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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 March 2008 (13.03.2008)

PCT

(10) International Publication Number
WO 2008/029162 A1

(51) International Patent Classification:
A61B 3/117 (2006.01)

(21) International Application Number:
PCT/GB2007/003393

(22) International Filing Date:
7 September 2007 (07.09.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0617605.1 7 September 2006 (07.09.2006) GB

(71) Applicant *(for all designated States except US)*: **THE QUEEN'S UNIVERSITY OF BELFAST** [GB/GB]; University Road, Belfast BT7 1NN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **STITT, Alan, W.** [GB/GB]; Centre for Vision Science, School of Biomedical Science, Faculty of Medicine and Health Sciences, Queen's University of Belfast, Belfast BT12 6BA (GB). **MACGARVEY, John, J.** [GB/GB]; Centre for Vision Science, School of Biomedical Science, Faculty of Medicine and Health Sciences, Queen's University of Belfast, Belfast BT12 6BA (GB).

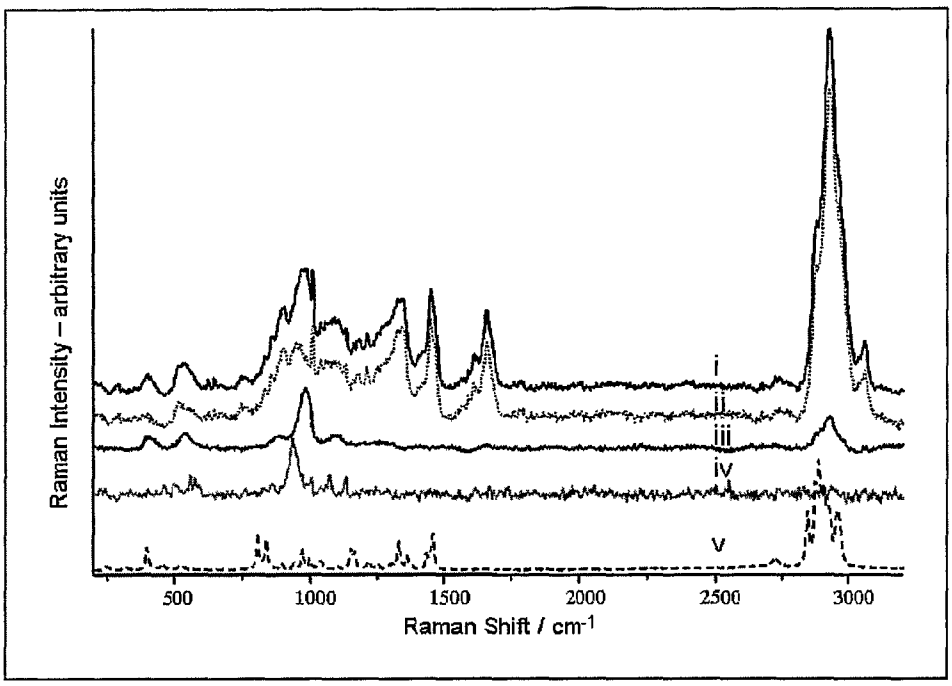
(74) Agent: **MURGITROYD & COMPANY**; Scotland House, 165-169 Scotland Street, Glasgow G5 8PL (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: BIOMARKERS



(57) Abstract: There is described a non-invasive system for the detection and measurement of disease-relevant biomarkers, in particular chemical modifications (adducts) called Advanced Glycation Endproducts (AGEs), in the eye and a method of detecting said biomarkers for the diagnosis of retinopathy or age-related macular degeneration (AMD). These adducts have been determined to be indicators for disease initiation and progression of diabetic retinopathy, human ageing in the context of the eye, and AMD.

WO 2008/029162 A1

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*

Biomarkers

Field of the Invention

The present invention relates to a non-invasive system for the detection and measurement of disease-relevant biomarkers in the eye and a method of detecting said biomarkers for the diagnosis of retinopathy or age-related macular degeneration (AMD). More particularly, the present invention provides a system and method for detecting chemical modifications (adducts) called Advanced Glycation Endproducts (AGEs) for use as indicators for disease initiation and progression of diabetic retinopathy. These same adducts are also established biomarkers for human ageing and, in the context of the eye, can be useful for monitoring ageing of the eye. AGEs are linked to AMD and this invention hopes to establish a non-invasive "risk-index" for predicting eyes at risk of age-related disease.

Background

Various changes in the retina are known to cause problems with vision. One example of such changes are age-related changes in the retina occurring due to chemical modifications that form irreversible AGE adducts which accumulate during the aging process and are experimentally linked to the initiation and progression of AMD. This is a disease which generally occurs above the age of 70 and represents the leading cause of blindness in the Western world.

AGEs are associated with ageing, but these adducts also accumulate at an accelerated rate in diabetes. In particular, diabetic retinopathy, which is the commonest microvascular complication of diabetes, is the prevailing

cause of registrable blindness in patients of working age in developed countries.

A) Diabetic retinopathy:

Diabetic retinopathy begins with discrete background changes typified by vascular closure and neural retina defects which can progress to moderate and severe stages characterised by non-proliferative diabetic retinopathy (NPDR). More severe proliferative diabetic retinopathy (PDR) stages are characterised by growth of new blood vessels on the retina and posterior surface of the vitreous. Macular oedema, characterised by retinal thickening from leaking blood vessels, can develop at all stages of retinopathy. Pregnancy, puberty, blood glucose control, hypertension and cataract surgery can accelerate these changes, but the disease remains complex and multifactorial in nature.

Sight-threatening Macular oedema and pre-retinal neovascularisation can be treated or contained to some extent by pan-retinal laser photocoagulation or vitreoretinal surgery. However, these interventions do not restore lost vision and are at the expense of functional retina and visual performance. Since these treatments are aimed at preventing vision loss and retinopathy can be asymptomatic in the early stages, it is important to identify and treat patients early in the disease. To achieve this goal, patients with diabetes should be routinely evaluated by opthalmoscopy and fundus photography to detect treatable disease. Guidelines suggest that eye examinations for diabetic patients can help to alleviate retinopathy (Fong, D.S., et al., Diabetic retinopathy. Diabetes Care, 2003. **26**(1): p. 226-9.) but there remains a pressing need for better diagnostic parameters that can predict the initiation and progression of this condition.

Formation and accumulation of AGEs has been suggested to contribute to diabetic eye disease. AGE modifications to the epsilon-amino groups of proteins can form directly from reaction of glucose with the epsilon-amino groups, but it has become increasingly appreciated that this sugar is much less reactive than alpha-oxaloaldehydes such as glyoxal, (GO), methylglyoxal (MGO) and 3-deoxylglcosone (3-DG). The concentrations of these alpha-oxaloaldehydes are increased in plasma during hyperglycaemia and arise from both chemical and metabolic pathways. At present, AGEs are detected and the level of AGEs is determined using ELISA techniques mostly in biopsy specimens or post-mortem samples. At present there is no way to measure AGEs in the retina non-invasively. Some indication is possible from vitreous samples obtained post-operatively but this material can only be obtained during complex vitreo-retinal surgery.

B) Age-related macular degeneration:
Ageing of the retina is characterised by progressive dysfunction leading to atrophy of the outer retina. Early in the disease there is no visual loss but a proportion of these persons will go on to develop the two late-stage manifestations of AMD. These are geographic atrophy (dry AMD) and uncontrolled proliferation of blood vessels in the sub-retinal space (wet AMD). These late manifestations result in severe irreversible loss of central vision.

At present, the precise pathogenesis of AMD is ill-defined, but changes in the chemical composition, physical structure and hydrodynamics of structures in the outer components of the retina during ageing are thought to be important in its development. There are also indications that pro-inflammatory processes increasing with age in certain susceptible individuals may play an important role in progression to AMD, in unison

with cumulative protein and lipid modifications in the oxygen-rich outer retina which appear to be central to the degenerative process.

Ageing remains the common risk factor for AMD initiation and progression. Through analysis of post-mortem tissue and experimental investigation it has been established that AGE-modification of structural proteins in the retina, as a subject approaches 70 years of age, could be an important pathogenic factor linked to AMD. It is established that modification of the ε-amino groups on proteins through advanced glycation reactions occurs during ageing (Baynes, J.W., The role of AGEs in aging: causation or correlation. Exp Gerontol, 2001. **36**(9): p. 1527-37.), leading to formation of adducts such as pentosidine, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and hydroimidazolone (Thorpe, S.R. and J.W. Baynes, Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids, 2003. **25**(3-4): p. 275-81.; Thornalley, P.J., A. Langborg, and H.S. Minhas, Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J, 1999. **344 Pt 1**: p. 109-16.)

Such AGE-modified proteins are recognised as important instigators of age-related dysfunction causing responses such as protein crosslinking, enzymatic dysfunction and loss of receptor recognition (Thornalley, P.J., The enzymatic defence against glycation in health, disease and therapeutics: a symposium to examine the concept. Biochem Soc Trans, 2003. **31**(Pt 6): p. 1341-2). AGE-modification can alter the key complement regulatory protein CD59 and increase inflammatory responses (Cheng, Y. and M.H. Gao, The Effect of Glycation of CD59 on Complement-Mediated Cytolysis. Cell Mol Immunol, 2005. **2**(4): p. 313-7.) and is an important pathogenic step in age-related neurodegeneration, including Alzheimer's disease (Munch, G., J. Gasic-Milenkovic, and T. Arendt, Effect of

advanced glycation endproducts on cell cycle and their relevance for Alzheimer's disease. J Neural Transm Suppl, 2003(65): p. 63-71.).

It has been shown that AGE adducts have an important pathogenic role in the neural retina and RPE (Stitt, A.W., Advanced glycation: an important pathological event in diabetic and age related ocular disease. Br J Ophthalmol, 2001. **85**(6): p. 746-53.; McFarlane, S., et al., Characterisation of the advanced glycation endproduct receptor complex in the retinal pigment epithelium. Br J Ophthalmol, 2005. **89**(1): p. 107-12..; Zhou, J., et al., Mechanisms for the induction of HNE- MDA- and AGE-adducts, RAGE and VEGF in retinal pigment epithelial cells. Exp Eye Res, 2005. **80**(4): p. 567-80.; Yamada, Y., et al., The expression of advanced glycation endproduct receptors in rpe cells associated with basal deposits in human maculas. Exp Eye Res, 2006. **82**(5): p. 840-8.; Schutt, F., et al., Proteins modified by malondialdehyde, 4-hydroxynonenal, or advanced glycation end products in lipofuscin of human retinal pigment epithelium. Invest Ophthalmol Vis Sci, 2003. **44**(8): p. 3663-8.; Howes, K.A., et al., Receptor for advanced glycation end products and age-related macular degeneration. Invest Ophthalmol Vis Sci, 2004. **45**(10): p. 3713-20.; Handa, J.T., et al., Increase in the advanced glycation end product pentosidine in Bruch's membrane with age. Invest Ophthalmol Vis Sci, 1999. **40**(3): p. 775-9.; Ishibashi, T., et al., Advanced glycation end products in age-related macular degeneration. Arch Ophthalmol, 1998. **116**(12): p. 1629-32.).

Recently AGE formation has been linked with age-related chronic inflammation at the outer retina in an in vivo model (Tian, J., et al., Advanced glycation endproduct-induced aging of the retinal pigment epithelium and choroid: a comprehensive transcriptional response. Proc Natl Acad Sci U S A, 2005. **102**(33): p. 11846-51.).

Advanced glycation has been implicated in basement membrane (BM) thickening (Charonis AS and Tsilibary EC, Structural and functional changes of lamanin and type IV collagen after nonenzymatic glycation. Diabetes, 1992. **41**(suppl 2): p. 49-51.; Gardiner, T.A., H.R. Anderson, and A.W. Stitt, Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. J Pathol, 2003. **201**(2): p. 328-33.) and it is significant that Bruch's membrane is known to progressively thicken in older patients (Coffey, A.J. and S. Brownstein, The prevalence of macular drusen in postmortem eyes. Am J Ophthalmol, 1986. **102**(2): p. 164-71.) and accumulate sub-RPE deposits that are highly AGE-modified (Mullins, R.F., et al., Characterization of drusen-associated glycoconjugates. Ophthalmology, 1997. **104**(2): p. 288-94).

Summary of the invention

The present inventors have surprisingly determined that disease related AGE modified proteins in the eye can be detected using Raman spectroscopy and have further determined that Raman spectroscopy can be used to detect and monitor ocular AGE protein modifications in the living eye using a non-invasive, painless procedure. Suitably, the procedure may be ophthalmoscopic-based.

