AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST : STANDARD PATENT

I/We being the person(s) identified below as the Applicant(s), request the grant of a patent to the person(s) identified below as the Nominated Person(s), for an invention described in the accompanying standard complete specification.

Full application details follow:

[71/70] Applicant(s)/Nominated Person(s):

Toa Medical Electronics Co., Ltd.

of

2-1, Minatojimanakamachi 7-chome, Chuo-ku, Kobe-shi, Hyogo-ken, Japan

[54] Invention Title:

A reagent for analyzing leucocytes

[72] Name(s) of actual inventor(s):

Takashi SAKATA
Takashi MORIKAWA
Kinya UCHIHASHI
Tomomi HASHIMOTO

[74] Address for service in Australia:

DAVIES COLLISON CAVE, Patent Attorneys, 1 Little Collins Street, Melbourne, Victoria, Australia. Attorney Code: DM

Basic Convention Application(s) Details:

[31] Application Number	[33] Country	Code	[32] Date of Application
5-324860	Japan	JP	22 December 1993

DATED this TWENTIETH day of DECEMBER 1994

Keith L Davies

a member of the firm of DAVIES COLLISON CAVE for and on behalf of the applicant(s)

M063390 201294

685875

AUSTRALIA

PATENTS ACT 1990

NOTICE OF ENTITLEMENT

We, **Toa Medical Electronics Co. Ltd.**, the applicant/Nominated Person in respect of Application No. 81578/94 state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person would, on the grant of a patent for the invention to the inventors, be entitled to have the patent assigned to the Nominated Person.

The Nominated Person is entitled to claim priority from the basic application listed on the patent request because the Nominated Person made the basic application.

DATED this TWENTIETH day of MARCH 1995

a member of the firm of
DAVIES COLLISON
CAVE for and on behalf
of the applicant(s)

(DCC ref: 1712373)

AU9481578

(12) PATENT ABRIDGMENT (11) Document No. AU-B-81578/94
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 685875

(54) Title
A REAGENT FOR ANALYZING LEUCOCYTES

International Patent Classification(s)
(51)[6] G01N 033/50

(21) Application No. : 81578/94 (22) Application Date : 20.12.94

(30) Priority Data

(31) Number	(32) Date	(33) Country
5-324860	22.12.93	JP JAPAN

(43) Publication Date : 29.06.95

(44) Publication Date of Accepted Application : 29.01.98

(71) Applicant(s)
TOA MEDICAL ELECTRONICS CO., LTD.

(72) Inventor(s)
TAKASHI SAKATA; TAKASHI MORIKAWA; KINYA UCHIHASHI; TOMOMI HASHIMOTO

(74) Attorney or Agent
DAVIES COLLISON CAVE , 1 Little Collins Street, MELBOURNE VIC 3000

(56) Prior Art Documents
EP 598663
EP 444241

In those apparatuses, leucocytes are classified into their subgroups (lymphocytes, monocytes, neutrophils, eosinophils and basophils) by the difference of RF signal intensity (changes in electric impedances at high-frequency), DC signal intensity (changes in direct current caused by the difference in the electroconductivity between the suspended particles and the liquid medium in which the particles are suspended), intensity of fluorescence, intensity of scattered light, absorbance, depolarization of scattered light or the like. In the classification and counting of leucocytes, it is necessary to pretreat the blood and lyse the erythrocytes so that the difference among each subgroup of the leucocytes according to the above-mentioned parameters can be detected.

.../2

Claim

1. A reagent for analyzing leucocytes comprising

(a) at least one cationic or amphoteric surfactant in an amount sufficient for lysing erythrocytes and cause damage to a part of cell membranes of leucocytes;

(b) at least one organic compound selected from the group consisting of acidic dyes, 8-anilino-1-naphthalenesulfonic acid, 6-(p-toluidino)-2-naphthalenesulfonic acid, chromotropic acid, phthalic acid, naphthalenesulfonic acid, and their corresponding salts in an amount sufficient for making a morphological difference among leucocytes by combining with a cationic component in leucocytes;

(c) a nonionic surfactant; and

(d) a buffer for adjusting pH.

AUSTRALIA

PATENTS ACT 1990

COMPLETE SPECIFICATION

NAME OF APPLICANT(S):

Toa Medical Electronics Co., Ltd.

ADDRESS FOR SERVICE:

DAVIES COLLISON CAVE
Patent Attorneys
1 Little Collins Street, Melbourne, 3000.

INVENTION TITLE:

A reagent for analyzing leucocytes

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

BACKGROUND OF THE INVENTION.

1. Field of the Invention.

The present invention relates to a reagent for analyzing leucocytes, especially for classifying and counting leucocytes using a classifying apparatus for leucocytes in the field of a clinical test.

2. Description of the Prior Art.

In the field of a clinical test, it is important for the diagnosis of various diseases to classify and count leucocytes using the whole blood of patients.

For such a purpose, many apparatuses and methods have been reported.

In those apparatuses, leucocytes are classified into their subgroups (lymphocytes, monocytes, neutrophils, eosinophils and basophils) by the difference of RF signal intensity (changes in electric impedances at high-frequency), DC signal intensity (changes in direct current caused by the difference in the electroconductivity between the suspended particles and the liquid medium in which the particles are suspended), intensity of fluorescence, intensity of scat-

tered light, absorbance, depolarization of scattered light or the like. In the classification and counting of leucocytes, it is necessary to pretreat the blood and lyse the erythrocytes so that the difference among each subgroup of the leucocytes according to the above-mentioned parameters can be detected.

For such a purpose, for example, leucocytes are shrunk to a suitable size using a lysing agent or specific cells are stained. The lysing agent as used in shrinking leucocytes is an organic carboxylic acid, organic sulfonic acid or phenol (WO88/07187), or two kinds of cationic surfactants (WO 84/03771 and WO 84/02777). Examples of methods for lysing erythrocytes and staining specific cells are a method in which the blood is treated with a reagent comprising the combination of an erythrocyte lysing agent (saponin and/or sodium dodecylsulfate), a tertiary or quaternary ammonium salt, an alcohol, a polyoxyethylene sorbitan ester, glutaraldehyde or formaldehyde, alkylene glycol, a physiological salt and Chlorazol Black (Japanese Laid-Open Patent Publication Hei-03/266999 (corresponding to: FR8915166)) and a method in which blood is treated with a reagent comprising the combination of sodium dodecylsulfate, formaldehyde, sugar (or sugar alcohol) and a buffer, followed by subjecting to a peroxidase staining (Japanese Laid-Open Patent Publication Sho-62/071857 (corresponding to: U.S. Patent Nos. 4,801,549 and 4,978,624)).

In the above-mentioned WO 84/03771 and WO 84/02777, the classification and counting of leucocytes are carried out by means of the difference in the DC signal intensity, which is convenient but shows a disadvantage that leucocytes can be only classified into three subgroups.

Methods according to Japanese Laid-Open Patent Publication Sho-62/071857 and Hei-03/266999 can classify leucocytes into four or more subgroups, but require a treatment at a high temperature and an fixation using a toxic aldehyde.

Further, in the method of WO88/07187, leucocytes are detected by means of RF signal, DC signal or the intensity of scattered light which however is a drawback that the detectors are complicate, large in size and expensive.

SUMMARY OF THE INVENTION

An object of the present invention is to offer a reagent which can classify leucocytes at least into four subgroups using an inexpensive and simple apparatus, and without using such toxic agent as aldehydes.

The present invention provides a reagent for analyzing leucocytes comprising (a) at least one cationic or amphoteric surfactant; in an amount sufficient for lysing erythrocytes and causing damage to a part of cell membranes of leucocytes; (b) at least one organic compound selected from the group consisting of acidic dyes, 8-anilino-1-naphthalenesulfonic acid, 6-(p-toluidino)-2-naphthalenesulfonic acid, chromotropic acid, phthalic acid, naphthalenesulfonic acid, and their corresponding salts in an amount sufficient for making a morphological difference

AUSTRALIAN PATENT OFFICE

among leucocytes by combining with a cationic component in leucocytes; (c) a nonionic surfactant; and (d) a buffer for adjusting pH.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing leucocytes of the present invention which uses decyltrimethylammonium bromide as an ionic surfactant.

Fig. 2 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing leucocytes of the present invention which uses lauryltrimethylammonium chloride as an ionic surfactant.

Fig. 3 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses myristyltrimethylammonium bromide as an ionic surfactant.

Fig. 4 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses cetyltrimethylammonium chloride as an ionic surfactant.

Fig. 5 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses Alizarin Violet 3R as an organic compound.

Fig. 6 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses Alphazurine A as an organic compound.

Fig. 7 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses Guinea Green B as an organic compound.

