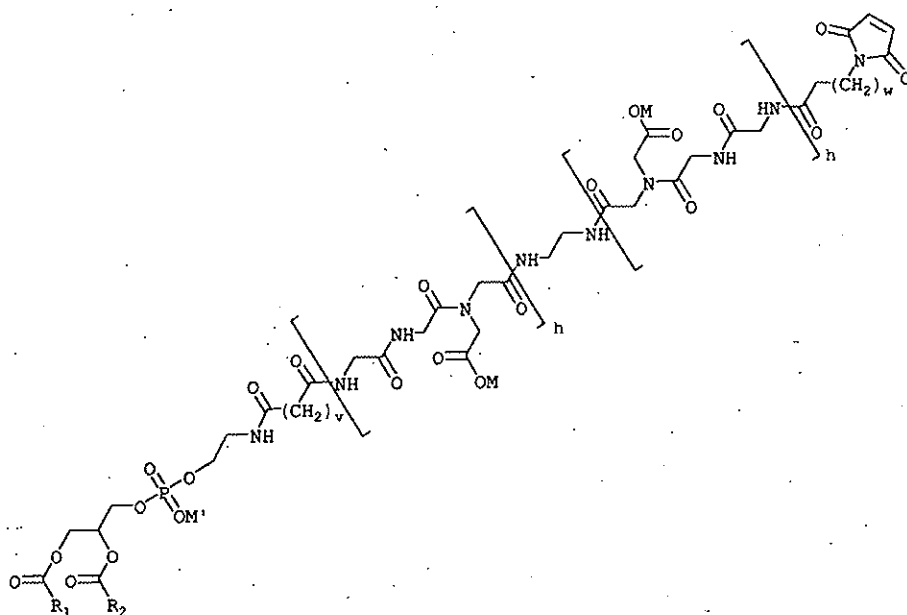
where F is a carbohydrate, x is the integer 2, 3 or 4, y is the integer 1, 2 or 3, and R₂ is O of a substituted hydroxyl of the carbohydrate.

- 10) The functional lipid construct of claim 8 of the structure:



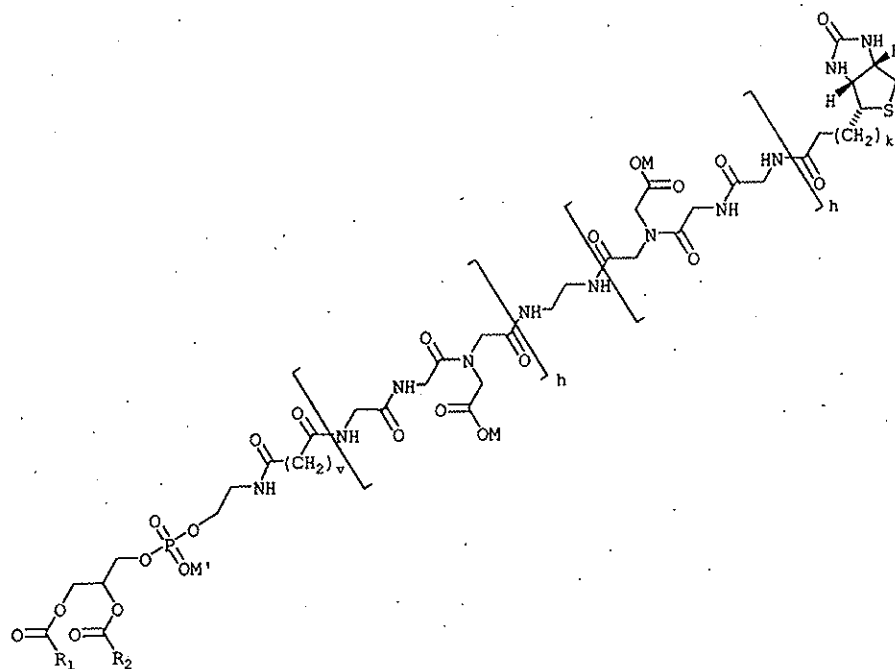
where F is a peptide, w is the integer 1 or 2, and R₃ is S of a substituted sulfhydryl of a Cys residue of the peptide.

