(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 February 2002 (28.02.2002)

PCT

(10) International Publication Number
WO 02/16950 A1

(51) International Patent Classification[7]: G01N 33/68

(21) International Application Number: PCT/US01/24279

(22) International Filing Date: 3 August 2001 (03.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/645,784 24 August 2000 (24.08.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/16950 A1

(54) Title: DRY POWDER FORMULATION FOR PROTEIN LABELING

(57) Abstract: The proteins in a biological sample that is sought to be analyzed for its protein composition by a chromatographic procedure are coupled to a dye in an unusually efficient manner by combining the sample with a dry powdered mixture containing the dye, a buffering agent, and in preferred embodiments, a denaturing agent as well. The dry powdered form of the mixture avoids deterioration or decomposition of the dye, and the combination of components in the mixture allows the dye to couple to the proteins in a relatively uniform and highly controllable manner when the powder mixture and sample are heated together and held at an elevated temperature until some or all of the powder dissolves.

DRY POWDER FORMULATION FOR PROTEIN LABELING

BACKGROUND OF THE INVENTION

The analysis of biological samples to determine the identity and amounts of various proteins is performed by a wide variety of separation techniques, many of which involve the use of dyes to enable the clinician to locate, identify, and quantitate the proteins. The dyes are detectable and readable either by visual observation or by machine processes. Included among the many types of dyes that are used for this purposes are those that emit colors in the visible range or the ultraviolet range to the eye without treatment or activation, and fluorescent dyes which emit upon excitation.

The dyes are most commonly applied to the proteins after separation has occurred and while the proteins are isolated into individual spots or bands in the gel. Applying the dyes in this manner and removing excess dye is a labor-intensive and time-consuming procedure that is subject to handling difficulties, operator error and various nonuniformities. In addition, many of the dyes are insoluble in water, and must first be dissolved in an organic solvent before being applied to the protein spots or bands in the gel. As an alternative, proposals have been made to apply the dyes to the proteins in the sample before the separation is performed. Concerns that arise in these proposals include the effect of the dyes on the apparent mobilities of the proteins, particularly when the proportion of dye to protein is nonuniform, and the instability of the dyes in aqueous media.

One of the areas in which these dyes are used is the electrophoresis of proteins under denaturing conditions. Denaturing electrophoresis, and particularly SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel eletrophoresis), is a highly effective and efficient means of analyzing proteins in a sample, since it identifies proteins independently of the potentially interfering influences of tertiary or quaternary shape or the complexity of their subunits. The concerns raised by the use of dyes apply with

particular force to denaturing electrophoresis in view of its widespread use and effectiveness.

SUMMARY OF THE INVENTION

It has now been discovered that dyes can be applied to biological samples in an unusually efficient manner from a dry powder mixture which contains a typical protein dye in powder form and a powdered non-nucleophilic buffering agent, and in preferred embodiments of the invention, a powdered denaturing agent as well. The coupling of the dye to the proteins in the aqueous biological sample is achieved by combining the powder mixture with the sample and heating the mixture to an elevated but non-boiling temperature for a period of time sufficient to dissolve the solid materials. The result is a substantially consistent relative staining of the proteins in the sample, i.e., an approximately even amount of dye among the various proteins in the sample, and substantially no change in the apparent mobilities of the proteins, i.e., a small enough amount of dye on each protein to only minimally affect its separation characteristics relative to the other proteins in the sample. The solution thus prepared is ready for loading onto a separation medium where it can be separated by conventional protein separation procedures known in the art. These procedures include the various forms of electrophoresis as well as other protocols. The individual protein bands or spots that result from the separation are discernable either by machine reading or by visual observation.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Included among the various dyes that can be used in the practice of this invention are electrophilic-activated forms of fluorescent dyes including, but not limited to, succinimidyl esters, vinyl sulfones, etc., of xanthenes, cyanines, coumarins, benzimides, phenanthridines, ethidium dyes, acridine dyes, carbazole dyes, phenoxazine dyes, porphyrin dyes, and quinoline dyes. Fluoresceins and rhodamines are particular types of xanthene dyes. Specific examples are 6-carboxyfluorescein, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, N,N,N',N'-tetramethyl-6-carboxyrhodamine, 6-carboxy-X-rhodamine, 5-carboxyrhodamine-6G, 5-carboxyrhodamine-6G, tetramethylrhodamine, Rhodamine Green, and Rhodamine Red. Umbelliferone is an example of a coumarin. Hoechst 33258 is an example of a benzimide dye. Texas Red is

an example of a phenanthridine dye. Examples of cyanine succinimidyl ester dyes are sulfoindocyanine succinimidyl esters, (carboxyalkyl)cyanines succinimidyl esters, and BODIPY succinimidyl esters (Molecular Probes, Inc.).

The buffering agent serves to limit the rate of hydrolysis of the dye and thereby the rate at which the dye becomes available for coupling to the protein when the powdered mixture is combined with the biological sample. Suitable buffering agents are those that are non-nucleophilic and that do not compete with the protein for coupling to the dye. Preferred agents are those that have a pH range of about 8.0 to about 9.5, and those that do not contain thiols or primary or secondary amines. Examples of suitable buffering agents are sodium borate, sodium carbonate, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES).