The inventors have shown that there are high levels of CML and pentosidine in human Bruch's membrane that is related to patient age (Figure 9). Advantageously this provides for the non-invasive detection and / or measurement of a diagnostic parameter which can predict the initiation and progression of retinopathy and / or macular degeneration in susceptible individuals.

Accordingly, a first aspect of the invention provides a method for measuring at least one AGE adduct in the eye comprising the steps:

- providing a laser light source which generates radiation at a wavelength that produces a Raman response with a wavelength shift for at least one AGE adduct to be detected,
- directing radiation from the laser radiation source into an eye from which at least one AGE is to be measured,
- collecting elastically and inelastically scattered spectra from ocular tissue,
- determining the difference in the intensity and spectral widths of the inelastically scattered spectra from the elastically scattered spectra from said eye, and
- providing a quantifiable Raman spectrum/spectra for said AGE(s).

The Raman effect occurs when light interacts with the electron cloud of the bonds of a molecule and excite the molecule. The relaxation of the molecule from this excited state causes Raman scattering. The energy difference between incident and scattered photons can be expressed as wave numbers cm^{-1}. Raman spectroscopic methods analyse the inelastic scattering of photons caused by changes in the vibrational energy of molecules upon excitation with monochromatic or laser light. The light scattered from the sample is collected and split into its different energies which are then recorded to give a Raman spectrum. Since Raman spectra information is very specific for the chemical bonds in molecules, it provides a fingerprint by which the molecule can be identified.

The laser radiation source provides light of a wavelength that produces a Raman response for an AGE wherein the wavelength shift detected allows

the identification and quantification of the amount of AGE-mediated protein modification present in the eye.

Suitably the eye may be *in situ* in the subject during the method. This is advantageous as the system and method of the invention allow for non-invasive testing for the presence of AGE in the eye of a subject.

Alternatively, the eye is removed from the subject prior to detection / measurement of AGE, for example, post mortem analysis of the subject.

In embodiments of the method, a step of filtering out said elastically scattered light can be included.

Suitably, in embodiments of the invention, the laser radiation source generates a light with a wavelength in the range 514 nm to 785 nm. In particular embodiments the laser radiation source generates light of wavelength in the range 600 nm to 650 nm. In particular embodiments the laser radiation source generates light of about 633 nm.

Suitably the radiation, (light) scattered from the eye is collected to allow detection of Raman shifts characteristic of AGEs. In embodiments radiation scattered from the eye is collected to detect Raman shifts in the range 500 cm^{-1} to 3500 cm^{-1}, preferably in the range 600 cm^{-1} to 1200 cm^{-1} or in the range 700- 1800cm^{-1}. In preferred embodiments radiation scattered from the eye is collected to allow detection of Raman shifts from the ranges of 600 cm^{-1} to 650 cm^{-1}, the range 350 cm^{-1} to 550 cm^{-1}, or in the range 800 cm^{-1} to 1100 cm^{-1}.

In particular embodiments, Raman spectra from molecules in the eye allow detection of Raman Shifts of about 633 cm^{-1}, of AGE-specific doublet

bands at about 400 cm^{-1} and 540 cm^{-1}, and of triplet of bands at about 880,980 and 1090 cm^{-1}.

In embodiments of the method, the method can include the step of examining the eye or a part thereof using an optical microscope.

In particular embodiments, the method may comprise the further steps of:

- providing at a second later time point a laser radiation source which generates radiation at a wavelength that produces a Raman response with a wavelength shift for an AGE to be detected;
- directing radiation from the laser radiation source into an eye from which an AGE is to be measured at a later time point;
- collecting elastically and inelastically scattered radiation from the eye at a later time point,
- determining the difference in the intensity and spectral widths of the inelastically scattered radiation from the elastically scattered radiation from said eye at later time point, and
- providing a quantifiable Raman spectrum for said AGE detected at said later time point.
- comparing the quantifiable Raman spectrum for said AGE at a first time point with the quantifiable Raman spectrum for said AGE determined at the later time point.

As will be appreciated by those of skill in the art, the step of comparing can utilise raw data, but this data will typically be processed to allow clarification of a particular Raman spectral fingerprint to allow quantification and robust identification.

This is advantageous as it allows changes in the amount of AGE modifications in the eye to be monitored over time, for example during a

time period over which the subject ages or during a time period in which the eye has been provided with a test agent, for example a candidate therapeutic agent.

Suitably the method may be used to determine a range of Raman spectra and / or the quantifiable profiles deriveable therefrom from an individual(s) known not to suffer retinopathy and / or macular degeneration or predisposition to retinopathy and / or macular degeneration. This/these Raman spectra and the quantifiable profiles deriveable therefrom may be considered to be a standard profile for a healthy individual.

Suitably the method may be used to determine several Raman spectra and / or the quantifiable profiles deriveable therefrom from an individual known to suffer retinopathy and / or macular degeneration or a predisposition to retinopathy and / or macular degeneration. These Raman spectra and / or the quantifiable profiles deriveable therefrom may be considered to be a standard spectral profile for an individual known to suffer retinopathy and / or macular degeneration or predisposition to retinopathy and / or macular degeneration.

Suitably, in the method, the radiation, for example light, from the laser radiation source may be directed onto the cornea, the lens and / or vitreous ocular tissues of a subject. In embodiments the light from the laser radiation source is directed to parts of the posterior segment of the eye including the vitreous and various layers of the neural retina. In embodiments of the method the light from the laser radiation source is directed to the retinal pigment epithelium, in particular the underlying pentalaminar structure known as Burch's membrane.

In specific embodiments the laser radiation source is directed to the Burch's membrane and innermost layers of the underlying choroid (BM-Ch) at a resolution of 3 micrometer maximum and a depth of 4 micrometers (optical section of about 4 μm).

In embodiments, at least one AGE detected can be selected from the group consisting of pentosidine, fructosyl-lysine, pyrraline, CML (carboxymethyllysine), CEL (carboxyethyllysine), argpyrimidine, glyoxal lysine dimer (GOLD) and advanced lipoxidation endproducts (ALEs) such as malondialdehyde (MDA), hydroxynonenal (HNE) hydroimidazolones N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H), N_{d}-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1) and N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine (3DG-H1), methylglyoxal-derived lysine dimer (MOLD), 3-deoxyglucosone-derived lysine dimer (DOLD) and acrolein-modified protein.

Suitably at least one AGE detected is selected from the group comprising pentosidine, pyrraline, arginine-hydroimidazonlone, CML (carboxymethyllysine), CEL (carboxyethyllysine), GOLD, argpyrimidine and advanced lipoxidation endproducts (ALEs) such as MDA-, HNE- and acrolein-modified protein.

In embodiments, methylglyoxal-derived hydroimidazolnlone and at least one other AGE can be measured and considered in combination. In embodiments, MG-H2 (methylglyoxal-derived hydroimidazolnlone) and at least one of pentosidine and CML (carboxymethyllysine) are considered in combination. In embodiments, all three of MG-H (methylglyoxal-derived hydroimidazolnlone), pentosidine and CML (carboxymethyllysine) are considered in combination.

The system and method may be used to detect individuals who have retinopathy and / or macular degeneration, but have not yet developed problems with their vision and / or prior to the appearance of any other symptoms.

Prediction of the onset of the disease permits early counselling and intervention. Early detection of the disease enables patient treatment and management at an early stage.

According to a second aspect of the present invention there is provided a method to determine retinopathy and / or macular degeneration in a subject comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE adduct in an eye from a test subject with an unknown status in relation to retinopathy and / or macular degeneration; and
- comparing said quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to retinopathy and / or macular degeneration with at least one standard quantifiable Raman spectrum for said AGE for a healthy individual,

wherein the presence of a difference in intensity in areas and / or the peaks between the spectra is indicative of the presence of retinopathy and / or macular degeneration in the subject with previously unknown status.

As will be appreciated, in the step of comparing, quantifiable vibrational spectrum and / or the Raman spectra may be compared.

In an alternative embodiment of the method at least one quantifiable Raman spectrum for an AGE obtained from an eye of a test subject with an unknown status in relation to retinopathy and / or macular degeneration, is compared with a quantifiable Raman spectrum for said AGE considered to be a standard spectrum / spectra for an individual known to suffer retinopathy and / or macular degeneration

wherein at least one commonality in areas and / or peaks between the spectra is indicative of the presence of retinopathy and /or macular degeneration in the subject.

According to a third aspect of the present invention there is provided a method of determining a predisposition of a subject to retinopathy and / or macular degeneration comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE from an eye from a test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration; and
- comparing the quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration with the quantifiable Raman spectrum for said AGE considered to be a standard spectrum for a healthy individual,

wherein the presence of a difference in intensity in areas and / or the peaks between the spectra is indicative of a predisposition to retinopathy and / or macular degeneration in the subject with previously unknown status.

In an alternative embodiment of the method the at least one quantifiable Raman spectrum for an AGE measured in an eye from a test subject with

an unknown status in relation to a predisposition to retinopathy and / or macular degeneration is compared with a quantifiable Raman spectrum for said AGE considered to be a standard spectrum / spectra for an individual known to suffer a predisposition to retinopathy and / or macular degeneration,

wherein at least one commonality in areas and / or peaks between the spectra is indicative of a predisposition to retinopathy and / or macular degeneration in the subject.

Typically, multiple differences or areas of commonality between a quantifiable Raman spectrum for an AGE from a test subject and the respective quantifiable Raman spectrum for said AGE from a healthy subject or a quantifiable Raman spectrum for said AGE from an individual known to suffer from retinopathy and / or macular degeneration and / or a predisposition to retinopathy and / or macular degeneration are observed.

In particular embodiments in an eye from an individual known to suffer from retinopathy and / or macular degeneration and / or have a predisposition to retinopathy and / or macular degeneration the level of AGE is at least three times greater than the level of AGE present in an eye from an individual who does not suffer from retinopathy and / or macular degeneration and / or have a predisposition to retinopathy and / or macular degeneration.

In particular embodiments in an eye from an individual known to suffer from retinopathy and / or macular degeneration and / or have a predisposition to retinopathy and / or macular degeneration the level of AGE is at least five times greater than the level of AGE present in an eye from an individual who does not suffer from retinopathy and / or macular

degeneration and / or have a predisposition to retinopathy and / or macular degeneration.

As will be appreciated by those in the art, the analysis and comparison of quantifiable Raman spectrum for an AGE may be performed by a computer.

Accordingly, a fourth aspect of the present invention provides a computer storage medium comprising a computer program, which when executed on a processor causes processor to:

i) receive subject data corresponding to a quantifiable Raman spectrum for an eye of a subject;

ii) access standard data corresponding to at least one standard quantifiable Raman spectrum for an AGE from a data storage device;

iii) compare the subject data and the standard data; and

iv) determine from the comparison of Step (iii) whether the presence of an AGE in the eye of the subject is indicated.

The program, when executed, may cause the processor to compare the subject data to a plurality of standard data sets to determine if variation of the AGE indicated in Step (iv) has occurred from previously recorded subject data.

The program, when executed, may cause the processor to compare the standard data to a plurality of subject data sets, recorded for the same subject, to determine if variation of the AGE indicated in Step (iv) has occurred from previously recorded subject data.

The program, when executed, may cause the processor to generate result data corresponding to the indication of the presence, or of the indication of the variation, of the AGE, and to output the result data to a user terminal.

In particular embodiments, in the step of comparing the quantifiable Raman spectrum for an AGE present in an eye of a subject with at least one standard quantifiable Raman spectrum for said AGE, a quantative measurement of the amount of AGE present in the eye of the subject with reference to the standard can be determined. For example, wherein the standard is provided from a individual known not to suffer from retinopathy and / or macular degeneration and / or have a predisposition to retinopathy and / or macular degeneration, it can be determined if the level of AGE present in the sample is above a threshold value, for example at least three times, at least four times, at least five time greater than the level of AGE present in an eye from an individual who does not suffer from retinopathy and / or macular degeneration and / or have a predisposition to retinopathy and / or macular degeneration.

As will be appreciated, based on the results of the comparison, a diagnosis as to whether the subject from which the Raman spectra has been obtained suffers from retinopathy and / or macular degeneration and / or has a predisposition to retinopathy and / or macular degeneration can be obtained.

Suitably, in embodiments of the second, third or fourth aspects of the invention, the quantifiable Raman spectrum for an AGE provided / compared may be obtained from a method wherein the laser radiation source is directed onto the cornea, the lens and / or vitreous ocular tissues of a subject. In particular embodiments the radiation is directed to parts of the posterior segment of the eye including the vitreous and various layers

of the neural retina. In particular embodiments the laser radiation source can be directed to parts of the eye other than the vitreous. In embodiments of the method the laser radiation source can be directed to the retinal pigment epithelium, in particular the underlying pentalaminar structure known as Burch's membrane. In specific embodiments the profile provided can be obtained from a method wherein the laser radiation source is directed to the Burch's membrane and innermost layers of the underlying choroid (BM-Ch) at a maximum depth of 4 micrometers.