- 11) The functional lipid construct of claim 8 of the structure:



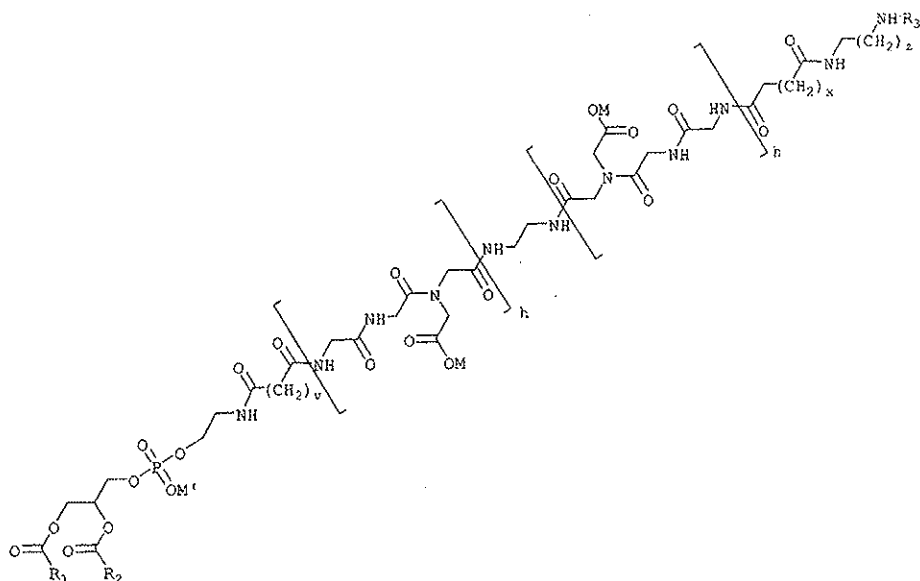
where F is the chemically reactive group maleimide and w is the integer 1 or 2.

- 12) The functional lipid construct of claim 8 of the structure:



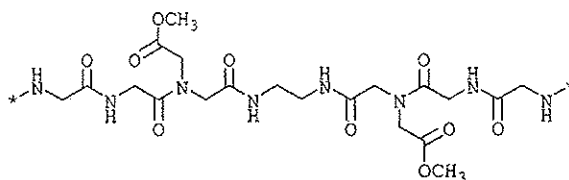
where F is the conjugator biotin and k is the integer 2, 3 or 4.

- 13) The functional lipid construct of claim 8 of the structure:

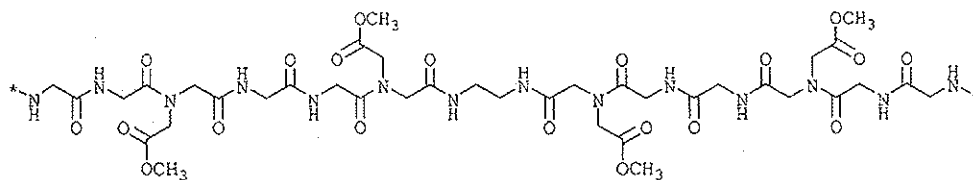


where F is the fluorophore of fluorescein (or one of its derivatives), x is the integer 2, 3 or 4, z is the integer 3, 4 or 5, and R₃ is C of the thiocyanate substituent of the isothiocyanate derivative of fluorescein (or one of its derivatives).

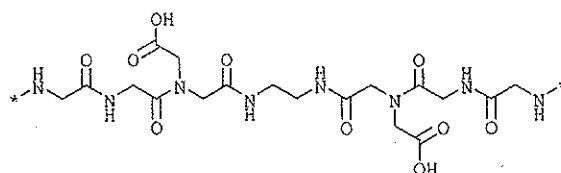
- 14) The functional lipid construct of any one of claims 8 to 13 where the substructure is:



