**Introduction**

Glycation is a non-enzymatic reaction between a protein and a reducing sugar, such as glucose. This reaction generates advanced glycation end products (AGEs) via a chain of intermediate reactions that produce Schiff bases and Amadori products. AGEs are implicated in the pathology of several age-related diseases, and many AGEs and their intermediate products, such as 3-deoxyglucosone (3DG), are thought to have a strong relationship with aging and lifestyle diseases. Several AGEs exhibit a characteristic fluorescence at 370 nm/440 nm, and fluorescence intensity can be measured to examine the formation of AGEs under different treatments.

Fluorescence is also a potential method of measuring cumulative AGE deposition in human skin non-invasively, in clinical studies. Previous studies have measured average fluorescence intensity between 420-600 nm, although Koetsier measured fluorescence at 350-420 nm on the skin of diabetes mellitus patients but did not find any specific fluorescence peak. Except for pentosidine (λ\text{max}: ex. 335 nm, em. 385 nm), no fluorescent AGEs have been detected on human skin.

The identification of specific fluorescence spectra derived from AGEs and emitted from skin is difficult because other fluorescent material, such as NADH, may also be present. A direct comparison of the fluorescence spectra from in vitro samples and in vivo human skin may help distinguish AGEs derived fluorescence. Here we compared the fluorescence spectra of specific proteins, skin glycation models, and human skin.
Subjects and Methods

Preparation and measurement of in vitro models

AGEs were produced by incubating protein and bovine skin samples in glucose reaction solutions for up to six days. Human Serum Albumin (HSA) (Sigma Chemical Co. Ltd, MO, USA) was incubated with and without glucose at 60 °C [7]. The glucose (+) (G (+)) reaction solution contained 0.1 M phosphate buffer (PBS) (pH 7.4), 40 mg/ml HSA, 2.0 M glucose solution, and distilled water at a 5:2:1:2 volume ratio. Earlier research has demonstrated this solution produces the pentosidine. The glucose (-) reaction (G (-)) solution contained 0.1 M PBS, 40 mg/ml HSA, and distilled water at a 5:2:3 volume ratio. Reaction solutions were incubated at 60 °C for 0, 3, 6 days.

Quinine sulfate solution was used as a positive controls for fluorescent material at 0, 5, 50, 500 µg/mL. The concentration of pentosidine produced by the HSA-Glc (+) model was measured by ELISA using the commercial kit ‘FSK Pentosidine’ (Fushimi Medicine Manufacture Place, Marugame, Kagawa, Japan) [8].

AGE positive or negative bovine skin, which contains collagen, were produced by incubating skin samples in a glucose positive (G (+)) solution (2 mol/L glucose and 0.1 M PBS) or a glucose negative (G (-)) solution, at 60 °C for 0, 2, 4 days [9]. Samples of bovine skin with known fluorescence were produced by adding 100 µL quinine sulfate to 50 mm² samples of half-dried, untreated bovine skins, and the fluorescence spectrum measured after 30 minutes.

Fluorescence intensity of the all samples was measured at different wavelengths with an LS50 fluorometer (Perkin-Elmer Inc., USA). The samples were decanted into 1-cm cells and fluorescence measured at 10 nm slit-width and 200 nm/min.

Clinical study

The skin fluorescence of 11 healthy men and women (five men and six women mean age: 30.1 ± 11.6 years old) was measured with an AGE Reader™ (DiagnOptics, Groningen, Netherlands) [15-17]. Excitation light (wavelength 300-420 nm) was projected onto 1 cm² of skin inside the upper arm, approximately 10 cm above the elbow, and the intensity of emitted light (420-600 nm, which is auto fluorescence (AF)) measured with a fluorometer. The skin AF (arbitrary units [AUs]) was 0.161 µg/mL (measured by ELISA).

Fluorescence spectrum of solution samples

At an excitation frequency of 370 nm, quinine sulfate exhibited a peak fluorescence of 440 nm (Fig. 1(a)). The HSA-Glc (+) model exhibited a peak intensity at 430 nm, and the HSA-Glc (-) model exhibited low fluorescence intensity at 430 nm (Fig. 1(b)). At an excitation frequency of 370 nm, pentosidine exhibited a peak 444 nm (Fig. 2). When a sample of diluted pentosidine was measured to test the detection sensitivity of pentosidine, the detection sensitivity at 370/440 nm was about 1/42.6 times more than at 335/385 nm. The concentration of pentosidine in the HSA-Glc (+) reaction liquid was 0.161 µg/mL (measured by ELISA).