Suitably the quantifiable Raman spectrum for an advanced glycation endproduct can be of at least one AGE selected from the group comprising pentosidine, pyrraline, arginine-hydroimidazonlone, CML, CEL, argpyrimidine and advanced lipoxidation endproducts (ALEs) such as MDA-, HNE- and acrolein-modified protein. In particular embodiments, the profile can be of advanced glycated endproducts measured selected from the group consisting of pentosidine, pyrraline, arginine-hydroimidazonlone, CML, CEL, argpyrimidine and advanced lipoxidation endproducts (ALEs) such as MDA-, HNE- and acrolein-modified protein.

Suitably the retinopathy detected by the method may be diabetic retinopathy. Alternatively the retinopathy detected by the method may be age related macular degeneration.

According to a fifth aspect of the present invention there is provided a system for the *in-situ* non-invasive measurement of AGEs in an eye comprising a Raman spectroscope comprising;

- at least one laser radiation source adapted to generate radiation at a wavelength that produces a Raman response with a wavelength shift for an AGE to be detected;

- laser radiation source directing means capable of directing radiation into an eye;
- radiation collecting means for collecting elastically and inelastically scattered radiation; and
- detection means to measure the difference in intensity and spectral width(s) of the inelastically scattered radiation from the elastically scattered radiation.

Advantageously, non-invasive evaluation of robust markers of disease progression in the eye(s) of diabetic patients would provide an important basis for recognising retinopathic risk.

Whilst a system has been described for use for non-invasive measurement of the carotenoids at the back of the retina in patients (Gellermann, W., et al., In vivo resonant Raman measurement of macular carotenoid pigments in the young and the aging human retina. J Opt Soc Am A Opt Image Sci Vis, 2002. 19(6): p. 1172-86.; Gellermann, W. and P.S. Bernstein, Noninvasive detection of macular pigments in the human eye. J Biomed Opt, 2004. 9(1): p. 75-85.), there has been no suggestion of a modifying such a system to vary stimulation wavelength in order to detect and quantify different groups of AGE adducts in various segments of the eye.

In particular embodiments of the system, the system includes an optical microscope.

In one embodiment the radiation generating source is an argon laser. In a particular embodiment the radiation generating source is a 1.0 mW argon laser spot (at various wavelengths, in particular wavelengths in the range 600 nm to 650 nm, more particular wavelengths around 633 nm capable of providing a spot of 8mm in diameter. Suitably the laser radiation source

may be a Raman confocal microscope. Suitably the Raman confocal microscope may use 633 nm excitation (20 mW), with x 100 objective and a slit width of 150μm. Suitably collection of spectra may be at 700-1800cm^{-1}.

Suitably the laser radiation source directing means may be an opthalmoscope. Alternatively mirrors, lenses or suitable combinations thereof may be used.

Suitably the radiation directing system may allow radiation to be directed onto the cornea, lens, vitreous, retinal tissues, the retinal epithelium (RPE), particularly within the underlying pentalaminar substrate known as Burch's membrane in the living eye. Suitably, this can allow for rapid, non-invasive evaluation of various ocular structures.

Suitably, the radiation collecting means can be, for example, but not limited to a fibre-optic collection bundle, lenses, mirrors or combinations thereof can collect light of wavelengths in the range less 514 nm to 785 nm. The radiation collecting means may collect light at wavelengths selected from at 514 nm, 633 nm or 785 nm.

In one embodiment the radiation collecting means is a fibre-optic collection bundle.

The range of wavelengths used can be determined to be appropriate to measure the AGE which is being considered.

In embodiments of the invention, the system can include filtering means, for example, but not limited to halographic filters, prisms, dielectrics, monochromators or combinations thereof.

Suitably a photodetector, including but not limited to, at least one CCD, or a photomultiplier may be used to measure the collected light.

The system can be used to detect AGE modified proteins to allow diagnosis of retinopathy and / or macular degeneration. Additionally or alternatively, the system can be used in the testing and monitoring of individuals believed to be at risk from retinopathy and / or macular degeneration. For example, individuals suffering from diabetes or individuals with a family history of diabetes may be tested in order to enable early intervention to prevent onset or development of the diabetes retinopathy.

The invention provides a method to characterise associations between diabetes, or age-related risks and AGEs. Further, following appropriate identification, quantification and validation of AGE-unique Raman spectra, the method provides a unique and important diagnostic tool for preventing blindness.

Throughout this specification, references to AGE should be understood to refer to intracellular and extracellular proteins of the eye, particularly, but not limited to, proteins of the cornea, lens and / or vitreous linked to one or more molecules of glucose, metabolites or related reducing sugars in the form of glucitol adducts, Schiff base adducts or Amadori products.

In a further aspect, there is provided a kit for carrying out a method of the invention said kit comprising at least;

- a system according to the fifth aspect of the invention, and

- at least one standard spectrum for comparison with the spectrum generated from an eye of an test subject and optionally,
- instructions on how the system is to be used and / or the results generated are to be interpreted.

Preferred features and embodiments of each aspect of the invention are as for each of the other aspects mutatis mutandis unless context demands otherwise.

The present invention will now be described by way of example only, with reference to the accompanying drawings, wherein:

Figure 1 shows pentosidine accumulation in Bruch's membrane choroid - HPLC analysis of pentosidine in human BM-Ch was performed and typical elution profiles for a younger (A, 41 year old) and older (B, 82 year old) sample showing a comparatively smaller pentosidine peak in the young sample are shown. Confirmation that the peak identified as pentosidine was correct was carried out by spiking authentic pentosidine in the samples, and observing an increase in the area units of those peaks (Aii, Bii). Standard pentosidine (0,4 pmol) eluted at a comparable time (C). Pentosidine content in BM-Ch for various age-cohorts (D). **$p<0.005$;

Figure 2 shows immunolocalisation and quantification of AGEs in Bruch's membrane - CML immunoreactivity was determined in sections of Bruch's membrane–choroid complex. Representative sections are shown from a 60 year old (A) and a 86 year old donor (B). In both cases the antibody localised CML to Bruch's membrane with greatest intensity (arrow). There was also significant deposition in the extracellular matrix of the choroid (*). Isotype (C) or secondary omission controls showed no evidence of

staining (original magnifications: x200). CML and CEL were quantified in Bruch's membrane–choroid complex by GC-MS from donors aged 0-50 years (hatched bars), 51-79 years (white bars) and >80 years (black bars) (D). There was a statistically significant increase in CML in the oldest compared to the youngest cohort, but no difference among the cohorts in CEL. * $P<0.05$;

Figure 3 shows Raman analysis of AGEs- Comparisons were made between the Raman signals obtained from i) AGE-modified BSA and ii) unmodified BSA. The difference between the two signals (subtraction spectrum iii) corresponds to the signal from AGE modifications. iv) equivalent Raman subtraction spectrum of CML-BSA which shows marked superficial similarly to AGE-BSA spectrum shown, but significant band shifts. v) Raman signal obtained from pure pentosidine;

Figure 4 shows Raman analysis of Bruch's membrane - The dominant Raman spectral signals identified using PCA, which closely match the signal for i) heme, ii) collagen and iii) AGE (arrow) , arginine/lysine (*) and peptide bonds (†). These spectra are the average of the extreme PC scores for selected chemically significant components. In i) and ii) a small contribution from collagen and from heme and collagen signals, respectively, has been subtracted for clarity;

Figure 5 shows Raman analysis of pentosidine in Bruch's membrane - Raman spectrum of pentosidine compared with ii) the regression coefficients selected by the uncertainty test as most correlated with pentosidine concentration. The bands of the Raman signal which positively contribute to the regression coefficients match bands which occur in the spectrum of pentosidine;

Figure 6 shows Age prediction using Raman spectral analysis of AGEs -

A) PLS-regression plot for the prediction of pentosidine concentration from Raman spectra of Bruch's membrane.
B) Plot of predicted pentosidine concentration against age. The difference between samples over 60 and under 60 is significant ($p<0.05$).
C) Validation PLS regression plot predicts the concentration of CML (dotted line) and CEL (solid line) adducts in Bruch's membrane using Raman spectroscopy;

Figure 7 shows Gender differences in AGEs within Bruch's membrane during ageing.
a) Gender score (0= male, 1=female) and b) age score (0=under 60, 1= over 60) predicted from the Raman signal of human donor Bruch's membranes. The predicted score is significantly different between the sexes and between the age groups ($p<0.00001$). The average age of each gender and the gender composition of the two age groups are not significantly different ($p>0.05$). c) most significant PC used to separate the genders and the age groups. The positive bands match the Raman signals of AGE and heme, while the negative bands match those of collagen;

Figure 8 depicts Raman spectra from retinal layers of human Bruch's membrane as well as characteristic spectra for pentosidine as a typical age-accumulated AGE on long-lived structural proteins wherein the composite spectral profile has placed the pentoside standard onto the clinical results – There are common areas and peaks around 1000 cm^{-1}, which is the position for Phe (aromatics) and also at 1200-1400 cm^{-1} there is an amide linkage. Pentosidine also has a histidine and CH2 deformation at around 1400 cm^{-1} common to the clinical samples. Older samples shown indicators of protein crosslinking and presence of pentosidine. The two older samples are two eyecups (A and B) from one patient and this would indicate that in this case there are no great variations between individual eyecups from analysis using this method:

Figure 9 shows a table of high levels of CML and pentosidine in human Bruch's membrane in relation to patient age – this is indicated as AGE/ALE content in human Bruch's membrane samples analysed using GC-MS. Data is expressed as Mmol/mol Lysine. In AMD terms, "young" is defined as less than 60 years of age;

Figure 10 shows Averaged predicted age against recorded age using Raman data that was preprocessed with orthogonal signal correction (OSC). Plots of the pentosidine level predicted from the OSC Raman signal of donor Bruch's membranes against the recorded/measured values ($R^2 = 0.705$). The OSC Raman signal showed a significant systematic change with age, and correlated well with the measured pentosidine level;

Figure 11 shows Raman spectra obtained from of selected pure AGEs using a Raman confocal microscope with 633 nm or 785nm excitation (20 mW), a 50 objective, and a slit width of 150µm; and

Figure 12 shows Raman spectra obtained from of selected pure AGEs using a Raman confocal microscope with 633 nm or 785nm excitation (20 mW), a 50 objective, and a slit width of 150µm.

EXAMPLE 1

AGEs in Bruch's membrane were quantified from post-mortem eyes and age-related correlations were established. Confocal Raman microscopy was used to identify and quantify AGEs in Bruch's membrane in a non-destructive, analytical fashion. Bruch's membrane and the innermost layers of the underlying choroid (BM-Ch) were dissected from fresh post-mortem eye-cups (n=56). AGE adducts were quantified from homogenized

tissue using reverse-phase HPLC and GC/MS in combination with immunohistochemistry. For parallel Raman analysis, BM-Ch was flat-mounted on slides and evaluated using a Raman confocal microscope and spectra analysed by a range of statistical approaches. Quantitative analysis showed the AGEs pentosidine, carboxymethyllysine (CML) and carboxyethyllysine (CEL) occurred at significantly higher levels in BM-Ch with age. ($P<0.05$-0.01). Defined Raman spectral "fingerprints" were identified for various AGEs and these were observed in the clinical samples using confocal Raman microscopy. The Raman dataset successfully modelled AGEs and not only provided quantitative data that compared with conventional analytical approaches, but also provided new and complementary information via a non-destructive approach with high spatial resolution. In particular, it was shown that the Raman approach could be used to predict chronological age of the clinical samples ($P<0.001$) and a difference in the Raman spectra between genders was also highly significant ($p<0.000001$).

Materials and Methods

Clinical samples

Human donor eyes (n = 56 age range 32-92 years) were obtained from patients of varying age and gender. None of the patients had been diagnosed with AMD and a range of causes of death were documented. Since this was an ageing study, the eyes were classified into young (<50 years old, n=8), intermediate (50-70 years, n=27) and old (70+ years, n=21). Eyes were retrieved approximately 4 hours post-mortem and immediately placed on ice. The anterior portion of the eye was removed and RPE layer brushed off as described previously (Rozanowska, M., Jarvis-Evans, J., Korytowski, W., Boulton, M. E., Burke, J. M., and Sarna,

T. (1995) Blue light-induced reactivity of retinal age pigment. In vitro generation of oxygen-reactive species. J Biol Chem **270**, 18825-18830).