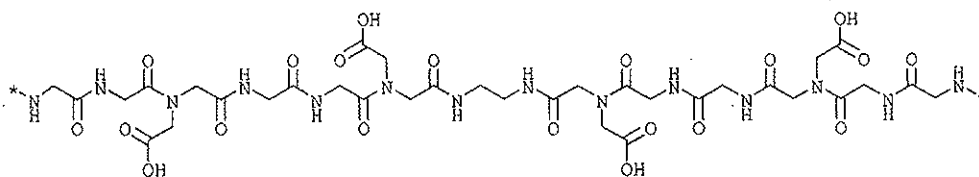
designated MCMG(1);



designated MCMG(2);

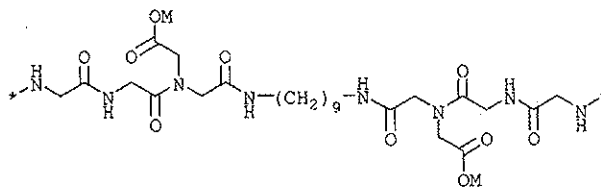


designated CMG(1); or



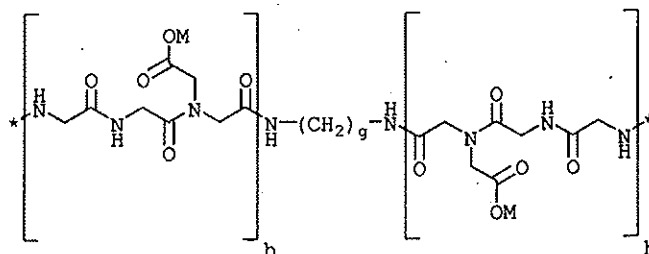
designated CMG(2).

- 15) A water soluble **peptide-lipid construct** of the structure F-S-L where F is a peptide, L is a diacyl or a dialkyl lipid, S is a spacer linked to F via a sulphide bond and includes the substructure:



where g is the integer 1, 2 or 3, M is a monovalent cation or substituent, and * is other than H.

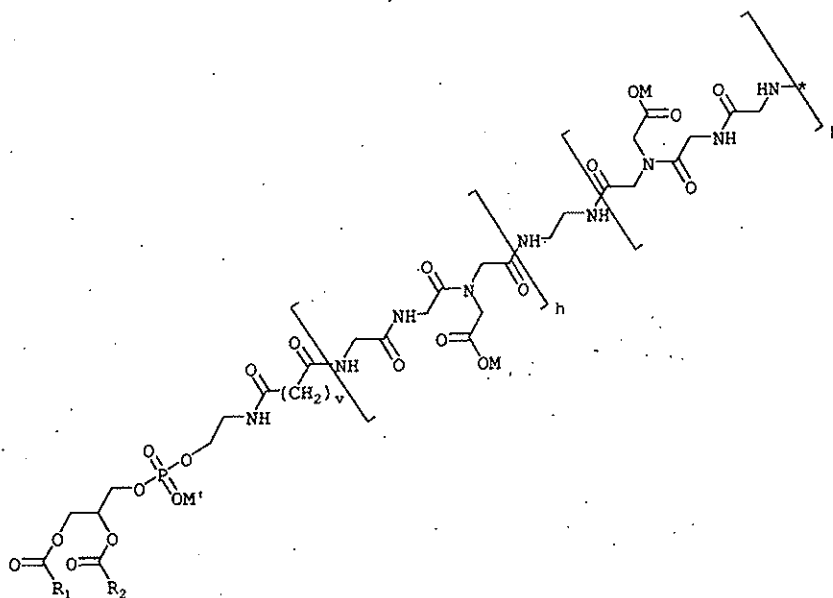
- 16) The peptide-lipid construct of claim 15 where the substructure is:



where h is the integer 1, 2, 3 or 4.

