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Noninvasive biosensor for cathepsin L in the stratum corneum

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Purpose: The objective is to propose an on-site testing biosensor of cathepsin L (CatL) activity in the stratum corneum, which can be used for the evaluation of skin conditions noninvasively and easily.
Methods: The biosensor comprises of a disposable test strip and a desktop-sized reader (260 × 150 × 290 mm^3, 1.9 kg), incorporating a charge-coupled device image sensor (CCD) unit to measure the reflectance of the test strip. A novel immuno-chromatographic test strip was proposed for CatL analysis in the stratum corneum. In order to realize the test strip, a colloidal gold technique was selected as the molecular recognition method for the CatL. A human skin sample was collected noninvasively by adhesive tape stripping.
Results: Based on optimal assay conditions, the sensitivity of the biosensor was evaluated. It required 10 min from a sample dropping to appear the test line on the test strip. The optical density was proportion to the CatL. Bioanalytical validation indicated that, within the biosensor's detection limit (172.2 μU/mL), its accuracy (R^2 = 0.94), and precision (CV = 15%) approach more elaborate laboratory-based analyzers. In addition, the truncated sampling-reporting cycle (<15 min) allows speedy reporting of CatL levels.
Conclusion: It was indicated that this noninvasive and easy-to use biosensor might be a novel tool for the semi-quantitative analysis of CatL in the stratum corneum.

Key words: cathepsin L – stratum corneum – colloidal gold technique – immuno-chromatographic – tape stripping – biosensor

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THE NEED to transform the delivery of health care from a centralized, hospital-based system to a more distributed, patient-centric model has partly been propelled by the need for early diagnosis of skin diseases (1). The conceptual appeal of using a skin biomarker as a diagnostic and prognostic matrix to augment clinical examination derives from the relative ease of procuring stratum corneum samples. A noninvasive determination of skin surface proteolytic activity may be useful for the diagnosis of human disease and properties of skin (2).

The cathepsin family is one of metabolizing enzymes of skin cells and it includes aspartic protease cathepsin D (EC 3.4.23.5) and cysteine proteases cathepsin B (EC 3.4.22.1), H (EC 3.4.22.16) and L (EC 3.4.22.15) (3,4). Cathepsin L(CatL)is a lysosomal cysteine protease with a major role in intercellular protein catabolism (5). CatL also shows the most potent collagenolytic and elastinolytic activity *in vitro* of any of the cathepsins (6). CatL has been identified as a stratum corneum desquamation process-related enzyme (7). It is indicated that expression of CatL is involved in early cutaneous malignant melanoma, a major skin cancer (8). Although the number of the patients with skin cancer has increased every year, noninvasive diagnosis methods for skin cancer have not been established yet.

A substrate, benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide (Z-Phe-Arg-AMC; $C_{33}H_{36}N_6O_6$), has been served for the analysis of CatL activity in ultramicroassay (9–10). However, this method has some problems in that (i) Z-Phe-Arg-AMC acts as a substrate for both CatL and B, and (ii) it cannot be analyzed rapidly and easily.

We have been focusing on the development of noninvasive methods for measuring the CatL activity by analyzing painlessly collected stratum corneum using tape stripping (11–12).

The purpose of this research was to propose an on-site testing biosensor of CatL in the stratum corneum, which can be used noninvasively and easily. A novel immuno-chromatographic test strip was fabricated that can analyze CatL semi-quantitatively. To realize the test strip, a colloidal gold technique was selected as the molecular recognition method for the CatL. The colloidal gold technique makes a low-cost qualitative test possible by exhibiting the color reactions of enzyme-linked immunosorbent assay (ELISA) on a visible line on the test strip. The preparation procedures of the test strip are explained, and the structure and principles of the portable optical device for use in the determination of the color density of the test strip are elucidated. Finally, as a feasibility study of the biosensor, CatL levels were analyzed as a comparison of chronic photostress using younger and elder adults.

Methods

Chemicals and materials

Colloidal gold solution (30 nm particle diameter) was purchased from British Biocell International Co., Ltd. (EMGC30, Cardiff, UK). An anti-CatL rabbit polyclonal antibody (SA-362, Enzo Life Sciences International, Inc., Farmingdale, NY, USA) was used as the anti-CatL antibody. Anti-rabbit IgG antibody was purchased from R&D systems, Inc. (AF008, Minneapolis, MN). CatL was purchased from Merck KGaA Corporate Communications (219402, human liver; Darmstadt, Germany). Bovine serum albumin (BSA), D(+)-trehalose dehydrate, casein sodium and 2-amino-2-hydroxymethyl-1,3-propanediol (tris) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nitrocellulose membrane, sample pad, conjugate pad and absorption pad were purchased from Millipore Co. (Billerica, MA, USA). A disk (D-Squame D100, diameter of 22 mm; Cuderm Corporation, Dallas, TX, USA) was used for the collecting of stratum corneum (13–14). The protein assay kit was purchased from Bio-Rad Laboratories, Inc. (500-0120, Hercules, CA, USA).