All methods were carried out in accordance with the tenets of the Declaration of Helsinki for any research involving human tissue. In addition, informed consent was obtained from relatives prior to the study and ethical approval was obtained from Research Ethics Committees of all involved Institutions.

The posterior segments, with RPE removed, were stored at -80^{0}C. Prior to use, the samples were thawed on ice in phosphate buffered saline (PBS), pH 7.4. The retrieved eyecups were then dissected bilaterally and strips of Bruch's membrane-choroid (BM-Ch) adjacent to the macula (1 cm x 0.5 cm) dissected from the remaining eyecup and frozen and stored at -80°C.

Reference Compounds

Model AGEs were prepared by incubating bovine serum albumin (BSA fraction V, Sigma-Aldrich Company Ltd., Dorset, UK, at 10 mg/ml) in 0.5mol/L glucose solution for 8 weeks at 37 °C, as described previously (Stitt, A. W., Li, Y. Mo, Gardiner, T. A., Bucala, R., Archer, D. B,. and Vlassare, Ho (1997) Advanced glycation end products (AGEs) colocalise with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats Am, J. Pathol. 150, 53-531.) CML-BSA and pentosidine were prepared as described (Wells-Knecht, M. C., Thorpe, S. R. and Baynes, J. W. (1995) Pathways of formation of glycoxidation products during glycation of collagen, Biochemistry34, 15134-15141; Ahmed, M. U., Brinkmann Frye, E., Degenhardt, T. P., Thorpe, S. R., and Baynes, J. W. (1997) N-epsilon-(carboxyethyl)lysine, a product of the chemical

modification of proteins by methylglyoxal, increases with age in human lens proteins. Biochem J 324 (Pt 2), 565-570).

Measurement of AGEs

Dissected BM-Ch were homogenised under liquid nitrogen and protein estimations conducted using the BCA Protein Assay method (Pierce, Rockford, IL, USA). Pentosidine and CML were quantified using HPLC and GC/MS respectively, as previously described (Degenhardt, T. P., Grass, L., Reddy, S., Thorpe, S. R., Diamandis, E. P., and Baynes, J. W. (1997) Technical note. The serum concentration of the advanced glycation end-product N epsilon-(carboxymethyl)lysine is increased in uremia. Kidney Int **52**, 1064-1067). Briefly, for pentosidine analysis, dissected BM-Ch were reduced with sodium borohydride in sodium borate buffer (pH 9.2) followed by protein precipitation with trichloroacetic acid (TCA). Lipids were extracted from the re-suspended protein pellet with ice-cold methanol: ether (3:1) after which reverse-phase HPLC (RP-HPLC) was conducted. Limits of detection and linearity range of the HPLC were 0.025-1.6 pmoles (r = 0.98). In some cases, a fixed amount of standard pentosidine (0.1-0.4pmol) was assayed in a 'mixing experiment' as previously described (Degenhardt, T. P., Grass, L., Reddy, S., Thorpe, S. R., Diamandis, E. P., and Baynes, J. W. (1997) Technical note. The serum concentration of the advanced glycation end-product N epsilon-(carboxymethyl)lysine is increased in uremia. Kidney Int **52**, 1064-1067). Results were expressed as pmol pentosidine/mg protein as calculated from the BCA assay.

GC/MS analysis (n=9) for CML and CEL was carried out on reduced BM-Ch samples processed as outlined above. The GC/MS was conduced as previously described (Dunn, J. A., McCance, D. R., Thorpe, S. R., Lyons, T. J., and Baynes, J. W. (1991) Age-dependent accumulation of N epsilon-

(carboxymethyl)lysine and N epsilon-(carboxymethyl)hydroxylysine in human skin collagen. Biochemistry **30**, 1205-1210) and the AGE concentrations were expressed as mmol/mol lysine. For parallel CML immunohistochemistry, BM-Ch samples were fixed in 4% (w/v) paraformaldehyde (PFA) (Sigma-Aldrich Company Ltd., Dorset, UK) and processed according to a previously published protocol with slight modifications (Stitt, A., Gardiner, T. A., Anderson, N. L., Canning, P., Frizzell, N., Duffy, N., Boyle, C., Januszewski, A. S., Chachich, M., Baynes, J. W., and Thorpe, S. R. (2002) The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. Diabetes **51**, 2826-2832). Briefly paraffin sections were permeabilised using phosphate buffered saline (PBS)-Tween (0.1%), blocked with 5% normal goat serum (NGS) and stained with polyclonal anti-CML antibody. Some were treated with PBS alone or an IgG rabbit polyclonal (DakoCytomation Ltd., Glostrup, Denmark) as negative and isotype controls. Immunoreactivity was detected with the secondary antibody horse radish peroxidase (HRP) conjugated goat anti-rabbit (1:100) and then stained with 3-amino-9-ethylcarbazole (AEC) and chromogen substrate (DakoCytomation Ltd., Glostrup, Denmark. The sections were counterstained with Mayer's haematoxylin (Sigma-Aldrich Company Ltd., Dorset, UK), mounted in an aqueous mounting medium (DakoCytomation Ltd., Glostrup, Denmark) and visualised by bright-field microscopy (Lucia G/G-F, Nikon, Surrey, UK).

Confocal Raman Spectroscopy

Segments of BM-Ch measuring approximately 0.5cm^2 in diameter in the region of the central retina were micro-dissected from the sclera. With Bruch's membrane uppermost, the samples were then placed directly onto extra-white slides (Menzel-Glaser, Braunschweig, Germany) to minimize

the non-Raman background from the substrate. All specimens were pre-treated in this fashion in preference to the standardised procedure of storage in 4 % paraformaldehyde (PFA), which would be expected to introduce artefactual cross-linking of the tissues.

Raman analysis was performed using a Raman confocal microscope (Horiba Jobin-Yvon LabRam HR800, Villeneuve d'Ascq, France) using 633 nm excitation (20 mW), with x 100 objective and a slit width of 150µm. A confocal hole-size of 200µm was used to give a nominal z-axis resolution of 1.5µm. An area was selected and 100 spectra (10 x 10 grid) were accumulated in the 700- 1800cm^{-1} region for 30 seconds. The spectral intensity was normalised by dividing each point by the background-corrected average intensity between 1555 and 1670 cm^{-1} (baseline points at 1500 and 1725 cm^{-1}). This region was chosen so as to include both heme and protein signals. All spectral acquisition was carried out in Labspec software V4.16 (Jobin-Yvon, Villeneuve d'Ascq, France).

Non-Raman Background Correction

Raman spectra were obtained for the model AGEs pentosidine, AGE-BSA and CML-BSA in a cell-free fashion. In common with many other biological systems, a degree of non-Raman background fluorescence was present in the samples studied. These were corrected for in two stages. Principal Components Analysis (PCA) was initially conducted on the unprocessed Raman data and the dominant non-Raman signals were identified. Any residual Raman signals were subtracted using a chemically equivalent signal that had no significant background. The root mean square best fit polynomial line (degree = 9) was determined for each of these backgrounds in order to obtain a smooth noiseless line approximating the various background shapes within the dataset. The linear combination of these backgrounds which gave the most effective

background subtraction was calculated in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Any remaining background was removed in Labspec software V4.16 (Jobin-Yvon, Villeneuve d'Ascq, France) using a series of linear extrapolations between adjacent minima within the spectrum. Since there are no common baseline points available for heme and protein, an adaptive method of analysis was employed. Instead of analysing selected fixed points for baseline determination of samples, the minimum value within a restricted range of Raman shifts was selected, allowing the baseline point to be adjusted according to the spectral shape.

The absolute signal intensity of the Raman signal due to any given component of a sample is generally non-reproducible due to a high number of influencing factors including focal position, sample absorbency, laser power and path-length. In order to standardise the signal it is necessary to normalise the spectral intensity to an internal standard. In this study a number of normalisation routines were compared and the optimal routine for each component was selected. These routines included no normalisation, normalising against the average intensity of the whole spectrum, the intensity of each of the bands at 1003, 1450 and 1570 cm^{-1} and the average intensity across the 1550-1690 cm^{-1} region.

Statistical analysis

Raman spectral analysis was conducted using The Unscrambler V9.6 (Camo, Trondheim, Norway). PCA was carried out on the full data set in order to assess the overall signal variation within the data set and identify important spectral signals (Williams, P., and Norris, K., eds (2001) Near-Infrared Technology in the Agricultural and Food Industries, AACC Press,

St Paul, MN). A multivariate regression technique known as "Projection to Latent Structures" was used to calibrate the Raman signal against the chronological age, gender of the donor and analytically quantified AGEs. Models were constructed for each of the reference parameters and then used to predict the levels of those parameters in the remaining dataset. Multivariate analysis methods used included Principal Component Analysis (PCA), regression methods such as Partial Least Squares (PLS) and Discriminant Analysis (DA). In all the statistical analyses the spectra were mean-centred, and cross validation was used for validation of the model.

Statistical analysis of the Raman-predicted results, together with the HPLC and GC/MS data was performed using Microsoft Excel to calculate mean and standard deviation (SD). Subsequently, a one-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis, was conducted using the statistical analysis package SPSS. Data were considered significant at the 95% level ($p < 0.05$). Gender comparison was carried out using student's T-test (unpaired, unequal variances). As male samples were more numerous a randomly selected subset was used in this comparison with female samples.

AGEs in Bruch's Membrane-Choroid Complex

Prior to pentosidine analysis, an internal pentosidine model AGE standard was also run alongside each individual experiment, at 0.404pmol to confirm elution time (Fig 1C). A typical chromatogram for Bruch's membrane-choroid specimens from younger patients showed a small peak eluting close to the retention time for authentic pentosidine (Fig. 1Ai & 1Aii, depicting a confirmatory mixing experiment using synthetic pentosidine to 'spike' the sample and validate the pentosidine specific nature of the peak by increasing peak height). Chromatograms from older donors (~70 years) typically showed a larger pentosidine peak compared to the young

specimens (Fig. 1Bi and ii). In terms of grouped analysis of pentosidine in BM-Ch, there was a doubling in pentosidine content of samples from the groups aged 50-70 years when compared to young samples (≤50 years) ($p<0.01$) (Fig. 1D). The increase in pentosidine with age was greater than 4 fold with the oldest age group (>70 years) ($p< 0.005$).

CML-immunoreactivity occurred at Bruch's membrane but also deeper within the choroid. CML-intensity was increased in sections from older patients (compare Figures 2A with 2B); while isotype controls showed negative staining (Figure 2C). Quantification of CML using GC/MS demonstrated that older patients had significantly higher levels of this adduct in BM-Ch ($P<0.05$) (Figure 2D). GC/MS studies also quantified the related adduct carboxy-ethyl-lysine (CEL) but differences were not statistically significant (Figure 2D).

Confocal Raman Spectroscopy of AGEs

Prior to analysis of tissue samples, the Raman signals of a generalised AGE-albumin and an albumin specifically modified by CML were compared with native albumin with the difference between the Raman signals for the modified and unmodified albumins reflecting the Raman band shape of AGE adducts (Figure 3 i-iv). The Raman signal of pure pentosidine was also obtained for reference (Figure 3v) The Raman signal for the undefined range of adducts formed during the incubation of albumin in glucose showed a doublet of bands at 400 and 540 cm-1 as well as a triplet of bands at 880,980 and 1090 cm-1. While CML showed a superficially similar band-shape to the undefined AGE adduct, significant shifts in band positions were evident (Figure 3iv). The CML spectrum contained a doublet at 510 and 565 cm-1 and bands at 860, 930, 1000, 1065 and 1130 cm-1. In contrast, the spectrum of pentosidine (Figure 3v)

showed a much greater number of narrower bands covering a wider range of the spectrum. For further investigation, the 700-1800 cm-1 region of the spectrum was chosen for clinical sample analysis.

Confocal Raman Spectroscopy of Bruch's membrane

For Raman spectroscopic analysis measurements on BM-Ch were made at a maximum depth of 4μm. As will be appreciated by those of skill in the art, the depth chosen can be varied according to the nature "thickness" of the specific tissue being considered. This depth was chosen following pilot studies in which deeper optical sections resulted in Raman scattering from within the choroid. It was also found that high melanin content in the choroid swamped the Raman signal from overlying Bruch's membrane; thus all confocal measurements were taken at defined depth.

PCA of the Raman data revealed a number of significant spectral signals (Figure 4, and supporting information for the 1st 18 PCs) with associated band assignments (Table 1).

Table 1. Raman assignments of selected bands in the 700-1800 cm^{-1} region from Bruch's membrane.