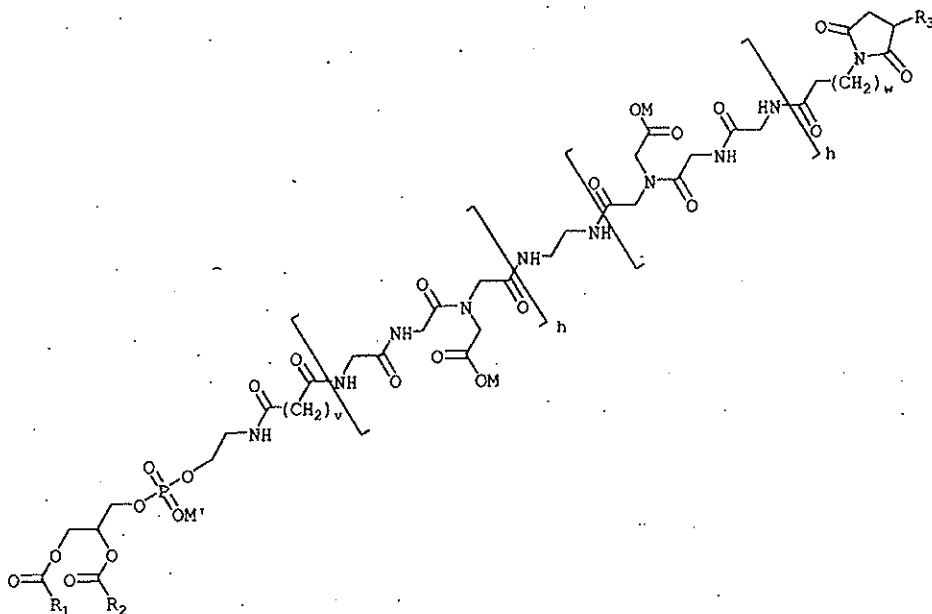
- 17) The peptide-lipid construct of claim 16 where g and h are the integer 2.
- 18) The peptide-lipid construct of claim 16 where M is H or CH₃.
- 19) The peptide-lipid construct of claim 16 where L is a diacylglycerophospholipid.
- 20) The peptide-lipid construct of claim 16 where L is phosphatidylethanolamine.

- 21) The peptide-lipid construct of claim 16 where the structure of the peptide-lipid construct includes the partial structure:



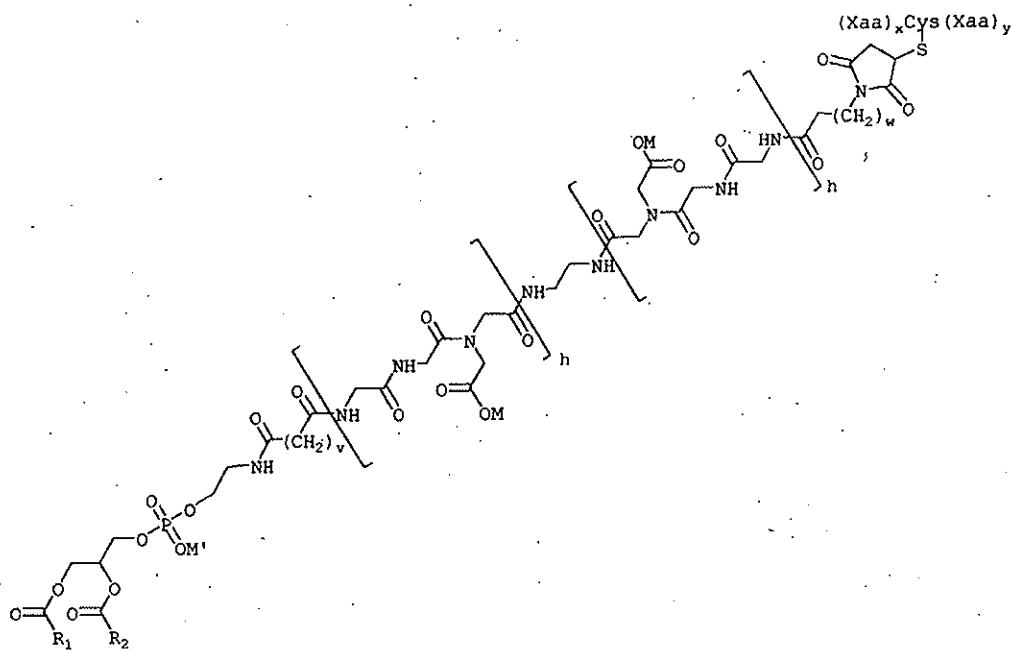
where v is the integer 3, 4 or 5, M' is a monovalent cation, and R_1 and R_2 are independently selected from the group consisting of: alkyl or alkenyl substituents of the fatty acids *trans*-3-hexadecenoic acid, *cis*-5-hexadecenoic acid, *cis*-7-hexadecenoic acid, *cis*-9-hexadecenoic acid, *cis*-6-octadecenoic acid, *cis*-9-octadecenoic acid, *trans*-9-octadecenoic acid, *trans*-11-octadecenoic acid, *cis*-11-octadecenoic acid, *cis*-11-eicosenoic acid or *cis*-13-docsenoic acid.