Fluorescence spectrum of bovine skin

After two or four days, samples of bovine skin incubated with glucose turned brown (Fig. 4(a)) and exhibited fluorescence at 451 nm; the fluorescence intensity increased with incubation period (Fig. 4(b)).

In the fluorescent-added (quinine sulfate solution, HSA-Glc (+), pentosidine) bovine skin samples, the fluorescence intensity of between 420-600 nm increased with the concentration of fluorescence agent and incubation period (Fig. 5).
Fig. 1. Fluorescence spectrum of quinine sulfate and HSA-Glc
Excitation wavelength was at 370 nm.
(a) Quinine sulfate (positive control for fluorescence).
(b) Human serum albumin incubated with glucose (G (+)) and no glucose (G (-)).

Fig. 2. Fluorescence spectrum of pentosidine
The maximum wavelength of 335/385 nm was observed the peak in the fluorescence wavelength of 444 nm at an excitation wavelength of 370 nm.
Identification of Advanced Glycation Endproducts derived fluorescence spectrum

![Graph showing normal and adjusted fluorescence spectrum](image)

**Fig. 3. Exploratory experiment of preproduction of fluorometer**
Subject was 28 year-old Japanese man
(a) Human skin fluorescence spectrum after irradiation by a normal LED for 25 msec and 5,000 msec
(b) Human skin fluorescence spectrum after irradiation for 5,000 msec by an LED fitted with a visible light cut-off filter.

**Measurement of CML content in skin stratum corneum**
After two or four days, samples of bovine skin incubated with glucose turned brown (**Fig. 4(a)**) and exhibited fluorescence at 451 nm; the fluorescence intensity increased with incubation period (**Fig. 4(b)**).

In the fluorescent-added (quinine sulfate solution, HSA-Glc (+), pentosidine) bovine skin samples, the fluorescence intensity of between 420-600 nm increased with the concentration of fluorescence agent and incubation period (**Fig. 5**).

**Clinical results for skin fluorescence**
The fluorescence exhibited by skin, after excitation irradiation 5,000 msec, and salient difference of fluorescence intensity among 11 subjects were exhibited at 451 nm and 485 nm (**Fig. 6(a)**). The correlation coefficient of age and skin AF was positive ($p < 0.05$) (age 500-700 nm) and skin AF 420-650 nm (**Fig. 6(b)**). In addition, the fluorescence intensity at 440 nm might be positively correlated with age ($r = 0.582, p = 0.060$) and with skin AF ($r = 0.832, p = 0.001$).
Fig. 4. Relation between fluorescence intensity and incubation period with glucose (N = 3)
(a) Appearance of bovine skin samples incubated with glucose in each time
(b) Fluorescence spectrum of bovine skin samples after irradiation for 3000msec. Δ Intensity means the difference between sample incubated with glucose (G (+)) and no glucose (G (-)).
Identification of Advanced Glycation Endproducts derived fluorescence spectrum

Fig. 5. Results of fluorescent sample added tests (N = 3)
(a) Quinine sulfate, (b) HSA-Glc (+), (c) pentosidine were added to bovine skin samples and incubated in glucose free solution. ΔIntensity means the difference in intensity between fluorescent-added and negative samples, irradiated at 1,000msec.
**Discussion**

**Fluorescence Spectrum of solution samples**

It has been previously reported that several AGEs physicochemical properties cause characteristic fluorescence at 370/440 nm \(^4\). Here we found peak fluorescence at 440 nm (430 nm) in glycated HSA sample (Fig. 1(b)). HSA-Glc (−) exhibited less fluorescence at 430 nm, which may have been generated by the original HSA reagent. The detection sensitivity at 370/440 nm was 1/42.6 times compared to 335/385 nm pentosidine (Fig. 2). No previous research has detected pentosidine at 370/440 nm.