Fig. 8 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses Alizarin Yellow-R as an organic compound.

Fig. 9 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses methyl alcohol as an alcohol.

Fig. 10 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses ethyl alcohol as an alcohol.

Fig. 11 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses 2-propanol as an alcohol.

Fig. 12 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses 2-phenoxyethanol as an alcohol.

Fig. 13 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses MES as a buffer.

Fig. 14 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses HEPES as a buffer.

Fig. 15 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses TRIS as a buffer.

Fig. 16 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses BC-25TX as a nonionic surfactant.

Fig. 17 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention which uses BC-25TX and HCO-60 are jointly used as a nonionic surfactant.

Fig. 18 is a scattergram showing the intensity of forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention.

Fig. 19 is a scattergram showing the volume on the principle of measuring electric resistance and the intensity of side scattered light measured with the reagent for analyzing the leucocytes of the present invention.

Fig. 20 is a scattergram showing the intensity of

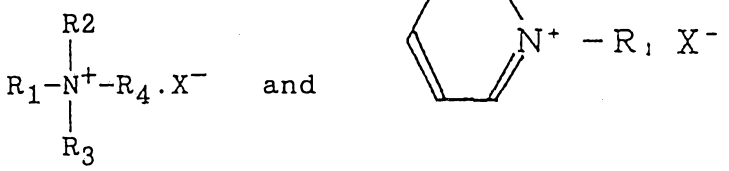
forward low-angle scattered light and the intensity of forward high-angle scattered light measured with the reagent for analyzing the leucocytes of the present invention.

Fig. 21 is an outline chart of an apparatus for measuring the samples treated with the reagent for analyzing the leucocytes of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The ionic surfactant in the reagent for analyzing leucocytes in accordance with the present invention comprises at least one cationic surfactant or amphoteric surfactant.

Preferred cationic surfactants are quaternary ammonium salt type or pyridinium salt type. The quaternary ammonium salt type and pyridinium salt type surfactants can be represented by the formula;



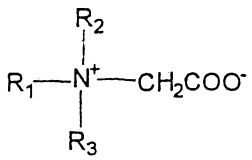
and

(R_1 is a C_{6-18} alkyl or alkenyl group; R_2 and R_3 are a C_{1-4} alkyl or alkenyl group; R_4 is a C_{1-4} alkyl or alkenyl group, or benzyl group; and X is a halogen atom). The total number of carbon atoms in the above formula is preferably in the range of 9-30.

Examples of the C_{6-18} alkyl or alkenyl group for R_1 are

hexyl, octyl, decyl, dodecyl or tetradecyl, or hexenyl, heptenyl or octenyl, preferably, a linear alkyl such as octyl, decyl or dodecyl. Examples of the C_{1-4} alkyl or alkenyl groups for R_2 and R_3 are methyl, ethyl, propyl or butyl, or propenyl, preferably, a C_{1-3} alkyl such as methyl, ethyl or propyl. Examples of the C_{1-4} alkyl and alkenyl groups for R_4 are methyl, ethyl, propyl or butyl, or propenyl, preferably, methyl, ethyl or propyl.

The amphoteric surfactant can be represented by the following formula;



(R_1, R_2 and R_3 are the same as defined above).

The total number of carbon atoms in the above three formula is preferably in the range of 9-30.

The above-mentioned cationic or amphoteric, referred to hereinafter in the description as the ionic surfactant, is used in a sufficient amount to lyse erythrocytes and cause damage to a part of cell membranes of leucocytes. Specifically, it is suitable to use about 30-5,000 mg/liter to the total volume of the regent, preferably about 50-3,000 mg/liter or, more preferably about 100-2,000 mg/liter, though it can be suitably modified depending upon the type of surfactant used or the like. Suitable amounts (concentrations) for each of the ionic surfactants in the reagent are given in Table 1. The surfactant may be used singly or in a combination of

AUSTRALIAN PATENT OFFICE

two or more surfactants.

Table 1.

Surfactant	Amount (mg/liter)
Octyltrimethylammonium bromide (OTAB)	1000-5000
Decyltrimethylammonium bromide (DTAB)	200-3000
Lauryltrimethylammonium chloride (LTAC)	150-2000
Myristyltrimethylammonium bromide (MTAB)	100-1500
Cetyltrimethylammonium chloride (CTAC)	50-1000
Stearyltrimethylammonium bromide	50- 500
Cetyldimethylethylammonium bromide	50- 500
Laurylpyridinium chloride	50- 500
Lauryldimethylaminoacetic acid betaine	500-3000
Stearyldimethylaminoacetic acid betaine	500-3000

The ionic surfactant ~~is required to~~ preferably possess a hemolytic activity of such an extent that makes pores in cell membrane of leucocytes which can pass an organic compound as discussed later but is not potent to make the cell nuclei naked. As the ionic surfactants, conventional cationic surfactants (e.g., LTAC, MTAB and CTAC) are usable but are used in far smaller amount than an amount for making the cell nuclei naked, so as to inhibiting their own homolyti· activities. Ionic surfactants possessing low hemolytic activity also are usable because it is sufficient to pull a part of membrane out. The hemolytic activity of the ionic surfactant is in proportion to the carbon numbers of the hydrophobic group and, the more the carbon numbers, the more the hemolytic activity, therefore cationic surfactants having a low hemolytic activity such as DTAB and OTAB or amphoteric surfactant are preferably used.

In addition to the ionic surfactant, the reagent in

accordance with the present invention contains an organic compound having an anionic group which makes a morphological difference among leucocytes by combining with a cationic component in leucocytes. The organic compound may be an acidic dye. They are not particularly limited but nearly all kinds of acidic dyes can be used. Further, because there is no necessity of measuring the absorbency and the fluorescence intensity other organic compounds other than dyes are applicable as well. Examples of the acidic dyes are Amido Black [Colour Index No. 20470], Alizarin Cyanine Green F [CI No. 61570], Acid Green 27 [CI No. 61580], Acid Blue 62 [CI No. 62045], Direct Red 31 [CI No. 29100], Brilliant Sulphaflavine [CI No. 56205], Alizarin Yellow R [CI No. 14030], Acid Blue 129 [CI No. 62058], Acid Green 25 [CI No. 61570], Chromotrope 2R [CI No. 16570], Coomassie Brilliant Blue R-250 [CI No. 42660], Carmine Acid [CI No. 75470], Coomassie Brilliant Blue G-250 [CI No. 42655], Carmoisine B [CI No. 14720], Direct Blue 86 [CI No. 74180], Ethyl Red [2-(4-diethylaminophenylazo)benzoic acid], Para Rosaniline [CI No. 42500], Violamine R [CI No. 45190], Acid Yellow 34 [CI No. 18890], Acid Orange 51

AUSTRALIAN PATENT OFFICE

[CI No. 26550], Brilliant Crocein MOO [CI No. 27290], Guinea Green B [CI No. 42085], Acid Blue 29 [CI No. 20460], Rhodamine B [CI No. 45170], Sulforhodamine B [CI No. 45100], Lissamine Green B [CI No. 44090], Acid Blue 9 [CI No. 42090], Fast Green FCF [CI No. 42053], Azocarmine B [CI No. 50090], Aniline Blue [CI No. 42780], Alphazurine A [CI No. 42080], Alizaline Violet 3R [CI No. 61710], Acid Blue 41 [CI No. 62130], Bieblich Scarlet [CI No. 26905], Erythrosin B [CI No. 45430], Methyl Red [CI No. 13020], Methyl Orange [CI No. 13025], Orange I [CI No. 14600], etc. Examples of the organic compounds other than dyes are aromatic organic acids having hydrophobic group and acidic functional group or acids having hydrocarbon of at least six carbon atoms or having heterocylic ring. More specificaliy, their examples are 8-anilino-1-naphthalenesulfonic acid, 6-(p-toluidino)-2-naphthalenesulfonic acid, chromotropic acid, phthalic acid or naphthalenesulfonic acid, or its corresponding salt. The amount of such organic compound may be suitably chosen, depending upon the type of surfactant used or the like and is, preferably, about 50-5,000 mg/liter to the total volume of the regent or, more preferably about 100-3,000 mg/liter.