Raman Shift / cm-1	Assignment	Comments
740	Heme	
750	heme, collagen	arg, hpro
803	Pentosidine	
830	Collagen	Pro

838	Pentosidine	
850	Collagen	hpro, arg
860	Collagen	arg, lys
875	Collagen	Pro
880	AGE	
890	collagen, fat	terminal C-C bonds
910	Collagen	Pro
940	heme, collagen	C-C bonds, α-helix conformation in proteins
965	Collagen	Arg
970	heme, pentosidine	
980	AGE	
995	heme, collagen, pentosidine	arg, pro
1010-20	Collagen	arg, lys
1030	Collagen	
1050	Collagen	arg, lys, pro
1060	fat, collagen	C-C bonds, arg
1080-90	collagen, AGE, fat	C-C bonds in non-linear molecules, arg, hpro
1125	heme, collagen, fat	C-C bonds
1136	Collagen	arg, lys
1150	Pentosidine	
1160	collagen, pentosidine	lys, pro
1180	Collagen	Hpro
1215	Pentosidine	
1250	heme, collagen	Amide III (peptide bonds)
1270	Fat	=C-H bonds
1315	Collagen	arg, hpro, lys

1300,26,60	Pentosidine	
1340,65,95	Heme	porphyrin group, sensitive to redox reactions
1400-1500	heme, collagen, fat	CH2 groups
1430,1455	Pentosidine	
1435	Collagen	arg, lys
1460	Collagen	arg, lys
1527	Collagen	Arg
1545,75,1610	Heme	porphyrin group, sensitive to redox reactions
1550	Collagen	Pro
1575	Collagen	Lys
1585	Collagen	Arg
1605	Collagen	hpro, pro
1620	Collagen	arg, lys, pro
1650-80	collagen, fat	Amide I (peptide bonds), C=C

Table 1

The Raman signal was dominated by spectral features attributable to a protein with a significantly high proportion of proline and hydroxyproline and a random coil secondary structure (Figure 4ii). Signals characteristic of heme and an spectral signal similar to that of the undefined AGE adducts identified above, previously unreported in the literature, were also dominant in the dataset (Figures 4i & 4iii). In Bruch's membrane this AGE signal (arrowed in figure 4iii) was observed in association with collagen and heme, and when these spectra were subtracted, a signal closely matching that of a combination of arginine and lysine remained (asterisked in Figure 4iii). The variation in the heme signal was significantly greater (a factor of ten) than variation in any of the other constituents, accounting for

71 % of the total variation within the unprocessed data set, compared with 7% of the variation explained by the second component. This dominant signal was found to be unrelated to the parameters of interest here (i.e. donor age and pentosidine levels and gender; R^2 <0.1, see supporting information), but its influence was minimised by normalising the spectra in such as way that the variation due to heme was reduced within the dataset. While the least squares regression correlation between the heme signal and these parameters was poor, there was however a significant increase in the heme signal in females compared with males ($p<0.05$) and in over 60 years old compared with under 60 years old ($p<0.01$) (Table 2).

Table 2. Results of PLS regression analysis of Raman spectra of Bruch's membrane with various parameters. [a] Standard Error of Prediction [b] Standard Error of Estimate

	R^2	SEP[a]	SEE[b]	No. Factors	No. Donors
Age	0.630	5.17	5.50	10	41
Age Male	0.644	4.84	3.83	3	30
Age Female	0.686	7.18	7.89	6	11
Pentosidine	0.487	3.89	4.55	9	33
Pentosidine Male	0.907	0.84	1.603	7	16
Pentosidine Female	0.972	0.17	0.45	4	5
CML	0.906	0.49	0.89	3	6
CEL	0.751	0.15	0.19	5	6

Table 2

AGEs in Bruch's membrane and Raman comparative analysis

PLS-regression analysis showed a positive correlation for pentosidine concentration as determined by HPLC with that determined by Raman analysis (Figure 6A), though the correlation did not work effectively for both sexes simultaneously and required separate models to be created for the male and female samples to provide the optimum model performance (Table 2). Raman analysis of pentosidine showed significantly greater levels in patients over 60 years old compared with patients under 60 ($P<0.05$, Figure 6B). Notably, males showed ca. three times the level of this AGE compared with females for both the Raman-predicted ($p<0.001$) and HPLC measured datasets ($p<0.05$), while the level of pentosidine was 2.5 fold higher in over 60 males than under 60 males ($p<0.05$). (Table 3).

Table 3. Statistical significance of comparing Raman predicted, HPLC and GCMS measured parameters with gender and age groups (<60 vs. >60). The groups showing elevated levels are shown, with M= male, F= female, O= >60, Y= <60. Gender comparison of CML and CEL GCMS data could not be made due small number of female donors measured. n.s. not significant, $p>0.05$.

	p<(Gender)	High	p<(Age group)	High
Raman Heme signal	0.05	F	0.01	O
Raman predicted Pentosidine	0.001	M	0.05	O
HPLC measured Pentosidine	0.05	M	0.05	O
Raman predicted CML	0.0001	F	0.05	O

GCMS measured CML	n.a.		0.05	O
Raman predicted CEL	n.s.		n.s.	
GCMS measured CEL	n.a.		n.s.	
Overall Raman signal	0.00001	F	0.00001	O

Table 3

The positive contributions to the regression coefficients selected by the uncertainty test as most correlated with pentosidine concentration (Figure 6Aii) all match band positions that occur within the Raman signal of pure pentosidine (Figure 6Ai).

As with pentosidine, the validation PLS regression analysis for Raman spectra from CML and CEL revealed a positive correlation between GC/MS analysis and Raman spectroscopy (Figure 6C and Table 2) and a close match between the Raman signal of the adduct and the regression coefficients used to predict the quantity of that adduct in Bruch's membrane (Figure 6C). The Raman predicted CML level also showed a significant change with age and significantly elevated levels in females compared to males ($p<0.05$ and 0.0001 respectively). The Raman predicted CEL levels showed no significant variation with age or gender (Table 3).

Overall analysis of the Raman dataset confirmed the gender and age differences within the BM-Ch samples, with PLS regression against gender and age group revealing highly significant differences in the Raman signals of male vs female and under 60 vs over 60 (Figure 7A &

7B, $p<0.00001$ in each case). The regression coefficients used to separate the sexes is shown in Figure 7C, and it closely matches that of the AGE adduct in Figure 2C.

Example 2

For a typical Raman resonance spectral measurement to detect AGEs in the eye, a 1.0 mW argon laser spot (at various wavelengths) could be used to direct a 8mm diameter spot at the cornea, lens or vitreous for 0.25 seconds.

Alignment of the laser spot, such that suitable measurements of AGE at selected points in an eye may be made, could be performed by self alignment wherein a subject would be requested to self-align the spot by viewing the blue-green laser beam superimposed on a target. Raman backscattered light would be collected by a fibre-optic collection bundle and the resultant Raman spectrum analysed. Using this methodology, it would be likely that Raman peaks could be obtained with good signal to noise ratio.

Using the system described, the ocular exposure levels would stay below specified threshold levels and will remain limited to protect the retina from photochemically and thermally induced injury under measurement conditions. The photochemical limit for retinal injury is listed as 2.7 C_B J/cm^2 and in a typical single exposure, measurement with the described instrument (0.5 second ocular exposure with 0.5 mW light at 488 nm) a total laser energy of 0.25 mJ would be projected onto a 8 mm diameter spot at the cornea and 1 mm diameter spot on the retina. This would correspond to a retinal exposure level of 32 mJ/cm^2, which is ~480 times lower than the 15.5 J/cm^2 photochemical limit.

For the used ocular exposure the inventors calculate a level of 0.5 mJ/cm^2 considering that the light energy of 0.25 mJ is distributed over a spot size diameter of 8 mm at the cornea; therefore this exposure level is ~19 times below the thermal limit of 9.6 mJ/cm^2 for retinal injury.

High resolution Raman spectroscopic techniques have potential to make important contributions as a diagnostic tool. Raman spectroscopy is rapid and specific with no extensive sample preparation being necessary. Furthermore, the native Raman spectra of molecules can be probed without need for specific fluorescence labelling.

The generation of this data and the like would enable a comprehensive database of Raman spectra for disease-related AGE-modifications to be developed that could be used for detection in "at risk " patient groups as a reliable index for how the eye is accumulating AGE-moieties as it ages.

Raman spectra from retinal layers as well as characteristic spectra for pentosidine as a typical age-accumulated AGE on long-lived structural proteins (Figure 1) have been generated. In addition, the inventors have demonstrated various AGEs form in human ocular tissues. For example, preliminary Raman data has identified characteristic unique spectra at 633 nm from retrieved donor human eyecups. Confocal Raman microscopy shows higher levels of pentosidine in Bruch's membrane from older eyes and this is corroborated by the inventors HPLC data. Partial least squares regressional analyses show correlation between HPLC analysis and Raman spectroscopy ($p<0.005$).

Although the present invention has been particularly shown and described with reference to a particular example, it will be understood by those

skilled in the art that various changes in the form and details may be made therein without departing from the scope of the present invention.

5

Claims

1. A method for measuring at least one AGE adduct in an eye comprising the steps:

- providing a laser light radiation source which generates radiation at a wavelength that is capable of producing a Raman response with a wavelength shift for at least one AGE to be detected,
- directing radiation from the laser radiation source into an eye from which an AGE adduct is to be measured,
- collecting elastically and inelastically scattered radiation from the eye,
- determining the difference in the intensity and spectral widths of the inelastically scattered radiation from the elastically scattered radiation from said eye, and
- providing a quantifiable Raman spectrum for said AGE(s).

2. The method of claim 1 wherein said eye is removed from the body of a subject.

3. The method of claim 1 or claim 2 wherein the laser radiation source generates radiation with a wavelength in the range 514 to 785 nm.

4. The method of any preceding claim wherein in the step of collecting radiation, radiation scattered from the eye is collected to allow detection of Raman shifts in the range 500 cm^{-1} to 3500 cm^{-1}.

5. The method as claimed in claim 4 wherein radiation scattered from the eye is collected to allow detection of Raman shifts of about

633 cm $^{-1}$, of AGE-specific doublet bands at about 400 cm^{-1} and 540 cm^{-1}, and of triplet of bands at about 880 cm^{-1},980 cm^{-1} and 1090 cm^{-1}.

6. The method of any preceding claim wherein the method further comprises the steps of:
   - providing at a second later time point a laser radiation source which generates radiation at a wavelength that is capable of producing a Raman response with a wavelength shift for an AGE adduct to be detected;
   - directing radiation from the laser radiation source into an eye from which an AGE is to be measured at a later time point;
   - collecting elastically and inelastically scattered radiation from the eye at the later time point,
   - determining the difference in the intensity and spectral widths of the inelastically scattered radiation from the elastically scattered radiation from said eye at said later time point, and
   - providing a quantifiable Raman spectrum for said AGE detected at said later time point.
   - comparing the quantifiable Raman spectrum for said AGE at a first time point with the quantifiable Raman spectrum for said AGE determined at the later time point.

7. The method of claim 6 wherein between the first and second time point, a test agent is applied to the eye into which the radiation from the radiation source is directed.

8. The method of any preceding claim wherein in the step of directing radiation from the laser radiation source into an eye, the radiation is directed onto the cornea, the lens and / or vitreous

ocular tissues of the eye of a subject.

9. The method of any of claims 1 to 7 wherein in the step of directing radiation from the laser radiation source into an eye, the radiation is directed to parts of the posterior segment of the eye including the vitreous and various layers of the neural retina.

10. The method of any one of claims 1 to 7 wherein in the step of directing radiation from the laser radiation source into an eye, the radiation is directed into the eye of a subject to the retinal pigment epithelium.

11. The method of any one of claims 1 to 7 wherein in the step of directing radiation from the laser radiation source into an eye, the radiation is directed into the eye of a subject to the underlying pentalaminar structure known as Burch's membrane.

12. A method to determine retinopathy and / or macular degeneration in a subject comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE in an eye from a test subject with an unknown status in relation to retinopathy and / or macular degeneration; and
- comparing said quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to retinopathy and / or macular degeneration with at least one standard quantifiable Raman spectrum for said AGE for a healthy individual,

wherein the presence of a difference in intensity in areas and / or the peaks between the spectra is indicative of the presence of retinopathy and / or macular degeneration in the subject with previously unknown status.

13. A method to determine retinopathy and / or macular degeneration in a subject comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE in an eye from a test subject with an unknown status in relation to retinopathy and / or macular degeneration; and
- comparing said quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to retinopathy and / or macular degeneration with at least one standard quantifiable Raman spectrum for said AGE for an individual known to suffer retinopathy and / or macular degeneration,

wherein at least one commonality in areas and / or peaks between the spectra is indicative of the presence of retinopathy and /or macular degeneration in the subject.