- 22) The peptide-lipid construct of claim 21 where the peptide-lipid construct is of the structure:



where w is the integer 1 or 2, and R₃ is S of a substituted sulfhydryl of a Cys residue (Cys) of the peptide.

The peptide-lipid construct of claim 22 where the peptide-lipid construct is of the structure:



and the sum of x and y is greater than 5.

- 24) The peptide-lipid construct of claim 23 where F is a peptide including a proximal terminal sequence (PTS) selected to promote solubility of the peptide.
- 25) The peptide-lipid construct of claim 24 where the PTS of the peptide is selected from the group consisting of:

SerLysLysLysLysGly

AlaAlaAlaAla

GlySerGlySerGly

26) The peptide-lipid construct of claim 22 where the Cys residue is a terminal Cys residue of the peptide (Cys).

27) The peptide-lipid construct of claim 26 where the terminal sequence of the peptide is selected from the group consisting of:

GlyLysLysLysLysSerCys

AlaAlaAlaAlaCys

GlySerGlySerGlyCys

CysSerLysLysLysLysGly

CysAlaAlaAlaAla

CysGlySerGlySerGly

28) The peptide-lipid construct of claim 27 where the Cys residue is a terminal Cys residue of the peptide at the carboxy-terminus of the peptide.

29) The peptide-lipid construct of claim 15 where F is a peptide comprising an epitope of antigens selected from the group consisting of: Glycophorin A, Glycophorin B, or mutations thereof (including the MNS blood group system).

30) The peptide-lipid construct of claim 26 where F is a peptide selected from the List of Peptides where (Xaa)_z is:

GlyLysLysLysLysSer

AlaAlaAlaAla

GlySerGlySerGly

SerLysLysLysLysGly

AlaAlaAlaAla

GlySerGlySerGly

31) The peptide-lipid construct of claim 30 where F is a peptide selected from the group consisting of:

CysThrTyrProAlaHisThrAlaAsnGlu

GlnThrAsnAspLysHisLysArgAspThrTyrAlaAlaAlaAlaAlaCys
 GlnThrAsnAspLysHisLysArgAspThrTyrGlySerGlySerGlyCys
 GlnThrAsnAspMetHisLysArgAspThrTyrGlySerGlySerGlyCys
 SerSerGlnThrAsnAspLysHisLysArgAspThrTyrCys
 ThrTyrProAlaHisThrAlaAsnGluValCys
 ThrTyrProAlaHisThrAlaAsnGluCys
 ProAlaHisThrAlaAsnGluValCys
 SerGlnThrAsnAspLysHisLysArgAspCys

- 32) The peptide-lipid construct of claim 15 where L is a glycerophospholipid selected from the group consisting of: 1,2-O-diioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE).
- 33) A **method** of detecting reactive antibody in the serum of a subject including the steps of:
- Contacting a sample of the serum with a suspension of cells modified to incorporate a functional lipid construct (F-S-L) of any one of claims 9, 10 or 15 to 32 to provide a mixture;
 - Incubating the mixture for a time and at a temperature sufficient to allow agglutination; and
 - Determining the degree of agglutination of the cells in the mixture;

where:

F is a carbohydrate or peptide comprising an epitope for the reactive antibody.

34) The method of claim 33 where the method includes the intermediate step of:

- Adding an anti-subject globulin antibody to the mixture prior to determining the degree of agglutination of the cells of the mixture.