The reagent in accordance with the present invention further contains a nonionic surfactant. There is no particular limitation for the nonionic surfactant and nearly all kinds of nonionic surfactants usually used as solubilizer

may be used. Its examples are nonionic surfactants having polyoxyethylene glycol (POE), polypropylene glycol (POP) or a block copolymer of polyoxyethylene glycol-polypropylene glycol (POE-POP) as a hydrophilic component. When only one kind of nonionic surfactant is used, it may cause such side effect of lysing leucocytes in addition to the desired lysis of an insoluble substance formed between the ionic surfactant with a cell-constituting component in erythrocytes. In such case, it is preferable to use a combination of two or more nonionic surfactants such as those wherein the additional mole number of the hydrophilic group are different. Alternatively, the combined use of nonionic surfactants wherein the structures of the lipophilic groups are different can inhibit the side effect. The amount of the nonionic surfactant which is necessary for dissolving the insoluble substance varies depending upon the type of ionic surfactant used or the like and is generally in the range of about 0.5-10 g/liter to the total volume of the regent, preferably about 1-8 g/liter.

The reagent in accordance with the present invention further contains a buffer for adjusting pH. Usually, there is no particular limitation for the buffer so far as it is used for keeping pH constant and any buffer having a pKa of "a desired pH ± 2.0" may be used. The examples are MES, TRIS, HEPES, succinic acid, phthalic acid or citric acid

buffers. In the present invention, it is preferable to adjust pH of the reagent to about 5-11 whereby the amount used is around 5-100 mM for the reagent.

An alcohol may be further used in the reagent without particularly limitation. Preferable alcohols are those easily available at low cost in an industrial scale, such as alkanols (e.g. methyl alcohol and ethyl alcohol) and alcohols having an aromatic ring (e.g. phenethyl alcohol and 2-phenoxyethanol). With respect to its amount, about 5-20% to the entire amount of the reagent is preferable in the case of methanol and a very rough yardstick is that, when there is an increase of one carbon, the amount is preferably halved. In the case of 2-phenoxyethanol, the preferred amount is 0.05-1%.

A metal salt may be added in the reagent. Preferred examples thereof are alkali metal salts such as sodium chloride, potassium chloride and lithium chloride. Usually, it is not necessary to use the alkali metal salt but, in the case of the apparatus wherein the measurement is conducted using a signal of electric resistance as mentioned later, the alkali metal is needed to adjust the electroconductivity of the sample to the value which is suitable for the measurement. The preferred amount in such a case is in the extent that the electroconductivity of the solution is about 5-20 mS/cm.

The reagent for analyzing leucocytes in accordance with

present invention may be composed of two liquids comprising a first liquid and a second liquid. The first liquid contains at least one organic compound in a sufficient amount to make a morphological difference among leucocytes by combining with a cationic component in leucocytes. The second liquid contains at least one ionic surfactant in a sufficient amount to be able to lyse erythrocytes and cause damage to a part of the cell membrane of leucocytes. The first and second liquid contain a nonionic surfactant and a buffer for adjusting pH. Like a single liquid type, the liquid may contain the alcohol and the metal salt. Concentration of each of the components is made in such an extent that, when the first and the second liquids are mixed, the above-mentioned concentration for each of the components is resulted. The reagent composed of the two liquids may be improved on its stability in storage.

The reagent may be used by mixing with the whole blood. In that case, the mixing ratio of the reagent to the whole blood is in the range from 1 : 2 to 1 : 100 by volume. Incidentally, the blood sample diluted with a suitable diluent is also available.

Using the reagent for analyzing leucocytes in accordance with the present invention, the classification and counting of leucocytes can be suitably carried out especially with a small analyzing apparatus using a semiconductor

laser. That is, the reagent can be suitably used by means of an apparatus wherein leucocytes can be classified/counted only by receiving the two parameters of forward low-angle and high-angle scattered light. There is no particular limitation for the analyzing apparatus and, for example, an apparatus equipped with a simple detecting part as shown in Fig. 21 may be used. The apparatus of Fig. 21 is equipped with a device which detects a two-angle forward scattered light. That is, in the apparatus of Fig. 21, there is a semiconductor laser LD before the flow cell CELL via a contender lens L2 and a collimater lens L1. Further, there is a photodiode PD after the flow cell CELL via a collector lens L3 equipped with a beam stopper BS. In the apparatus, the intensity of the scattered light of low angles (about 1-5°) and high angles (about 6-20°) among the forward scattered light is measured, and by means of the difference among the intensity of the scattered light, leucocytes can be classified at least into four subgroups and counted.

By the reagent of the present invention, leucocytes can be conveniently classified/counted using a flow cytometer.

In accordance with the reagent of the invention:

(1) the ionic surfactant may lyse erythrocytes in whole blood, damage the cell membrane of leucocytes and accelerate the permeability of the organic compound through the cell membrane;

(2) the organic compound may adjust the morphological

information of the leucocytes in such a manner that is convenient for classifying leucocytes;

(3) the nonionic surfactant may solubilize the insoluble substance formed by combining of the ionic surfactant with the organic compound; and

(4) the buffer may keep pH of the reagent to a certain value.

Ionic surfactants usually hemolyze erythrocytes and dissolve the cell membrane of leucocytes to make its nuclei naked; therefore, the method of classifying leucocytes into three subgroups by determining the volume information of them has already been used widely as a simple and convenient means for classifying leucocytes. However, in this method, the hemolytic action is so strong that most of leucocytes are made naked whereby it is not possible to morphologically classify leucocytes into subtypes. On the other hand, the reagent of the present invention utilizes fundamentally the same principle of the action of the ionic surfactant as in the above mentioned method, i.e., the ionic surfactant lyses erythrocytes and damages the membrane of leucocytes. However, the homolylic action required in the reagent of the present invention is to draw out a part of cell membrane (possibly a part of the lipid components, resulting in forming pores in erythrocytes which can pass hemoglobin and small pores in leucocytes which can pass through the organic

compound, while the nucleus of leucocytes is not naked. As a result, hemoglobin is flown out of erythrocytes whereby the erythrocytes hardly scatter the light whereupon they can be discriminated from leucocytes by optical information. As a result of a partial flowing-out of cell membrane and the liquid in the leucocytes, the partly-damaged leucocytes significantly reduce the scattered light on the cell surface whereby the information in the cells can be obtained in more detail.

It has been well known that the ionic surfactant increases its solubilizing ability, depending upon the concentration at the concentration higher than the CMC of the surfactant. In the conventional method, leucocytes are made naked using the above-mentioned solubilizing ability of the surfactant, while, in the present invention, the ionic surfactant is used in a low concentration and, therefore, the solubilizing ability of the ionic surfactant is low whereby, quite unexpectedly, the positive charge in the ionic surfactant neutralizes the negative charge of anionic components (nuclei, granules, RNA, etc.) in the cells to make them insoluble. As a result, even when leucocytes are treated with the ionic surfactant, the shape of the cells hardly changes whereby the effect of keeping the cell shape which is similar to that achieved by fixing the cell by an aldehyde can be resulted. Consequently, the reagent of the present invention does not give an influence on analytical

result by change of treating conditions as period and temperature, differently from the known method for classifying leucocytes into three subgroups by use of the ionic surfactant. The sample treated with the reagent of the present invention is stable for a long period.

Furthermore, the organic compound acts to insolubilize the cationic component in leucocyte cells (e.g. granules [eosinophilic granules], protein, etc.) whereby the similar effect as the fixing with an aldehyde is obtained and, probably, the morphological difference (e.g. a difference in the scattered light) is made among leucocytes depending upon the amount of the cationic component. In other words, it is likely that, as a result of a neutralization of the positive charge of the cationic component in the cells to make them insoluble, their flowing-out to outside of the cells can be inhibited, the morphological information of leucocytes is maintained and the shape of leucocytes can be changed so as to be convenient for the classification.

In the present invention, the nonionic surfactant is used to solubilize the insoluble substance which is produced by a combination of the ionic surfactant with the organic compound.

Furthermore, in the present invention, the presence of the ionic surfactant in a small amount inhibits an excessive solubilization.

In the present invention, though the alcohol is not always necessary, if added, it is effective in terms of the following respects. The alcohol has an action of potentiating the action of an ionic surfactant selectively whereby the ionic surfactant even in a low concentration can cause damage to the cell membrane. In addition, it is likely that the alcohol denatures the protein contained in the cell to make it insoluble. Accordingly, when the alcohol is used, it is possible that the damage of the cell such as the loss of the cell membrane and granule is kept minimum and consequently that the desired damage of the cell membrane is caused. Therefore, it has an effect of maintaining the optical difference by the scattered light, etc. Moreover, it exhibits an action of accelerating the lysis of erythrocytes. Therefore, even though the membrane of erythrocytes may be hardly lysed in some diseases such as cirrhosis, the additional of the alcohol is effective in such a case, in turn, erythrocytes are lysed and the damage of leucocytes is inhibited. It is probable that the alcohol hurts the cell membrane of erythrocytes by a mechanism different from that of the ionic surfactant.

Preferred reagents of the reagent for analyzing the leucocytes in accordance with the present invention will be given as hereunder.