14. A method of determining a predisposition of a subject to retinopathy and / or macular degeneration comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE from an eye from a test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration; and
- comparing the quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration with the quantifiable Raman spectrum for said AGE considered to be a standard spectrum for a healthy individual

wherein the presence of a difference in intensity in areas and / or the peaks between the spectra is indicative of a predisposition to retinopathy and / or macular degeneration in the subject with previously unknown status.

15. A method of determining a predisposition of a subject to retinopathy and / or macular degeneration comprising the steps:

- providing at least one quantifiable Raman spectrum for an AGE from an eye from a test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration; and
- comparing the quantifiable Raman spectrum for said AGE obtained from the test subject with an unknown status in relation to predisposition to retinopathy and / or macular degeneration with the quantifiable Raman spectrum for said AGE considered to be a standard spectrum spectra for an individual known to suffer a predisposition to retinopathy and / or macular degeneration,

wherein at least one commonality in areas and / or peaks between the spectra is indicative of a predisposition to retinopathy and / or macular degeneration in the subject.

16. A computer storage medium comprising a computer program, which when executed on a processor causes processor to:

i) receive subject data corresponding to a quantifiable Raman spectrum for an eye of a subject;

ii) access standard data corresponding to at least one standard quantifiable Raman spectrum for an AGE from a data storage device;

iii) compare the subject data and the standard data; and

determine from the comparison of Step (iii) whether the presence of an AGE in the eye of the subject is indicated.

17. A system for the *in-situ* non-invasive measurement of AGEs in an eye comprising a Raman spectroscope comprising;

- at least one laser radiation source adapted to generate radiation at a wavelength that produces a Raman response with a wavelength shift for an AGE to be detected;
- laser radiation source directing means capable of directing radiation into an eye;
- radiation collecting means for collecting Raman spectra;
- detection means to measure the difference in intensity, and spectral width(s) of the inelastically scattered spectra (Raman) from the elastically scattered radiation.

18. The system as claimed in claim 17 wherein the radiation generating source is an argon laser.

19. The system as claimed in claim 18 wherein the radiation generating source is a 1.0 mW argon laser capable of providing a spot of 8mm in diameter.

20. The system as claimed in any one of claim 17 to 19 wherein the radiation collecting means are adapted to collect light of wavelengths in the range 514 to 785 nm.

21. A kit for carrying out a method of the invention said kit comprising at least;

- a system according to any one of claims 17 to 20, and

- at least one standard Raman spectrum in which at least one AGE has been measured, wherein the spectrum is considered to be a standard spectrum for an individual known to suffer from retinopathy and / or macular degeneration, and / or a predisposition to retinopathy and / or macular degeneration;
- or
- at least one standard Raman spectrum in which at least one AGE has been measured, wherein the spectrum is considered to be a standard spectral profile for a healthy individual with an AGE adduct Raman spectra profile or degree of AGE-modification of proteins,

for comparison with the spectrum generated from an eye of a test subject; and optionally

-instructions on how the system is to be used and / or the results generated are to be interpreted.

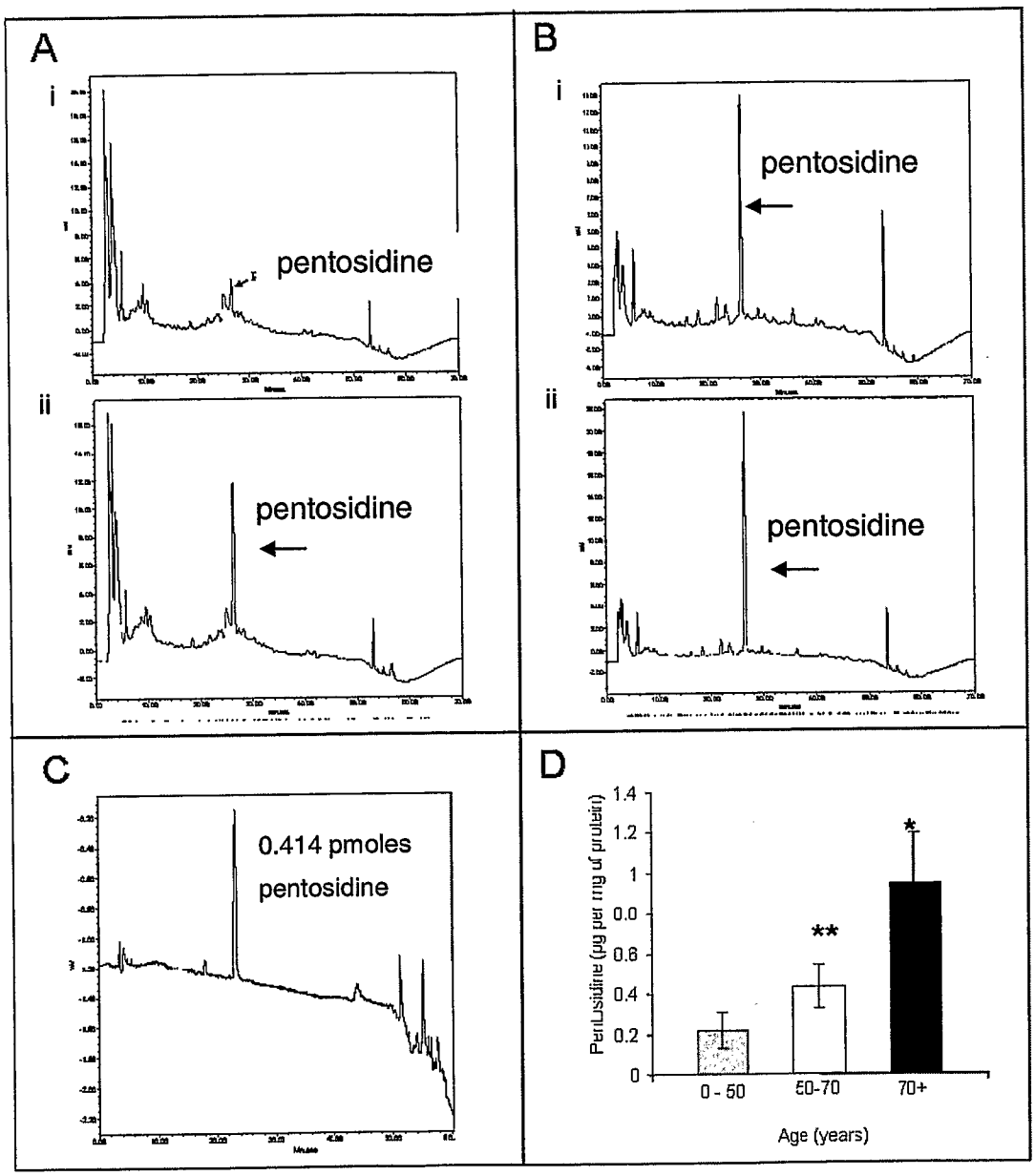

A
i
pentosidine
ii
pentosidine
B
i
pentosidine
ii
pentosidine
C
0.414 pmoles
pentosidine
D
PentLysidine (µg per mg of protein)
1.4
1.2
1
0.0
0.8
0.4
0.2
0
**
*
0 - 50
50-70
70+
Age (years)

Figure 1

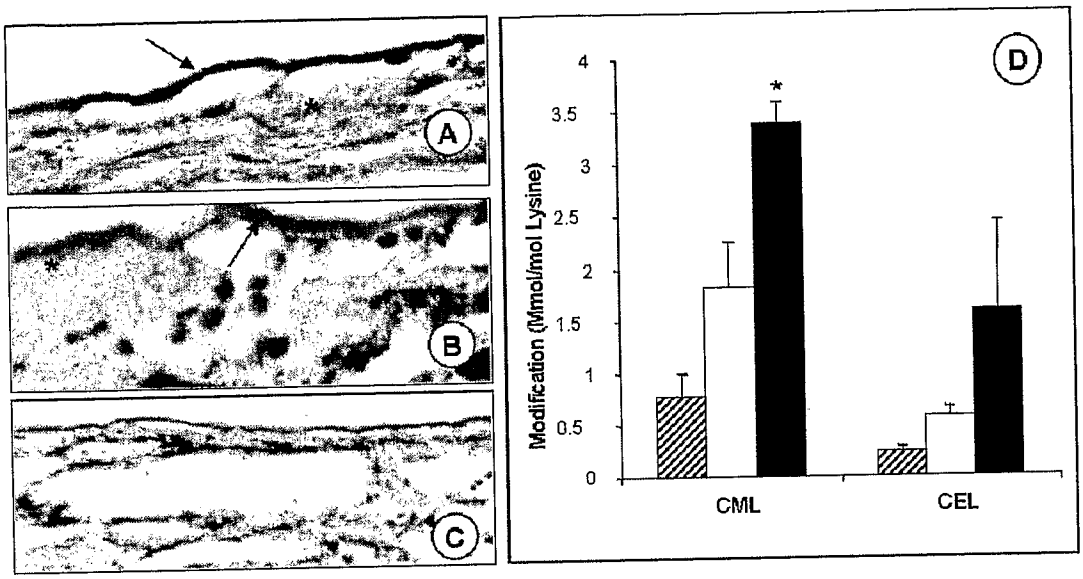


Figure 2

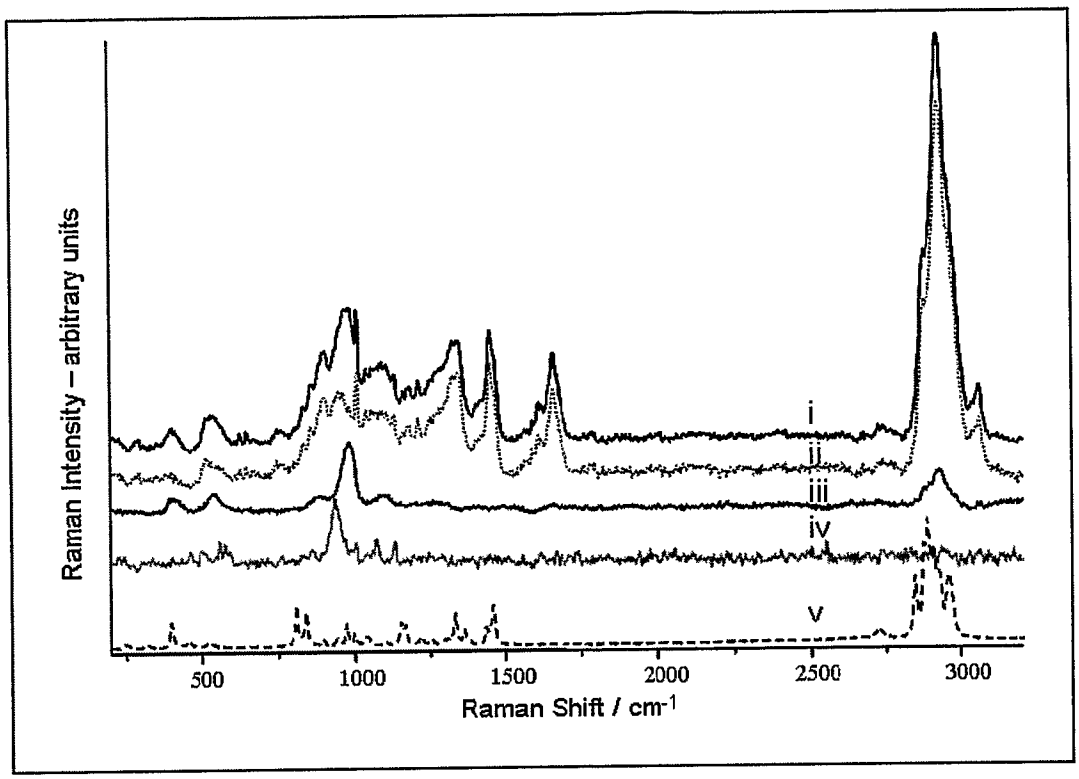

Raman Intensity – arbitrary units
i
ii
iii
iv
v
500
1000
1500
2000
2500
3000
Raman Shift / cm-1