In the preferred practice of the invention, the powder mixture includes a denaturing agent (also in powder form). This permits the clinician to perform protein denaturation and labeling in a single step. Conventional denaturing agents may be used, examples of which are sodium dodecyl sulfate (SDS), urea, and guanidine. The most preferred is SDS. Other substances may also be included on an optional basis. Examples are sugars (such as sucrose) for density adjustment and a tracking indicator to indicate the location of the moving solute front during the separation process. The tracking indicator can be any indicator that is inert with respect to the proteins and other components included in either the powder mixture or the biological sample. One example of such an indicator is Bromophenol Blue. A reducing agent (in solid form) can also be included. Examples of suitable reducing agents are dithiothreitol, dithioerythritol, and tris(carboxyethyl)phosphine. Other examples of all of these additives will be readily apparent to those skilled in the art.

The relative amounts of each component in the powder mixture are not critical to the invention and may vary. In a typical composition that contains both the active dye (i.e., the fluorescent, ultraviolet or visible dye that couples to the proteins) and an indicator dye (to show the location of the moving ion front), plus SDS as a denaturing agent, sucrose as a density adjusting agent, and sodium borate as the buffer, preferred amounts of each for a final volume of 1.0mL are as follows:

Active dye	2-100μg
Denaturing agent, such as SDS	0-20mg

Density adjusting agent, such as sucrose	0-100mg
Buffer, such as sodium borate	2-20mg
Indicator dye	0-200μg

The particle size of the powder mixture is not critical to the invention and can vary as well. A preferred particle size range is from about 5 microns to about 500 microns. The preparation and size control of the particles in the mixture is achieved by conventional methods, including precipitation, grinding, sieving, and other methods that will be readily apparent to those skilled in the art.

In use, the powder mixture is combined with the biological sample either immediately prior to or a short period of time before the sample is applied to the separation medium for analysis. The powder mixture can first be mixed with water to form a preliminary solution that is subsequently combined with the biological sample. In other embodiments of the invention, the powder mixture is combined directly with the sample, and the powder and sample are heated to a temperature high enough to gradually dissolve the dye yet low enough to avoid boiling of the sample. Preferred temperatures are in the range of from about 80°C upward, more preferably about 90°C to about 100°C, and most preferably about 95°C. The mixture is then held at that temperature for a sufficient period of time for the solid components to dissolve. In most cases, acceptable results will be achieved in a few minutes, preferably about five minutes. The solution is then cooled and loaded into the separation system.

The powder mixture of this invention can be used to prepare biological samples for a wide variety of separation procedures that involve the denaturing of proteins in the samples and the use of dyes for differentiation and identification of the different proteins. Included among these procedures are ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, molecular sieve chromatography, adsorption chromatography, exclusion chromatography, and various forms of electrophoresis, including isoelectric focusing and conventional electrophoresis in either a capillary, tube gel, or slab gel configuration, or microchannels on a chip. The invention is particularly useful in two-dimensional electrophoresis, where the first dimension is a linear separation in a rod-shaped or strip-shaped gel and the second is performed by placing the rod or strip along one edge of a slab gel for migration of the bands laterally out of the rod or strip and into the slab in a direction perpendicular to the

axis of the rod or strip. Separation media for the various forms of electrophoresis include polyacrylamide, cellulose, agarose, dextran, polyvinylalcohol, starch, silica gel, and polymers of styrene and divinylbenzene, as well as combinations of these materials. Polyacrylamide gel electrophoresis is of particular interest.

The foregoing is offered primarily for purposes of illustration. Further modifications and variations of the various parameters of the composition and method of this invention will be readily apparent to those skilled in the art and are included within the scope of the invention.

WE CLAIM:

1. A dry powder composition comprising:

(a) a dye capable of coupling to a protein,

(b) a non-nucleophilic buffering agent, and

(c) a protein denaturing agent.

2. A dry powder composition in accordance with claim **1** in which said protein denaturing agent is sodium dodecyl sulfate.

3. A dry powder composition in accordance with claim **1** in which said dye is a fluorescent dye.

4. A dry powder composition in accordance with claim **1** in which said buffering agent is sodium borate.

5. A dry powder composition comprising:

(a) a fluorescent dye capable of coupling to proteins,

(b) a non-nucleophilic buffering agent which maintains a pH within the range of about 8.0 to about 9.5 when dissolved in an aqueous solution,

(c) sodium dodecyl sulfate, and

(d) a tracking dye which is inert to proteins.

6. A process for the preparation of a protein-containing biological sample for electrophoretic analysis of the protein composition of said sample, said process comprising:

(a) combining said sample with a dry powder mixture comprising (i) a dye capable of coupling to a protein, (ii) a non-nucleophilic buffering agent, and (iii) a protein denaturing agent to form a suspension, and

(b) heating said suspension to dissolve at least a portion of said mixture.

7. A process in accordance with claim **6** in which said denaturing agent is sodium dodecyl sulfate.

8. A process in accordance with claim **6** in which step (b) comprises heating said suspension to a temperature of from about 90°C to about 100°C.

INTERNATIONAL SEARCH REPORT

Interna[illegible]al Application No
PCT/US 01/24279

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 352 246 A (HAEHNKE MANFRED ET AL) 4 October 1994 (1994-10-04)	1,4
A	abstract column 11, line 6-20 column 12, line 7-19 claims 1,14,17	2,3,5-8
A	US 4 649 193 A (MEININGER FRITZ ET AL) 10 March 1987 (1987-03-10) abstract column 30, line 49-66; examples 1,2,101-104	1-8
A	US 6 090 164 A (STECKELBERG JOACHIM ET AL) 18 July 2000 (2000-07-18) column 2, line 60 -column 3, line 22	1-8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2001	04/01/2002

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

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