Example 1.

Ionic surfactant	100–500 mg
Magnesium 8-anilino-1-naphthalenesulfonate (an organic compound)	2 g
BC30TX (a nonionic surfactant: polyoxyethylene (30) cetyl ether; Nikko Chemicals)	1 g
HEPES	10 mM
Methanol	100 ml
NaOH	sufficient for pH 7.0
Distilled water	q. s. to 1000 ml

The reagent (1 ml) of the above-mentioned composition in which various kinds of surfactants in various concentrations were used as an ionic surfactant was mixed with 30 microliters of blood and, after 30 seconds, the forward low-angle scattered light and the forward high-angle scattered light were measured using a flow cytometer.

Figs. 1–4 show the results wherein 750 mg/liter of decyltrimethylammonium bromide (DTAB), 500 mg/liter of lauryltrimethylammonium chloride, 500 mg/liter of myristyltrimethylammonium bromide and 100 mg/liter of cetyltrimethylammonium chloride were used as the ionic surfactant, respectively. In the drawings, L is lymphocyte, M is monocyte, N is neutrophil and basophil and E is eosinophil. In all of the ionic surfactants, nearly the same scattergrams were resulted whereby leucocytes were able to be classi-

fied and counted.

Example 2.

DTAB (a cationic surfactant)	1.5 g
An organic compound	0.3–3 g
BC30TX (a nonionic surfactant; Nikko Chemicals)	1 g
Citric acid	50 mM
Methanol	100 ml
NaOH	sufficient for pH 7.5
Distilled water	q. s. to 1000 ml

The reagent (1 ml) of the above-mentioned composition in which various kinds of organic compounds in various concentrations were used as the organic compound was mixed with 30 microliters of blood and, after 30 seconds, the forward low-angle scattered light and the forward high-angle scattered light were measured using a flow cytometer.

Figs. 5–8 show the results where 3 g/liter of Alizaline Violet 3R, 0.3 g/liter of Alphazurine A, 0.3 g/liter of Guinea Green B and 3 g/liter of Alizarin Yellow R were used, respectively. In all of the organic compounds used, nearly the same scattergrams were obtained whereby the leucocytes were able to be classified and counted.

Example 3.

DTAB	1.5 g
Brilliant Crocein M00 (an organic compound)	1 g
HEPES	10 mM
HCO50 (a nonionic surfactant; polyoxyethylene (50) hydrogenated castor oil; Nikko Chemicals)	4 g
Alcohol	q.s.
NaOH	sufficient for pH 7.0
Distilled water	q. s. to 1000 ml

The reagent (1 ml) of the above-mentioned composition in which various kinds of alcohols in various concentrations were used as the alcohol was mixed with 30 microliters of blood and, after 30 seconds, the forward low-angle scattered light and the forward high-angle scattered light were measured using a flow cytometer.

Figs. 9-12 show the results when 100 ml/liter of methyl alcohol, 50 ml/liter of ethyl alcohol, 25 ml/liter of 2-propanol and 2.5 ml/liter of 2-phenoxyethanol were used as the alcohol, respectively. The erythrocytes were well contracted in case any of the alcohols was used whereby the leucocytes were able to be counted giving nearly the same scattergrams. Thus, the leucocytes were able to be classified and counted.

Example 4.

DTAB	1.5 g
Magnesium 8-anilino-1-naphthalenesulfonate (an organic compound)	2 g
HCO50 (a nonionic surfactant; Nikko Chemicals)	4 g
Buffer	10 mM
2-Phenoxyethanol	2.5 ml
NaOH	q.s.
Distilled water	q. s. to 1000 ml

The reagent (1 ml) of the above-mentioned composition in which various kinds of buffers in various concentration were used for changing pH was mixed with 30 microliters of blood and, after 30 seconds, the forward low-angle scattered light and the forward high-angle scattered light were measured using a flow cytometer.

Figs. 13-15 show the results where MES (pH: 5.5), HEPES buffer (pH: 7.0) and TRIS buffer (pH: 8.5) were used, respectively. Though there was a tendency that the intensity of the scattered light of the leucocytes decreased as pH of the reagent goes up, it was still possible to classify and count the leucocytes.

Example 5.

DTAB	1.0 g
Magnesium 8-anilino-1-naphthalenesulfonate (an organic compound)	1.5g
BC-25TX (a nonionic surfactant; polyoxyethylene (25) cetyl ether; Nikko Chemicals)	4 g
HEPES	10 mM
Methanol	100 ml
NaOH	sufficient for pH 7.0
Distilled water	q. s. to 1000 ml

The reagent (1 ml) of the above-mentioned composition was mixed with 30 microliters of blood and, after 30 seconds, the forward low-angle scattered light and the forward high-angle scattered light were measured using a flow cytometer.

Fig. 16 shows the result where 3.0 g/liter of BC-25TX was added as a nonionic surfactant for solubilizing the precipitate formed by a combination of the components for constituting erythrocytes with the ionic surfactant. In Fig. 16, not only the effect for solubilizing the precipitate but also the influence on the leucocytes were noted. Thus, among the leucocytes, those W which were other than the eosinophils were contracted whereby it was not possible to classify into four groups.

Now, the nonionic surfactant used (i.e. BC-25TX) was

reduced to 2.0 g/liter and 1.0 g/liter of HCO-60 (Nikko Chemicals; additional mole number of polyoxyethylene was 60) which was a type of polyoxyethylene hydrogenated castor oil was added thereto whereby the result was that, as shown in Fig. 17, no precipitate was produced and a good scattergram classifying the leucocytes into four groups was achieved.

Example 6.

DTAB	1.5 g
Magnesium 8-anilino-1-naphthalenesulfonate (an organic compound)	2 g
HCO 50 (a nonionic surfactant; polyoxyethylene (50) hydrogenated castor oil; Nikko Chemicals)	4 g
PEN 4630 (a nonionic surfactant; polyoxyethylene (30) polyoxypropylene (6) 2-decyl tetradecyl ether; Nikko Chemicals)	4 g
Phthalic acid	50 mM
2-Phenoxyethanol	2.5 ml
NaOH	sufficient for pH 5.5
NaCl	30 mM
Distilled water	q. s. to 1000 ml

One ml of the reagent of the above composition and 30 microliters of blood were mixed and, after 30 seconds, a measurement was conducted using a flow cytometer.

Fig. 18 shows the result of the measurement of forward low-angle scattered light for determining the size informa-

tion and also the result of the measurement of forward high-angle scattered light for determining the morphological information. Furthermore, Fig. 19 shows the results of measuring the lateral scattered light for determining the morphological information and of measuring the volume by a principle of measurement by means of an electric resistance for determining the size information using a flow cytometer whereby the electric resistance signals were able to be measured as disclosed in the Japanese Laid-Open Patent Publication Hei-05/034,251. (which corresponds to U.S. Patent No. 5,264,369)

In the above-mentioned reagent composition the electroconductivity of the sample to be measured was adjusted to a suitable value by adding sodium chloride thereto for measurement on the principle of electric resistance.

The leucocytes were able to be clearly classified and counted by any of the measuring parameters.

Example 7.

A first liquid

Ammonium 8-amilino-1-nathphalenesulfonate (an organic compound) 3.0 g

HCO 50 (a nonionic surfactant; polyoxyethylene (50) hydrogenated castor oil; Nikko Chemicals) 5 g

PEN 4630 (a nonionic surfactant; polyoxyethylene (30) polyoxypropylene (6) 2-decyl tetradecyl ether; Nikko Chemicals) 1.3 g

Phthalic acid	2.5 g
Succinic acid 2Na $6H_2O$	13.5 mg
2-Phenoxyethanol	4.7 ml
NaOH	sufficient for the pH 5.3
NaCl	2.9 g
Distilled water	q. s. to 1000 ml

A second liquid

The second liquid had the same composition and concentration as the first liquid except that DTAB 6.9 g/liter was used in place of ammonium 8-anilino-1-nathphalene-sulfonate.

One ml of the first liquid of the above composition and 33 µl of blood were mixed, then 0.2 ml of the second liquid was added, and a mixture was reacted for 20 second at 35°C, followed by measuring the forward low-angle scattered light and the forward high-angle scattered light with a flow cytometer.

Fig. 20 shows the result. When the reagent consisted of the first and second liquids, a good scattergram was obtained whereby the leucocytes were able to countered into four groups.

As illustrated hereinabove, it is now possible with the present invention to provide a reagent composition which substantially comprises one aqueous solution and is capable of classifying and counting leucocytes by a simple procedure of just mixing the reagent of the present invention with a

blood sample. Accordingly, classification of leucocytes can be conducted only by measuring the two scattered light signals and an apparatus of a simple structure can be applied.