Figure 3

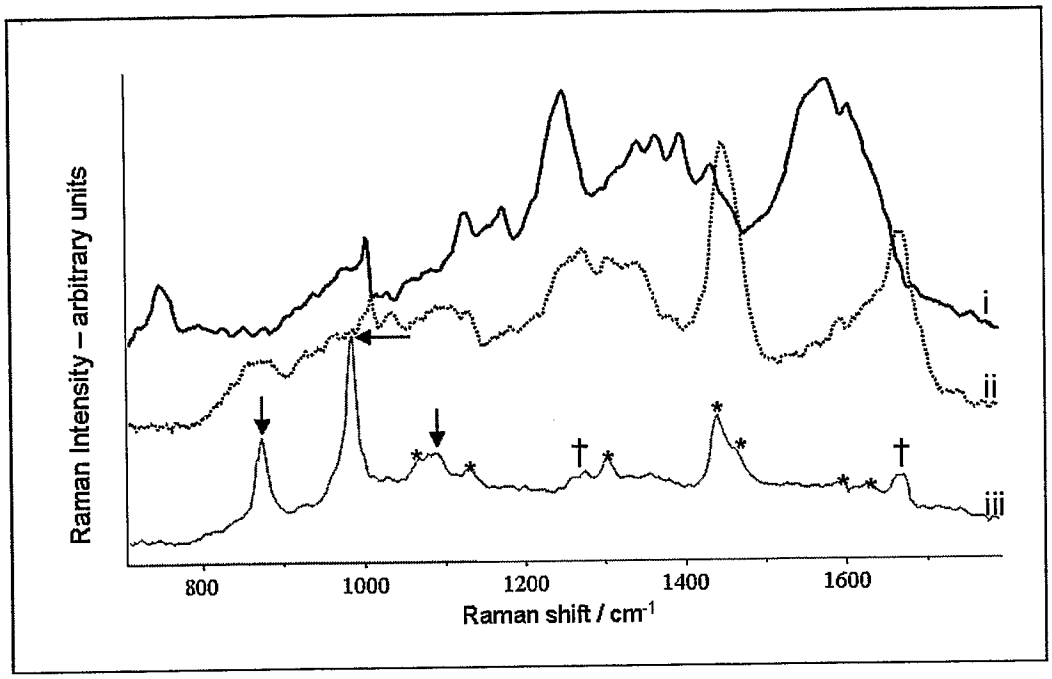

Raman Intensity – arbitrary units
i
ii
iii
800
1000
1200
1400
1600
Raman shift / cm-1

Figure 4

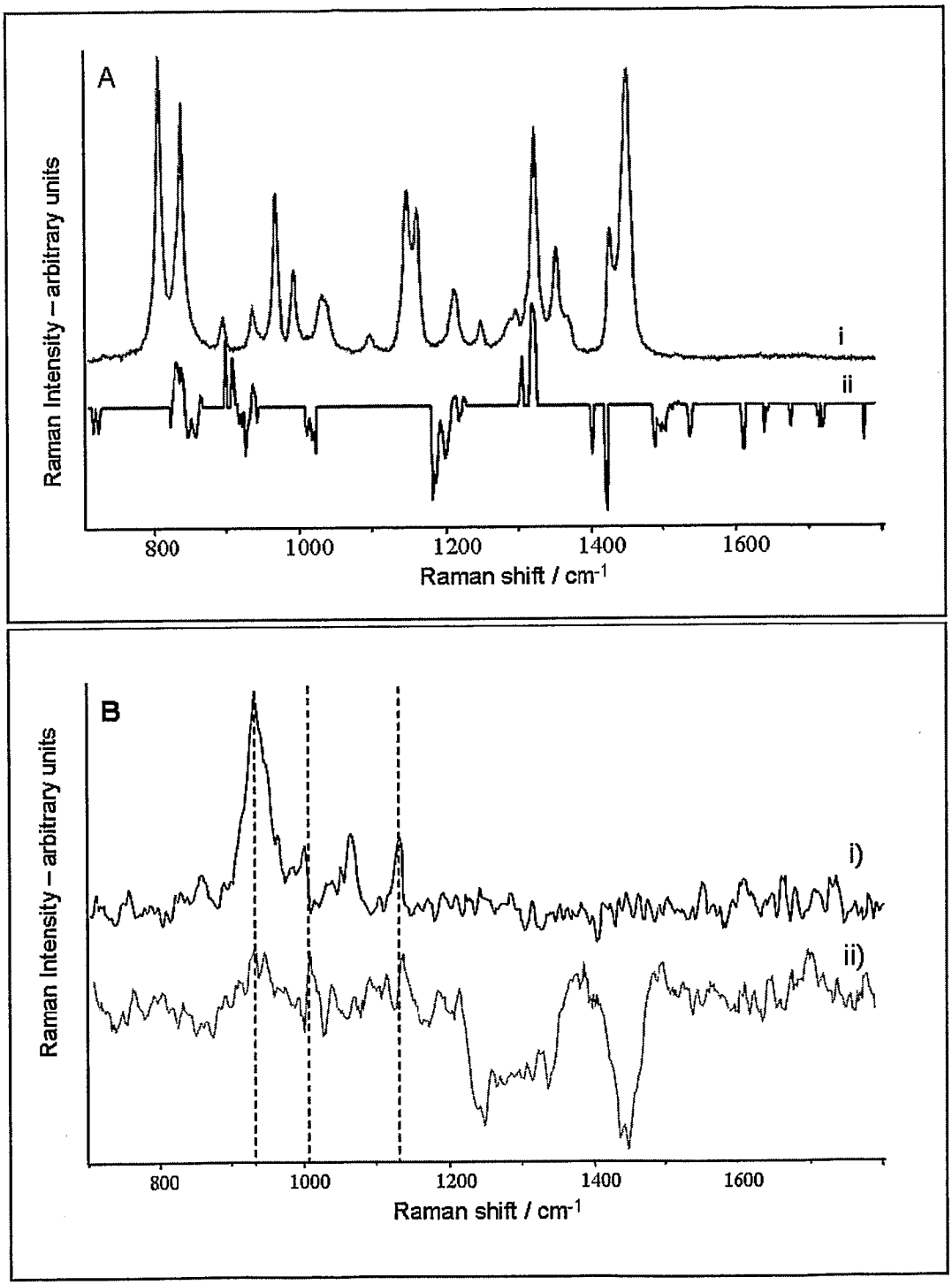

A
Raman Intensity – arbitrary units
i
ii
800
1000
1200
1400
1600
Raman shift / cm-1
B
Raman Intensity – arbitrary units
i)
ii)
800
1000
1200
1400
1600
Raman shift / cm-1

Figure 5

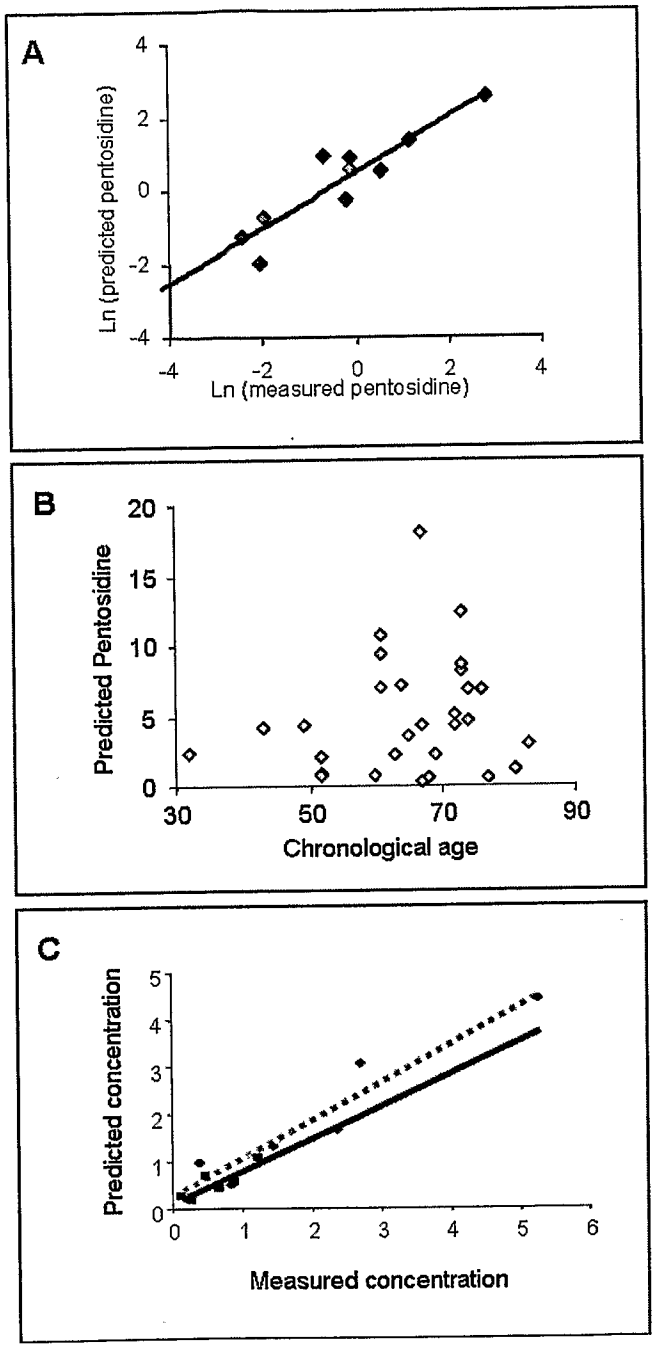

A
Ln (predicted pentosidine)
4
2
0
-2
-4
-4
-2
0
2
4
Ln (measured pentosidine)
B
Predicted Pentosidine
20
15
10
5
0
30
50
70
90
Chronological age
C
Predicted concentration
5
4
3
2
1
0
0
1
2
3
4
5
6
Measured concentration

Figure 6

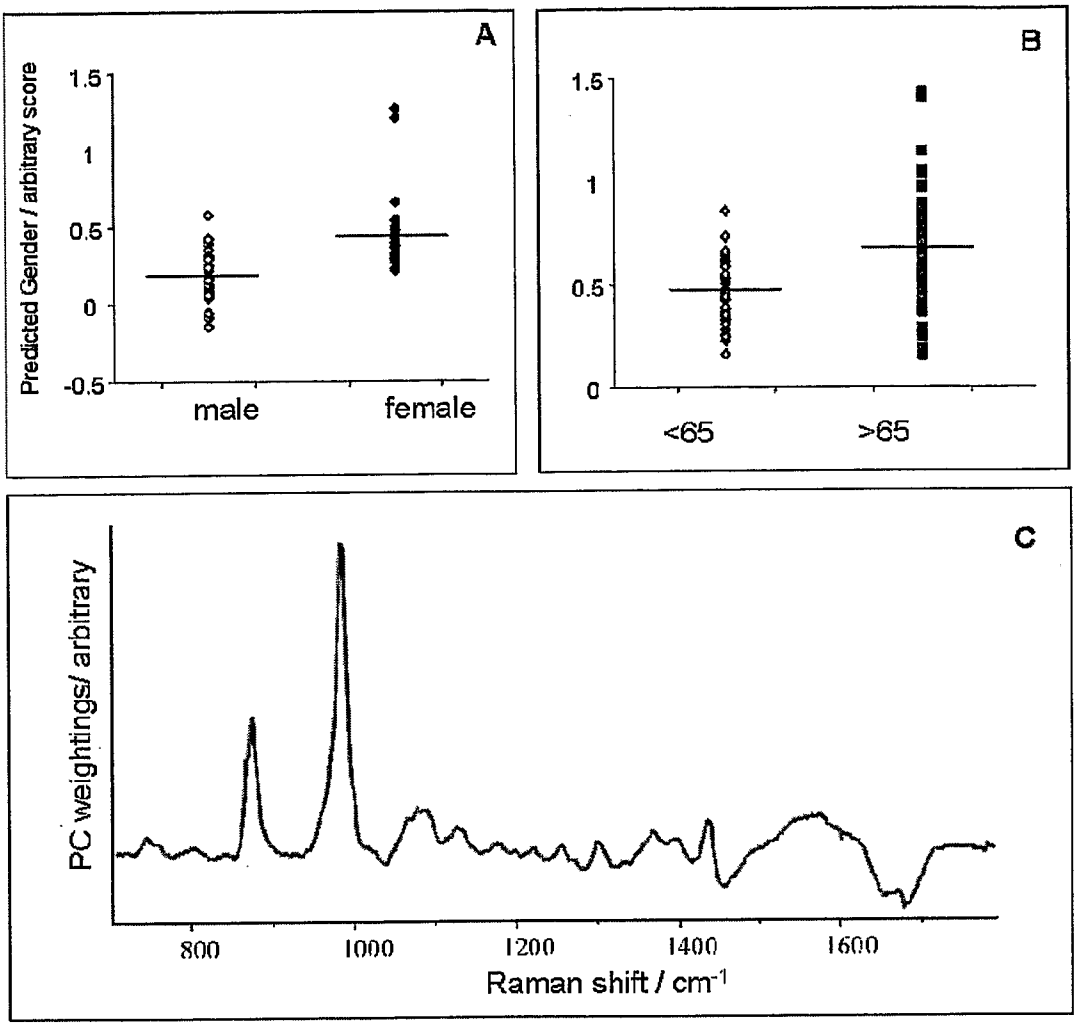

A
Predicted Gender / arbitrary score
1.5
1
0.5
0
-0.5
male
female
B
1.5
1
0.5
0
<65
>65
C
PC weightings/ arbitrary
800
1000
1200
1400
1600
Raman shift / cm-1