35) The method of claim 34 where the anti-subject globulin antibody is anti-human globulin (AHG) antibody.

36) The method of claim 33 where F is a peptide and the method includes the preliminary step of:

- Adding an amount of the peptide to the sample of the serum;

where the amount of the peptide is sufficient to neutralize non-specific agglutination or confirm specificity of the reactive antibody.

37) The method of claim 33 where the reactive antibody is reactive to an antigen selected from the group consisting of: Glycophorin A, Glycophorin B, or mutations thereof (including the MNS blood group system).

38) The method of claim 33 where the subject is a human.

39) The method of claim 33 where the cells are red blood cells.

40) A **method** of preparing a peptide-lipid construct of any one of claims 15 to 32 including the step of:

- Reacting a peptide including a Cys residue with a functional lipid construct of claim 11.

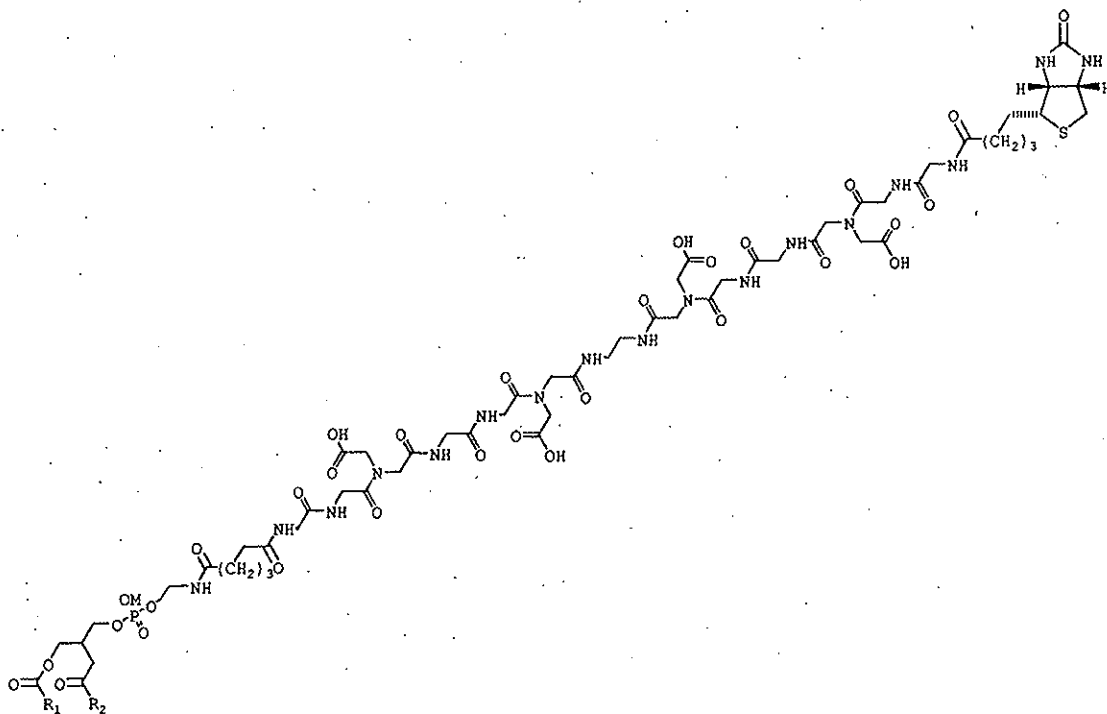
41) A **method** of effecting qualitative and quantitative changes in the functional moieties expressed at the surface of a cell including the step of:

- contacting the cell in vitro with a solution of a construct of any one of claims 1 to 32 for a time and at a temperature sufficient to allow the construct to incorporate into the cell.

42) A **method** of immobilizing one or more cells or multi-cellular structures including the steps of:

- Contacting the cells or multi-cellular structures with a solution of constructs of claim 12 for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells or multi-cellular structures to provide modified cells or multi-cellular structures; and
- Contacting the modified cells or multi-cellular structures with an avidin-coated substrate capable of being reversibly localized to a surface.