In addition, there is no need of using dangerous chemicals such as an aldehyde and, therefore, it is now possible to classify and count blood samples safely.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A reagent for analyzing leucocytes comprising

(a) at least one cationic or amphoteric surfactant in an amount sufficient for lysing erythrocytes and cause damage to a part of cell membranes of leucocytes;

(b) at least one organic compound selected from the group consisting of acidic dyes, 8-anilino-1-naphthalenesulfonic acid, 6-(p-toluidino)-2-naphthalenesulfonic acid, chromotropic acid, phthalic acid, naphthalenesulfonic acid, and their corresponding salts in an amount sufficient for making a morphological difference among leucocytes by combining with a cationic component in leucocytes;

(c) a nonionic surfactant; and

(d) a buffer for adjusting pH.

2. A reagent according to claim 1 in which the reagent further contains an alcohol.

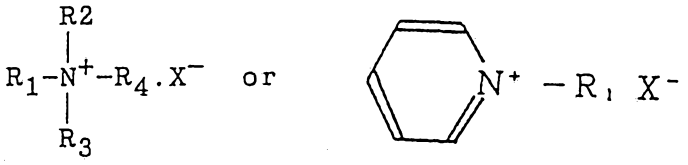
3. A reagent according to claim 2 in which the alcohol is methanol, ethanol, propanol or 2-phenoxy ethanol.

4. A reagent according to claim 1 or 2 in which the reagent further contains a metal salt.

5. A reagent according to claim 4 in which the metal salt is sodium chloride, potassium chloride or lithium chloride.

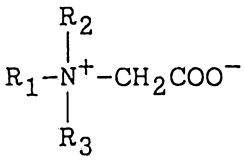
AUSTRALIAN PATENT OFFICE

6. A reagent according to claim 1 in which the cationic or amphoteric ~~ionic~~ surfactant is at least one cationic surfactant represented by the formula;



(R_1 is a C_{6-18} alkyl or alkenyl group; R_2 and R_3 are a C_{1-4} alkyl or alkenyl group; R_4 is a C_{1-4} alkyl or alkenyl group or benzyl group; and X is a halogen atom).

7. A reagent according to claim 1 in which the cationic or amphoteric ~~ionic~~ surface active agent is at least one amphoteric surfactant represented by the formula;



(R_1, R_2 and R_3 are the same as those defined in claim 6)

8. A reagent according to claim 1 in which the organic compound is a substance which has at least six carbon atoms and has both hydrophobic group and anionic functional group, is charged in negative in an aqueous solution and is able to modify the shape of leucocytes by combining with them.

AUSTRALIAN PATENT OFFICE

9. A reagent according to claim 8 in which the organic compound is at least one substance selected from the group consisting of 8-anilino-1-naphthlenesulfonic acid or salts thereof, 6-(p-toluidino)-2-naphthalenesulfonic acid or salts thereof, chromotropic acid, phthalic acid, naphthalenesulfonic acid and acidic dyes.

10. A reagent according to claim 9 in which the acid dye is Brilliant Crocein MOO, Alizaline Violet 3R, Alphazurine A, Guinea Green B, Alizarin Yellow R.

11. A reagent according to claim 1 in which a hydrophilic group of the nonionic surfactant is selected from the group consisting of polyoxyethylene glycol, polypropylene glycol and polyoxyethylene glycol-polycxypropylene glycol block copolymer.

12. A reagent according to claim 1 in which two or more nonionic surfactants are used.

13. A reagent according to claim 1 in which the nonionic surfactant is present in ~~contains~~ about 30-5,000 mg per liter of the reagent.

14. A reagent according to claim 1 in which the organic compound used is present in ~~contains~~ about 50-5,000 mg per liter of the reagent.

AUSTRALIAN PATENT OFFICE

15. A kit when used to prepare the reagent according to claim 1 composed of two liquids comprising a first liquid containing at least one organic compound in an amount sufficient for making a morphological difference among the leucocytes by combining with the cati nic charging components in the leucocytes and a second liquid containing at least one cationic or amphoteric surfactant in an amount sufficient for being able to lyse erythrocytes and cause damage to a part of the cell membranes of the leucocytes, the first and the second liquid contain a nonionic surfactant and a buffer for adjusting pH.

16. A reagent according to claim 1 in which the reagent is mixed with blood in the range from 1:2 to 1:100 by volume.

17. A reagent according to claim 1, substantially as hereinbefore described with reference to the drawings and/or Examples.

18. A method for classifying leucocytes into four groups comprising:

treating blood sample with a reagent for analyzing leucocytes as set forth in claim 1; and

obtaining a size and a morphological information of leucocytes.

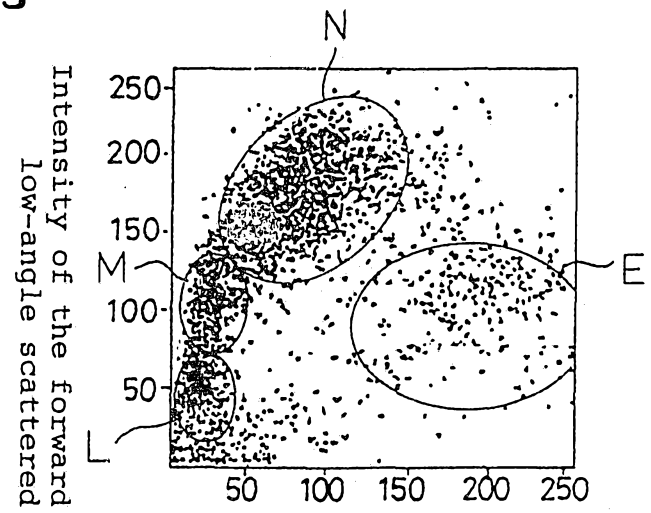
19. A method as set forth in claim 18 wherein the data on the size of leucocytes is obtained by determining forward low-angle scattered light or an electric resistance signal and the data on the form of leucocytes is obtained by determining forward high-angle scattered light or side scattered light.

DATED this 11TH day of SEPTEMBER, 1997
TOA MEDICAL ELECTRONICS CO. LTD.
by DAVIES COLLISON CAVE
Patent Attorneys for the Applicants

ABSTRACT

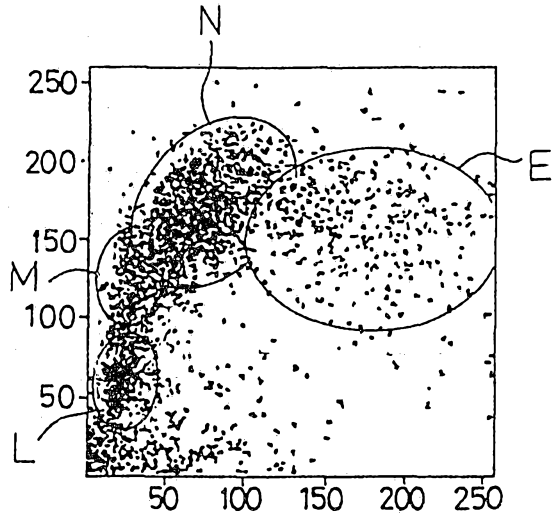
A reagent for analyzing leucocytes comprises (a) at least one ionic surfactant in an amount sufficient for lysing erythrocytes and cause damage to a part of cell membranes of leucocytes; (b) at least one organic compound having an anionic group in an amount sufficient for making a morphological difference among leucocytes by combining with a cationic component in leucocytes; (c) a nonionic surfactant; and (d) a buffer for adjusting pH.

