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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/71317 A1

(51) **International Patent Classification**[7]: **G01N 21/00**, 21/01, 21/17, 31/20, 33/544, 33/538, 33/567, 33/537, 33/543, 33/53, 33/546, 33/552, C12M 1/00, C12N 1/00, 1/20, 11/00, C12Q 1/68, C07H 21/04

(21) **International Application Number:** PCT/US01/08790

(22) **International Filing Date:** 20 March 2001 (20.03.2001)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
60/190,091 20 March 2000 (20.03.2000) 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, CR, CU, CZ, DE, DK, DM, DZ, 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, US, 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, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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 01/71317 A1

(54) **Title:** METHOD FOR DETECTING AN ANALYTE BY FLUORESCENCE

(57) **Abstract:** Two-dimensional and three-dimensional arrays of a polydiacetylene backbone having a substrate incorporated are used in chemical sensing methods to detect the interaction of an analyte with the substrate by monitoring the change in the fluorescence of the array.

METHOD FOR DETECTING AN ANALYTE BY FLUORESCENCE

DESCRIPTION

Technical Field

The present invention relates to a method for detecting the presence of an analyte in a sample, and more particularly, to a method involving the monitoring of the change in fluorescence. According to the present invention, an array incorporating a polydiacetylene backbone with a substrate incorporated in the array is employed.

Background of the Invention

Polydiacetylenes are conjugated polymers with backbones of alternating double and triple bonds formed from the 1,4-addition polymerization of 1,3-diacetylenes (Figure1). Polydiacetylenes generally absorb well in the visible region of the spectrum, and hence are highly colored, ranging from blue to yellow. There has been intense interest in the non-linear optic properties of polydiacetylenes and extensive study has been made of both the solvo-chromic properties of solubilized polydiacetylenes and the thermo-chromic properties of polydiacetylene films and single crystals. It is well known that to form polydiacetylene, the diacetylene monomers must be in an ordered packing to allow the polymerization to occur. It seems to be generally accepted, though the inventors are not bound herein, that disruption of the packing of the side chains can affect the conjugation length of the backbone, and hence the chromic properties.

Diacetylene monomers have been used to form various ordered systems, including crystals, liquid crystals, liposomes and films that were then polymerized to form the polymer. Liposomes have been made from monomers with two diacetylene chains and polar head groups (such as phosphotidylcholines, and its analogues) and from monomers with single diacetylene chains. The liposomes can be polymerized with UV light or γ-radiation. Monomer films have been formed by Langmuir Blodgett methods or cast from solvents and then also polymerized with UV light or γ-radiation. The choice of monomer structure, conditions of liposome or film formation, and polymerization conditions all

affect the conjugation length of the polydiacetylene backbone, and hence the color of the system. Upon heating, these polymerized systems can undergo a change in the effective conjugation length, from the longer length forms (blue and purple) to the shorter length forms (red and yellow). This change has been attributed to the side-chains moving and repacking upon being heated. Soluble polydiacetylenes show solvo-chromic behavior and polydiacetylene films often change color upon exposure to solvent vapors. Polydiacetylene films and liposomes formed from diacetylene surfactants also often change color with change in pH. In the case of the packed polymer arrays that form the films and liposomes, it is generally accepted that changes in the environment that affect the organization and packing of the side chains coming off the conjugated backbone can affect the conjugation length and hence the chromic and electronic properties of the polymer.

These polydiacetylene films and liposomes have been suggested for chromogenic assays that depend upon color change (Charych et al, US Patent 6,001,556; Charych et al, US Patent 6,180,135; Charych et al, US Patent 6,080,423; Charych, US Patent 6,183,772; Charych et al, US Patent 6,022,748). It has been hypothesized by Charych (Okada S. et al, *Acc. Chem. Res.*, 1998, *31*, 229-239) that binding to a ligand incorporated in a blue polydiacetylene films or liposomes perturbs the side chains of the polydiacetylene and hence the conjugation length of the polydiacetylene and changes the color of the film or liposomes to red. The color change is proposed to be measured either by eye or by a UV/VIS spectrophotometer and comparison of the absorbance at a wavelength above 600nm and the absorbance of a wavelength below 600nm.