Figure 7

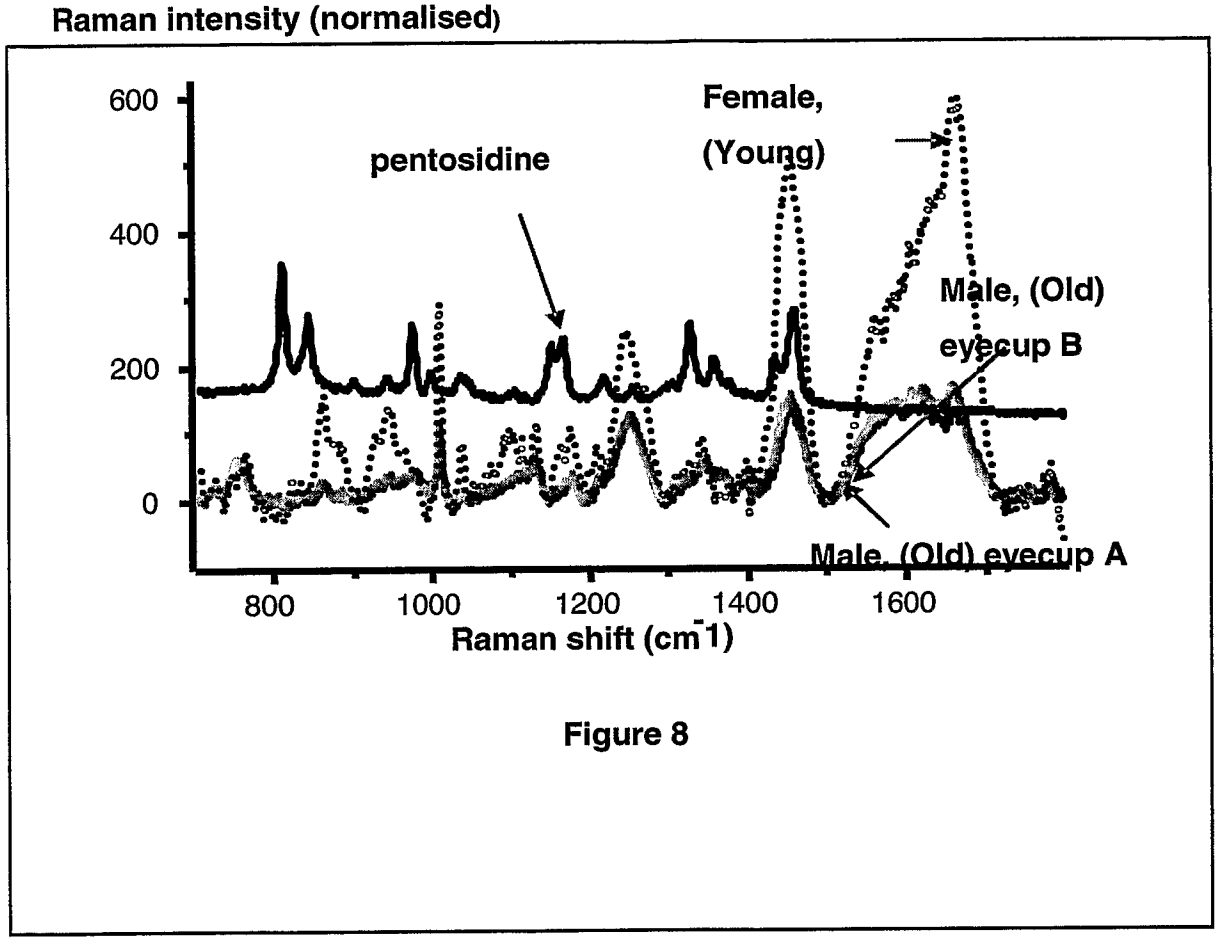

Raman intensity (normalised)
600
400
200
0
pentosidine
Female,
(Young)
Male, (Old)
eyecup B
Male, (Old) eyecup A
800
1000
1200
1400
1600
Raman shift (cm-1)

Figure 8

	CML	CEL	MDA
Young (< 60 years)	0.865	0.296	0.026
Old (>60 years)	2.461 +/- 1.081	1.447 +/- 1.168	0.164 +/- 0.141

Figure 9

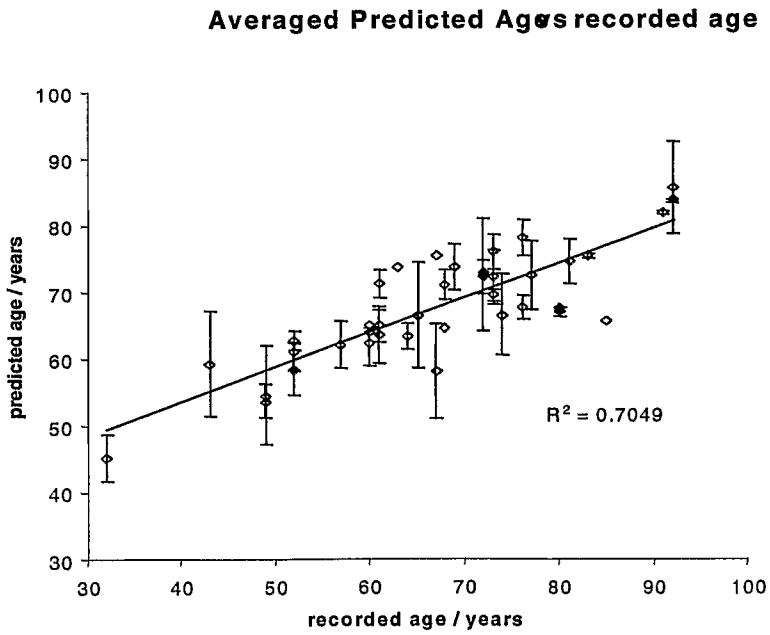


Figure 10

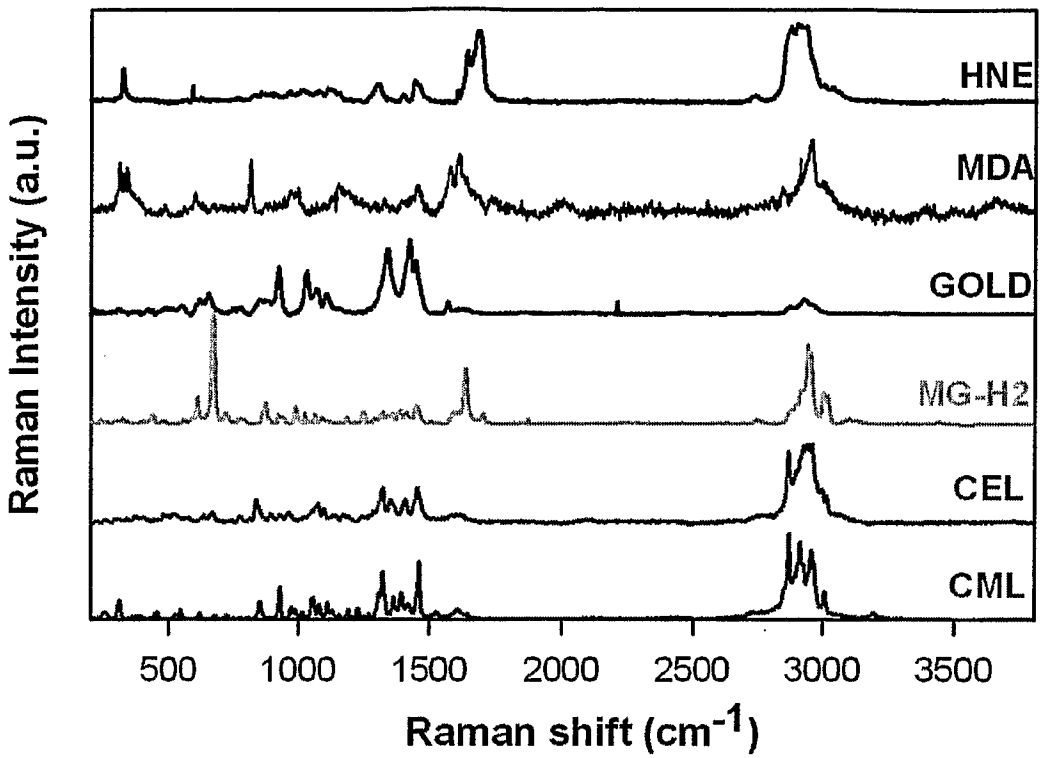

HNE
MDA
GOLD
MG-H2
CEL
CML
Raman Intensity (a.u.)
500
1000
1500
2000
2500
3000
3500
Raman shift (cm-1)

Figure 11

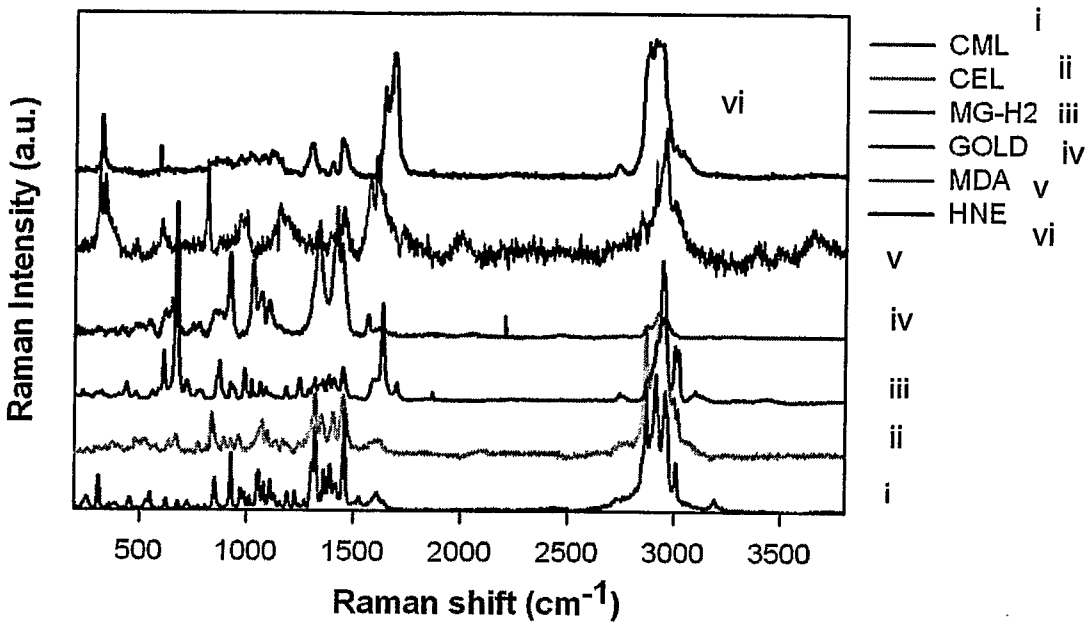

Raman Intensity (a.u.)
Raman shift (cm-1)
500
1000
1500
2000
2500
3000
3500
vi
v
iv
iii
ii
i
CML
CEL
MG-H2
GOLD
MDA
HNE
i
ii
iii
iv
v
vi

Figure 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/003393

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61B3/117

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)
A61B

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 721 583 B1 (DURKIN ANTHONY J [US] ET AL) 13 April 2004 (2004-04-13) column 1, lines 54-57,64-67 column 2, lines 1-5 column 4, lines 24-30,34-36,46-49 column 5, lines 15-30,47-49 column 6, lines 6-10 column 8, lines 23-31 column 9, lines 2-5 column 10, lines 43-46 figure 1	1,3-9, 11,16-21
Y		2,10
Y	US 6 424 850 B1 (LAMBERT JAMES L [US] ET AL) 23 July 2002 (2002-07-23) column 8, lines 65-67	2

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 January 2008	07/02/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Worms, Georg

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/003393

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 873 831 A (BERNSTEIN PAUL S [US] ET AL) 23 February 1999 (1999-02-23) column 7, line 30	10

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/003393

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 12-15
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2007/003393

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6721583 B1	13-04-2004	NONE	
US 6424850 B1	23-07-2002	US 6181957 B1	30-01-2001
US 5873831 A	23-02-1999	AT 220878 T	15-08-2002
		AU 6698798 A	29-09-1998
		CA 2263262 A1	17-09-1998
		DE 69806732 D1	29-08-2002
		DE 69806732 T2	03-04-2003
		EP 0966224 A1	29-12-1999
		JP 2001517978 T	09-10-2001
		WO 9840006 A1	17-09-1998