Fig. 1



Decyltrimethylammonium bromide
750mg/ℓ

Fig. 2



Lauryltrimethylammonium chloride
500mg/ℓ

Fig. 3

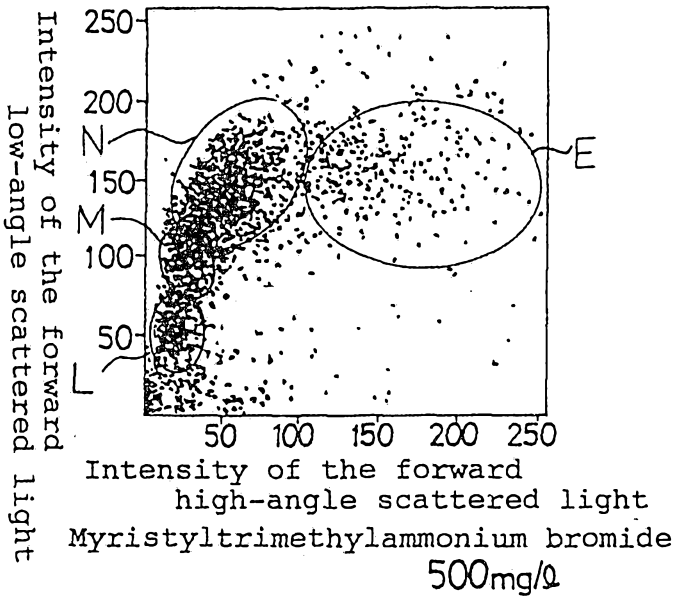

Intensity of the forward low-angle scattered light
250
200
N
150
M
100
50
L
E
50 100 150 200 250
Intensity of the forward high-angle scattered light
Myristyltrimethylammonium bromide
500mg/Ω

Fig. 4

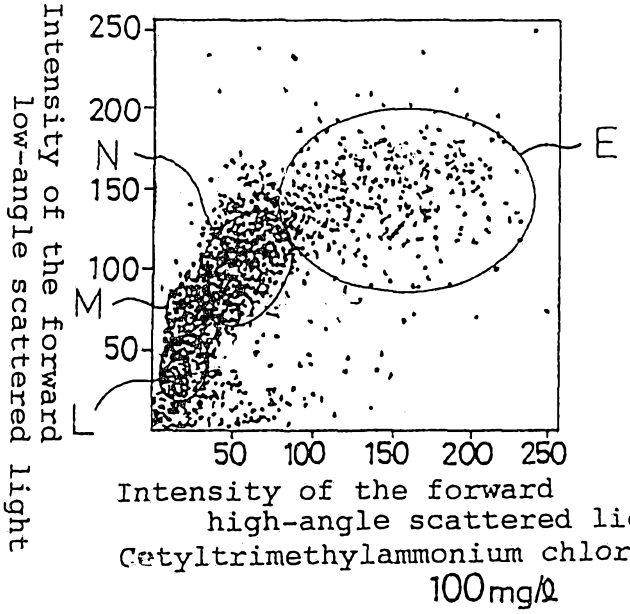

Intensity of the forward low-angle scattered light
250
200
N
150
100
M
50
L
E
50 100 150 200 250
Intensity of the forward high-angle scattered light
Cetyltrimethylammonium chloride
100mg/Ω

Fig. 5

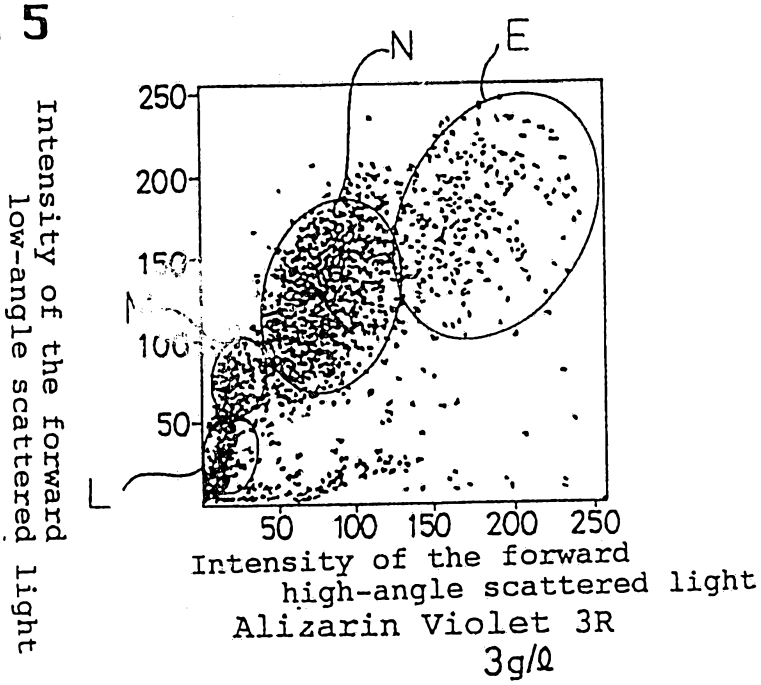

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
Alizarin Violet 3R
3g/ℓ

Fig. 6

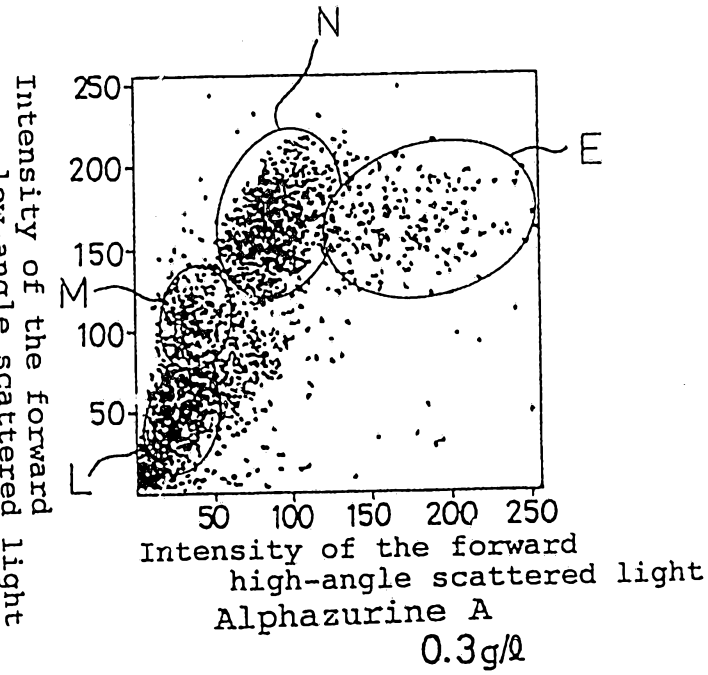
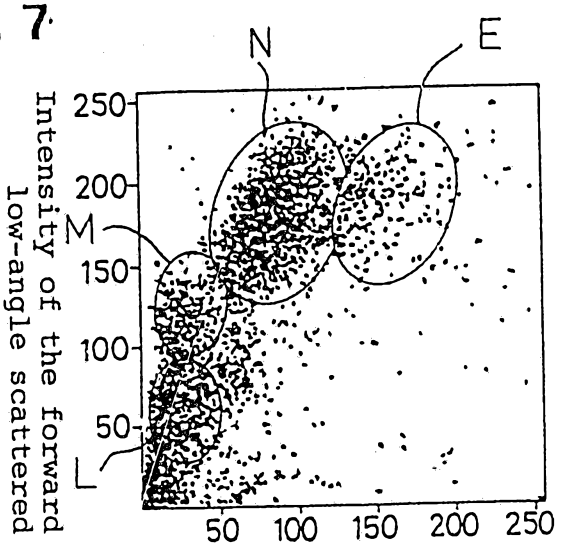
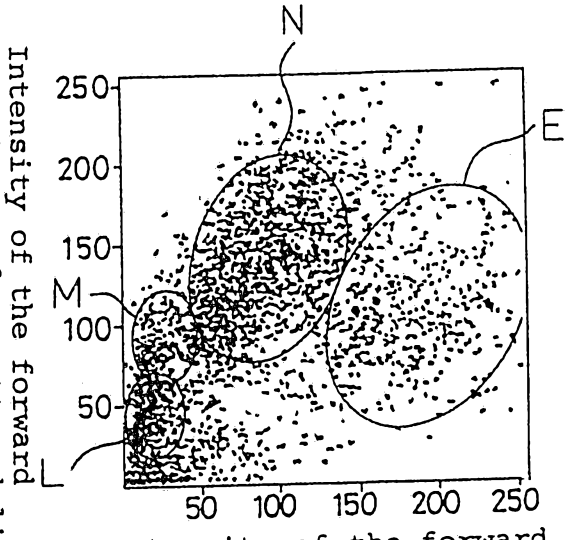