- 43) The method of claim 42 where the avidin-coated substrate is selected from the group consisting of: avidin-coated magnetic beads.
- 44) The method of claim 42 where the being reversibly localized to a surface is by application of a magnetic field.
- 45) The method of claim 44 including the steps of:
- Contacting the cells or multi-cellular structures with a dispersion of constructs of the structure:



for a time and at a temperature sufficient to allow an effective amount of the construct to incorporate into the cells or multi-cellular

structures to provide modified cells or multi-cellular structures;

- Contacting the modified cells or multi-cellular structures with avidin-coated magnetic beads; and
- Applying a magnetic field to localize the beads to a surface;

where R_1 and R_2 are both $(CH_2)_7CHCH(CH_2)_7$.

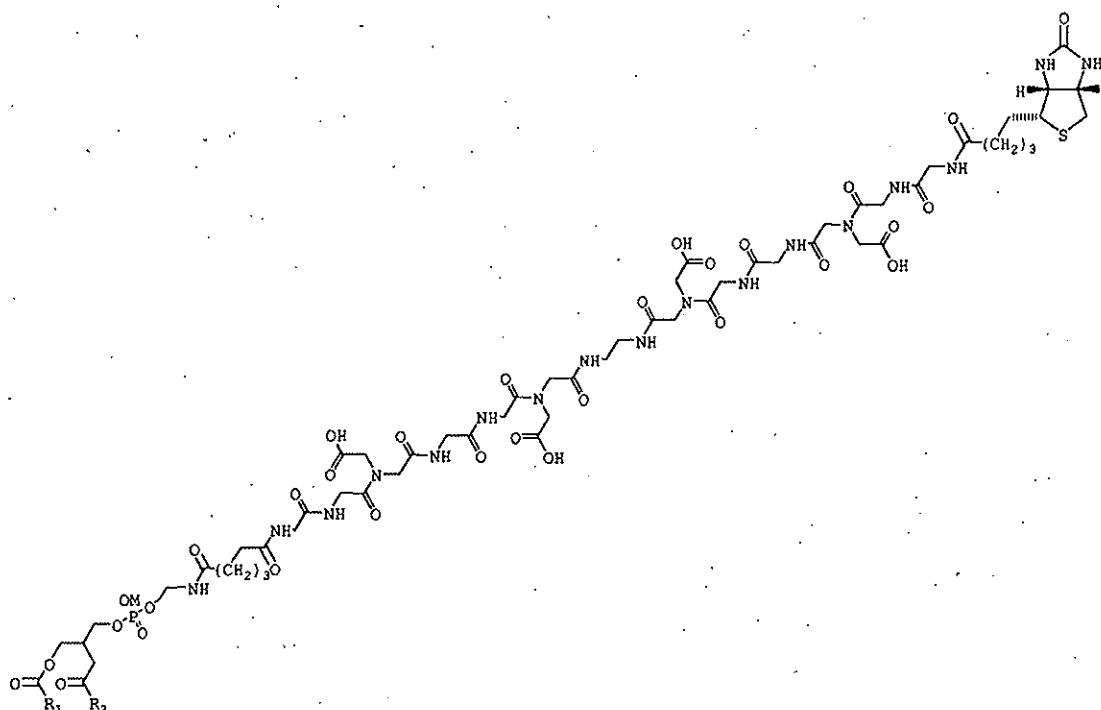
46) A **method** of promoting the aggregation of a first and second population of cells including the steps of:

- Contacting the first population of cells with a solution of constructs of claim 12 for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;
- Contacting the second population of cells with a solution of constructs of claim 12 for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;
- Contacting the modified cells of one of the populations with an excess of avidin; and

- Contacting the modified cells of the first and second populations.

47) The method of claim 46 including the steps of:

- Contacting the first population of cells with a solution of constructs of the structure:



for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the cells to provide modified cells of the first population;

- Contacting the second population of cells with a solution of the constructs for a time and at a temperature sufficient to allow an effective amount of the constructs to incorporate into the

cells to provide modified cells of the first population;

- Contacting the modified cells of one of the populations with an excess of avidin; and
- Contacting the modified cells of the first and second populations.