The phenomenon of fluorescence is distinct from the absorbance properties that give systems their color. In order to be fluorescent, the system must absorb one wavelength of light and then emit another. Upon absorbing the light, the system is excited to a higher energy state. It can then return to the ground state by a variety of mechanisms, most of which do not lead to fluorescence. These alternative, non-radiative, mechanisms for returning to the ground state lead to many strongly absorbing species to be non-fluorescent, and makes the prediction of which species will be fluorescent a difficult task and therefore not apparent to those skilled in the art.

For instance, while some organic systems with extended conjugation exhibit fluorescence, many more do not.

Along these lines, generally species absorb light in the ultraviolet and visible ranges. The ultraviolet wavelength range is approximately 190nm-380nm; the visible light range runs from approximately 380nm to 800nm. Upon absorption of the light the species move to a higher energy electronic excited state. What happens then determines if the species is fluorescent. If a species absorbs light at one wavelength, is excited to a higher energy state, and then emits light at a different wavelength and returns to the ground state, it is fluorescent (or phosphorescent). For fluorescence to occur the excited species must be capable of emitting light; generally for the fluorescence to be measurable the emitted light must be at a different wavelength than the excitation. The Stokes shift is the difference between the excitation and emission wavelengths. Most species that absorb light are not capable of light emission; they return to the ground state by a variety of non-radiative mechanisms. Furthermore, fluorescent species often absorb wavelengths of light that do not cause fluorescence, as well as absorbing wavelengths that do cause fluorescence. In short, absorbance of light is necessary for fluorescence but does not guarantee it.

On the other hand, color is an absorbance property; the colors we see are related to the wavelengths of light that the species is absorbing. For example if the species absorbs light primarily at 650 nm, we will see it as blue, while if it absorbs primarily at 550 nm, we will see it as red. Color arises from absorbance of light in the visible range. Most colored species are not fluorescent. If a colored species is fluorescent, it will normally appear one color, but when it is excited with the appropriate wavelength, it will glow with the color of the emitted light. For example, a fluorophore may look like an orange powder, but glow green under a UV lamp.

Polydiacetylenes can show fluorescence. However, their ability to fluoresce is dependent on the structural form and organization of the polymers (particularly the conjugation length and aggregation state), whether in solution, a film, or formed into liposomes or other three-dimensional structures.

It is known that polydiacetylene films have an intrinsic fluorescence when produced in the red or yellow form, and are not fluorescent (by conventional measurements) when the film is made in the blue form (Yasuda A. et al, *Chem. Phys. Lett.*, 1993, *209(3)*, 281-286). This fluorescent property of the films has been used for microscopic imagining of film domains and defects.

Ribi et al have suggested two sensors using polydiacetylene film fluorescence. The first sensor (Saul et al, U.S. Patent 5,415,999 and US Patent 5,618,735) uses a red, fluorescent, polydiacetylene film layered with a fluorescence modulation reagent non-covalently associated with the film that modulates the measured emission of the film, e.g. by absorbing the emitted light, in the presence of an analyte. The fluorescent state of the film does not change during the assay; rather the emission is obscured or revealed by the action of the fluorescence modulation agent. The second suggested sensor (Ribi, U.S. Patent 5,622,872) uses a film of specific composition for detection of an analyte by change in the fluorescence of a film of this composition. The films in the detection method claims comprise a polymerized film, polymerized from diacetylene monomers of the defined formulation $(A)_a(D)_aC_x(C \quad C)_2C_yLB$ wherein A is a functional group used to link the film to an underlying substrate, a is 0 or 1, C is carbon, x and y are 1 or greater and (x+y) is in the range of 4-32, D and L are bond or linking groups and B is a specific binding member which binds to a specific analyte, one terminus of each monomer is proximal to the underlying substrate and the other terminus comprising B (i.e. the film is a mono-layer with every polydiacetylene side-chain either terminating in proximity to the underlying substrate, or in a binding member). Neither Ribi nor others, to our knowledge, have suggested detection of analytes using three-dimensional arrays of polydiacetylenes (e.g. liposomes or tubules) and measuring the change in fluorescence arising from the interaction of the analyte and the polydiacetylene three-dimensional array.

Summary of Invention

The present invention provides a sensing method that measures fluorescent changes in polydiacetylene films as they convert from the non-fluorescent form (generally blue or purple) to the fluorescent forms (generally red to yellow). More

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