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
Alphazurine A
0.3g/ℓ

Fig. 7



Guinea Green B
0.3g/ℓ

Fig. 8



Alizarin Yellow-R
0.3g/ℓ

Fig. 9

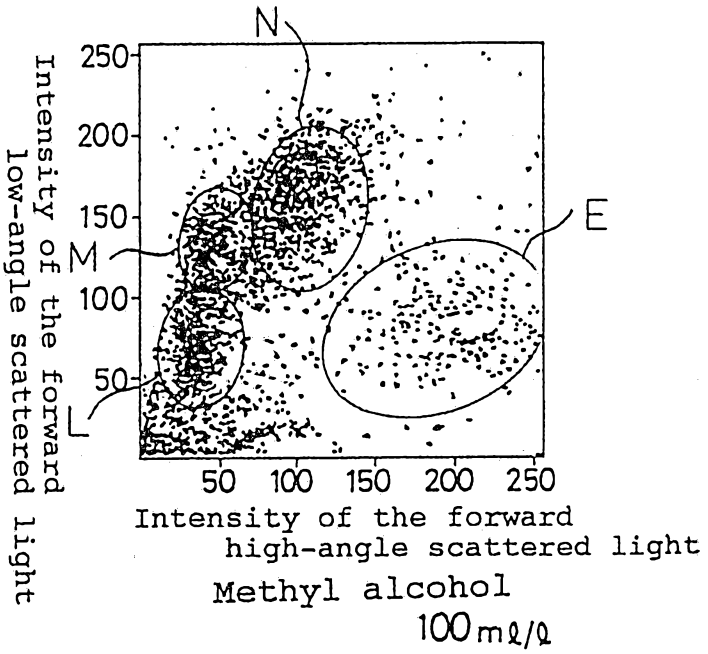

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
Methyl alcohol
100 mℓ/ℓ

Fig. 10

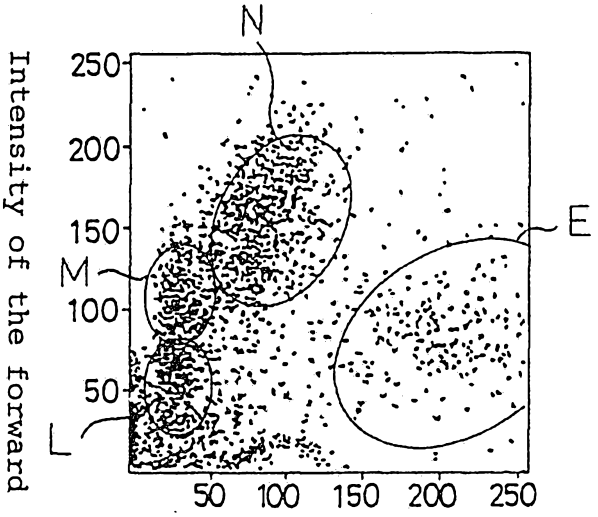

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
Ethyl alcohol
50mℓ/ℓ

Fig. 11

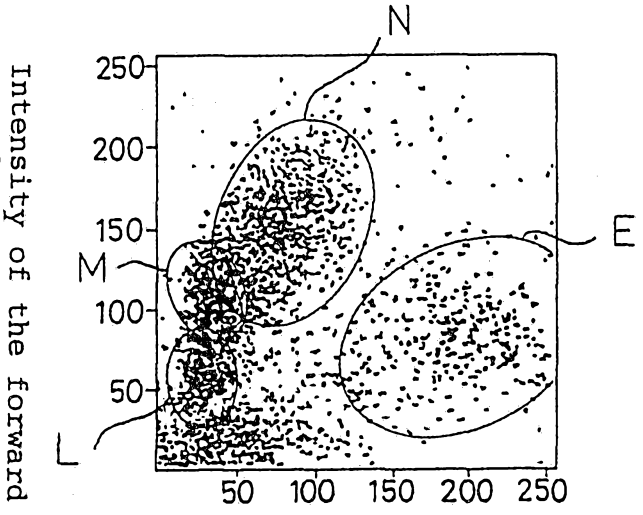

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
2-Propanol
25mℓ/ℓ

Fig. 12

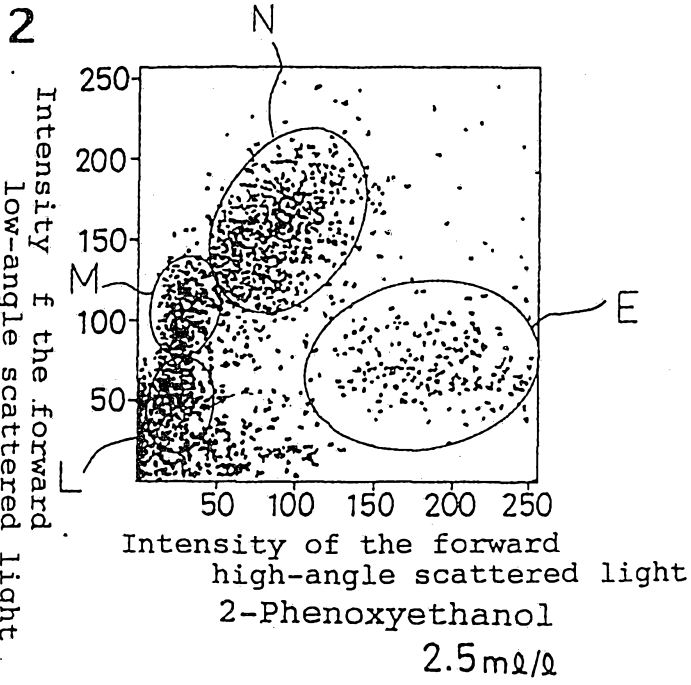

N
M
E
250
200
150
100
50
50 100 150 200 250
Intensity f the forward low-angle scattered light
Intensity of the forward high-angle scattered light
2-Phenoxyethanol
2.5mℓ/ℓ

Fig. 13

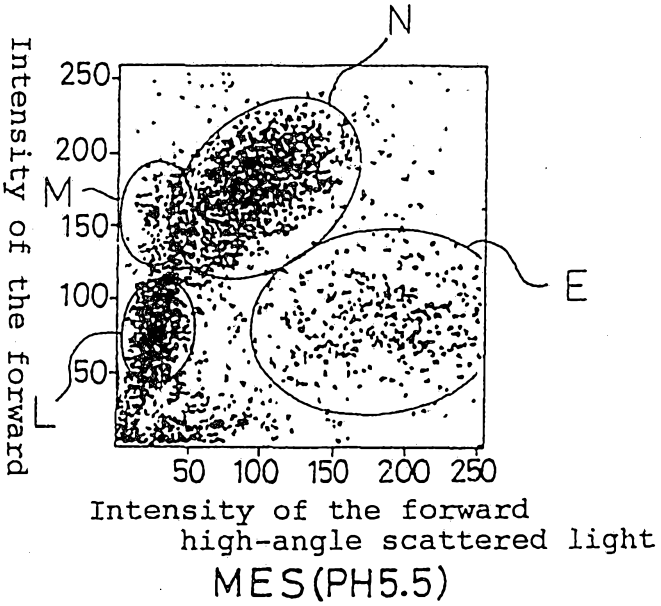

N
M
E
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
MES(PH5.5)

Fig. 14

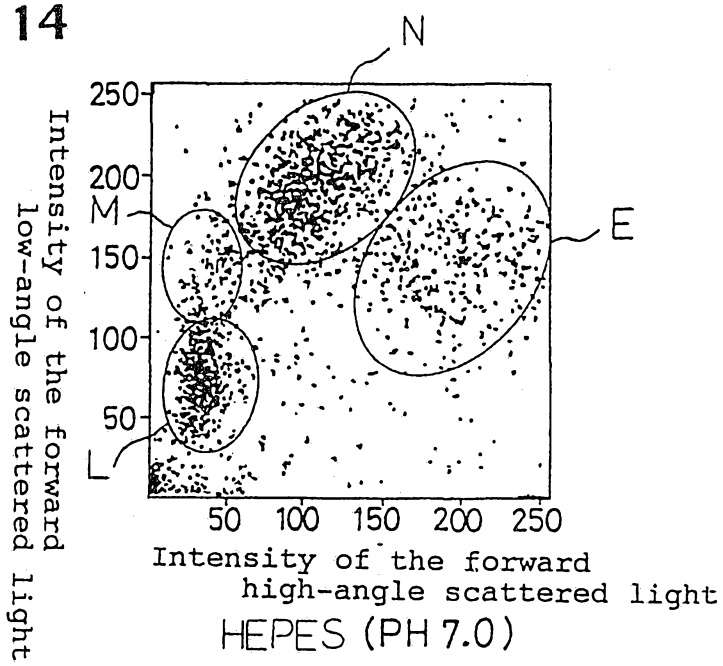

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
HEPES (PH 7.0)