Paul reported that relative fluorescence intensity at 370/440 nm was positively correlated with pentosidine in human skins taken by skin biopsy \(^19\). Our results suggest direct measurement of pentosidine at 370/440 nm may be possible. Although pentosidine is less common in plasma, the concentration of it increases with aging \(^2,20\). Since pentosidine binds with collagens in the dermis, a non-invasive fluorescent measurement method may provide information on the relationship between aging and disease. These results indicate an excitation wavelength fluorescence at 370/440 nm is suitable to measure AGEs derived fluorescence intensity.

Hori reported that the protein derived from various living tissues, such as collagen, elastin, and keratin, exhibits a similar increase in fluorescence intensity to glucose incubated albumin, that depends on incubation period \(^7\). The fluorescence at 370/440 nm may help measure glycation and the degree of aging in various living human tissues. We suggest the observed intensity at 370/440 nm represents ‘AGEs fluorescence’.

**Fluorescence spectrum of bovine skin sample**

Data from the bovine skin samples showed the fluorescence intensity at 451 nm was related to degree of skin glycation (Fig. 4(b)). Bachmann reported that the fluorescent substances detected from a living tissues at 360 nm of excitation light is only the fluorescence originating in the collagen detected at 440-460 nm at present \(^21\). The observed fluorescence intensity at 451 nm in the bovine skin sample is likely to be produced by reaction products from glycated collagen.

In the fluorescent-added samples, pentosidine derived fluorescence detected near 444 nm was also detected at 451 nm in the bovine skin sample (Fig. 5(c)). The result is significant because pentosidine is the only fluorescence AGEs reported from skin and is typical of AGEs linked with aging and some disease. Moreover, fluorometer used is unlikely to detect other lights, and can therefore be used to monitor AGEs fluorescence. In addition, the fluorescence spectrum of HSA-Glc (+) peak on the short wavelength side (near 430 nm) compared with the other fluorescence-added samples, implying fluorescence intensity at the short wavelength side had become a high value of intensity comparatively (Fig. 5(b)).

**Fluorescence spectrum of human skin**

In healthy people, differences in fluorescence are difficult to detect at this detection sensitivity, although an AGE Reader can distinguish the fluorescence spectrum of healthy people \(^23\), diabetes mellitus patients, and renal disease patients. Since the precise spectrum of fluorescent AGEs is not detectable in the human skins, a target cannot be extracted by the methods, so the mean intensity of the broad fluorescence region (420-600 nm) has been used to evaluate the degree of glycation in the body. This hinders the development of this field. Koetsier reported that a specific peak of intensity wasn’t discovered for diabetes mellitus patients in ex. 350-420 em. 420-600 nm \(^12\). Although the light intensity of a xenon lamp light source is stable and does not change with time, in a living body excitation light is easily dispersed and sensitivity falls. If a narrow-band of excitation light is used to avoid the dispersion, then the amount of excitation light will decline. The technical limit of the above measuring instruments is also mentioned as a subject.

The preproduction fluorometer was equipped with an
LED light source, and the half bandwidth of the emission spectrum is narrow because, and the fluorescence intensity higher than that provided by an AGE Reader. Moreover, the excitation light LED can be controlled by equipping with a visible light cut-off filter so that a pure fluorescence spectrum can be obtained. As a result, the spectra become detectable at peaks of 451 and 485 nm, which AGE Reader cannot detect in human skins (Fig. 3(b)). Thus, the preproduction fluorometer enabled a comparison between fluorescence AGEs produced in models and living tissue. However, one new problem is that the intensity of excitation light may exceed the gain value of a spectroscope used to measure fluorescence, and should be tested in future. Also, the intensity of excitation wavelength may need to be adjusted to adjust for differences in skin color 24,25).

Among healthy people, we observed two peaks at 451 nm and 485 nm in human skins were found (Fig. 6(a)). Since correlation with skin AF is so strong and the fluorescence region is similar to spectrum obtained from HSA-Glc (+), we suggest the system is detecting fluorescence from skin AGEs. In future, most effective wavelength to monitor a degree of glycation in the body may be found by considering the relation between fluorescence spectrum and biological information.

**Conclusion**

The in vitro experimental result demonstrated that fluorescence intensity detected at 370/440 nm was derived from AGEs. The fluorescence spectra of glycated HSA and human skin are similar, and thus measurement of AGEs fluorescence has potential to monitor the degree of glycation in the living tissue.

**Conflicts of Interest**

The authors have no conflicts of interest in this study.

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**References**

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