Fig. 15

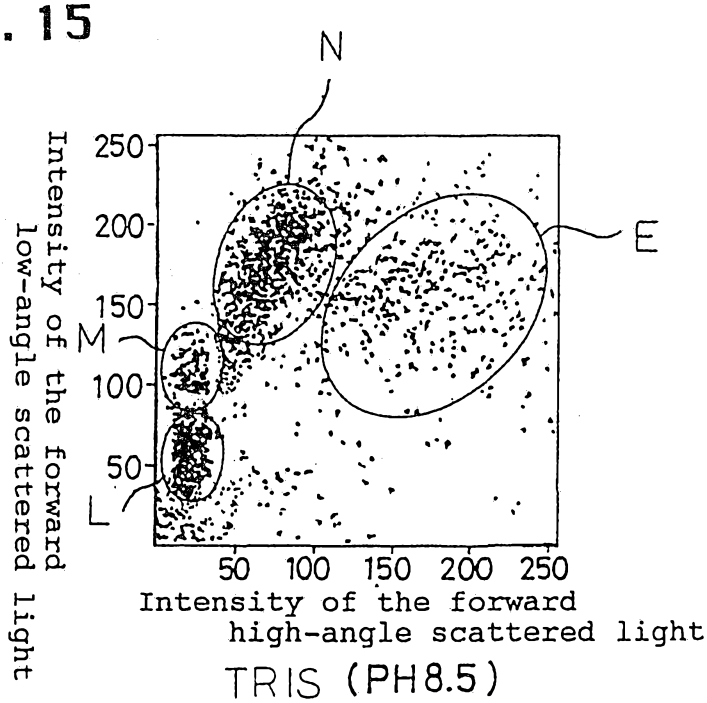

N
E
M
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light
TRIS (PH8.5)

Fig. 16

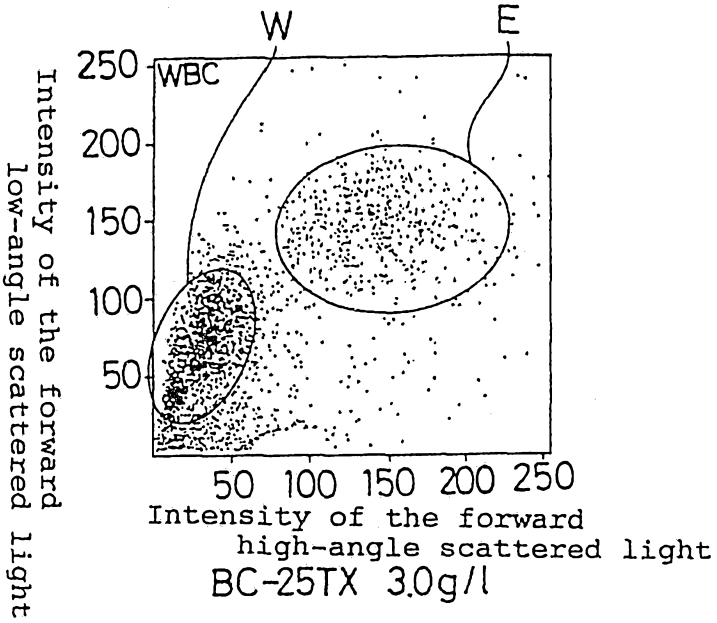

W
E
WBC
250
200
150
100
50
50 100 150 200 250
Intensity of the forward high-angle scattered light
Intensity of the forward low-angle scattered light
BC-25TX 3.0g/l

Fig. 17

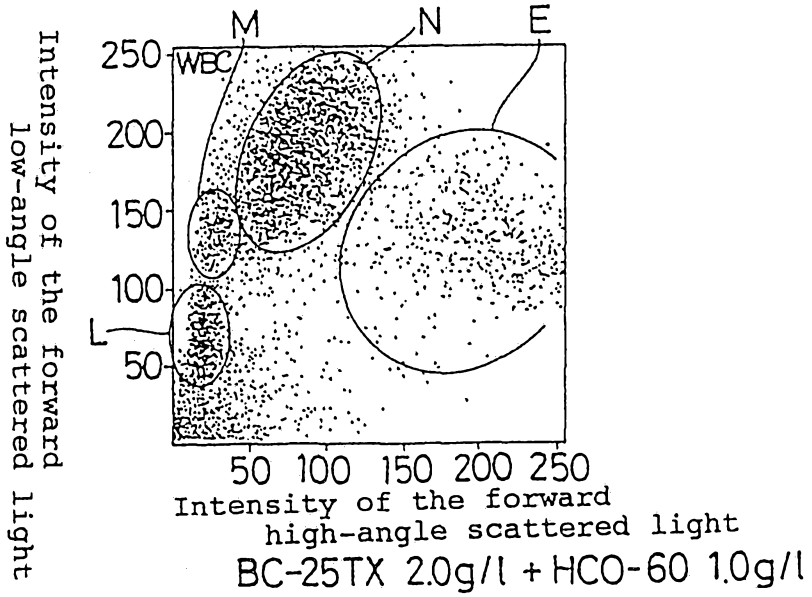

M
N
E
WBC
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward high-angle scattered light
Intensity of the forward low-angle scattered light
BC-25TX 2.0g/l + HCO-60 1.0g/l

Fig. 18

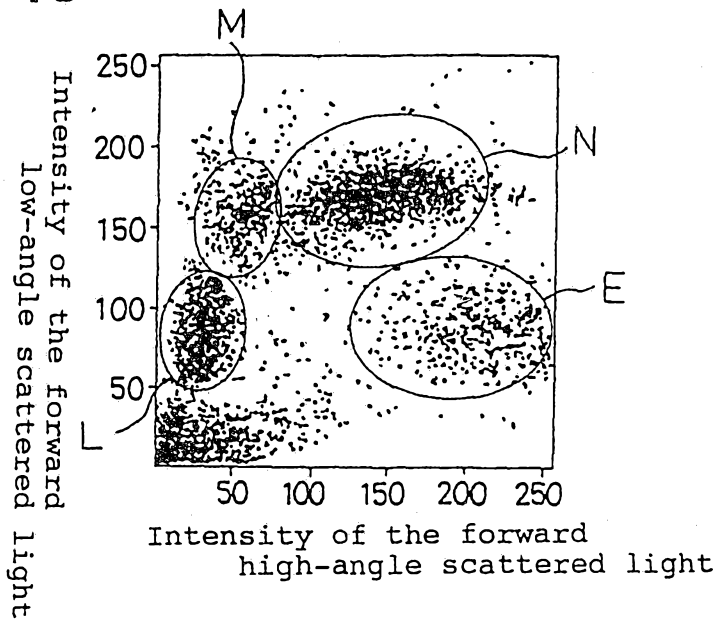

M
N
E
L
250
200
150
100
50
50 100 150 200 250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light

Fig. 19

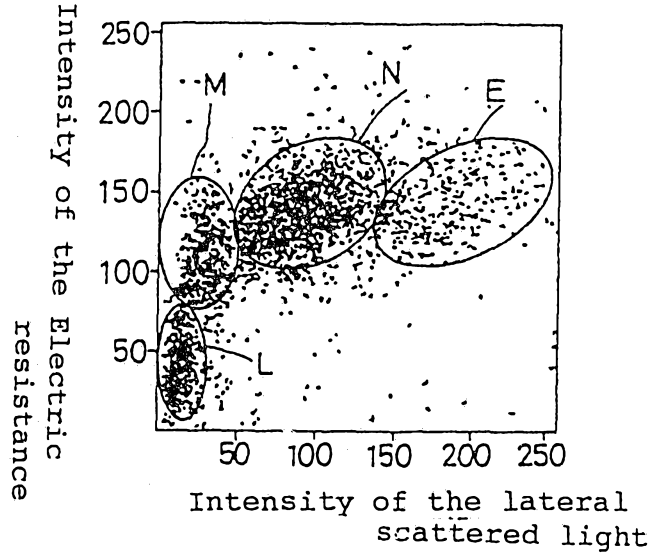

M
N
E
L
250
200
150
100
50
50 100 150 200 250
Intensity of the Electric resistance
Intensity of the lateral scattered light

Fig. 20

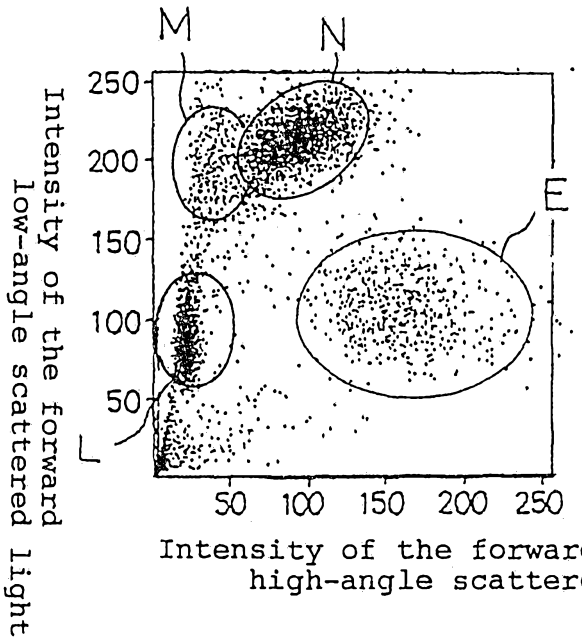

M
N
E
L
250
200
150
100
50
50
100
150
200
250
Intensity of the forward low-angle scattered light
Intensity of the forward high-angle scattered light

Fig. 21