Wavelength characteristics of colloidal gold

The wavelength characteristics of colloidal gold were measured using a spectrophotometer (V-570, Jasco Co., Tokyo, Japan). Colloidal gold solution was prepared at 0.5×10^{11}, 1.0×10^{11} and 2.0×10^{11} particles/mL using 10 mM tris-HCl buffer solution (pH 9.2). The transmittance, t, can be calculated using the following equation:

$$t = T/T_0 \quad (1)$$

where, T: the transmitted light at each concentration,

T_0: the transmitted light without sample solution (OD = $-\log t$).

Preparation and optimization of colloidal gold-antibody conjugate

A colloidal gold-antibody conjugate was synthesized according to the following protocol. First, 100 μL of anti-CatL antibody was added to 1 mL colloidal gold solution. After incubation at 4°C for 20 min, the mixture was added to 123 μL tris-HCl buffer solution (pH 9.2). After incubation at 4°C for 20 min, the mixture was centrifuged (14,000 g) at 10°C for 15 min. The sediment was resuspended in 400 μL tris-HCl buffer solution (pH 9.2) containing 5% (w/v) D(+)-trehalose dehydrate.

Preparation of immuno-chromatographic test strip

A fabricated immuno-chromatographic test strip for CatL analysis (test strip) consisted of a sample pad ($5 \times 10 \times 0.83$ mm^3), a conjugate pad ($5 \times 5 \times 0.41$ mm^3), a nitrocellulose membrane ($50 \times 5 \times 0.24$ mm^3) and an absorption pad ($5 \times 5 \times 0.83$ mm^3). To make the conjugate pad, a filter paper was dipped in the colloidal gold-antibody conjugate (secondary antibody) solution and then dried in an oven. The anti-CatL antibody and 2 μL of 0.5 mg/mL anti-rabbit IgG antibody were immobilized on the nitrocellulose membrane to make a test line (primary antibody) and a control line. Finally, the nitrocellulose membrane was blocked against nonspecific protein adsorption by immersion in 10 mM phosphate buffer solution (pH 7) containing 1% (w/v) casein sodium, and incubated for 30 min at room temperature (Fig. 1a).

To optimize the concentration of the colloidal gold-antibody conjugate, the reflectance of the conjugate pad with the colloidal gold-antibody conjugate (0.5×10^{11}, 1.0×10^{11}, and 2.0×10^{11} particles/mL) was measured using 0.5 mU/mL of CatL.

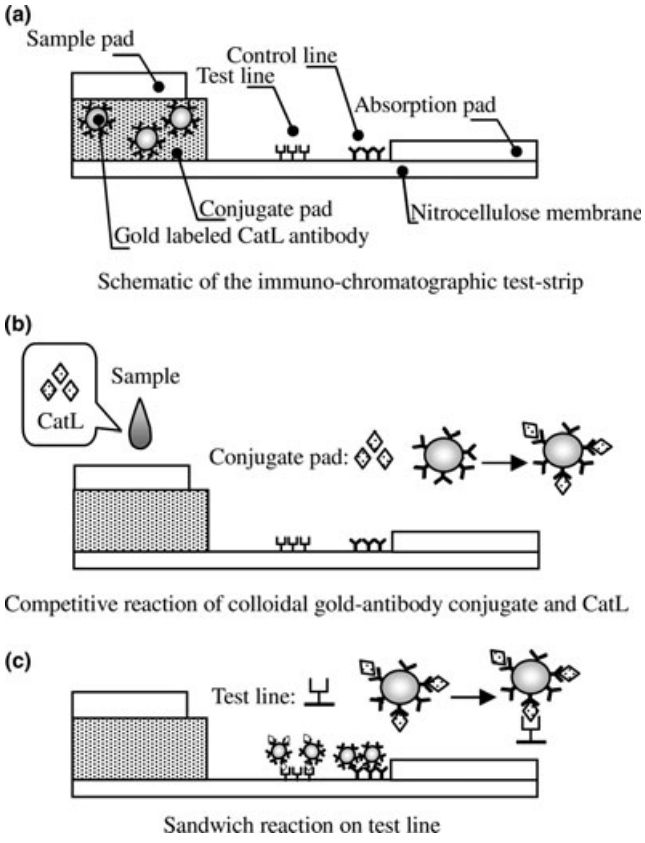

Fig. 1. Principle of colloidal gold-based immuno-chromatographic test strip. (a) Schematic of the immuno-chromatographic test strip. (b) Competitive reaction of colloidal gold-antibody conjugate and CatL. (c) Sandwich reaction on test line.

Collection of stratum corneum sample

The stratum corneum was collected by tape stripping method using the disk. First, the disk is affixed on the skin surface for 30 s, after that it is pulled off the skin. Immediately after, the next new disk is affixed on the same place. This operation is repeated four times to collect four disks from the same area because a few volumes of CatL can be collected at once. Two disks are inserted into 750 μL acetate buffer (4 mM, pH 5) containing 0.1% triton X-100. An ultrasonic wave is applied to the samples using an ultrasonic cleaner (2210DTH, Branson Ultrasonic Corporation, CT, USA) for 30 min at 37° C. The dissolved solutions are pretreated using a centrifugal filtration unit (Vivaspin 500; Sartorius Stedium Biotech S.A., Aubagne Cedex, France) to remove any contaminant proteins. The samples are centrifuged to 10-times concentration using a centrifugal evaporator (CE1, HitachiKoki Co., Ltd, Tokyo, Japan) for 4 h at 138 g, 37°C. Finally, to remove triton X-100 from the sample solution, it is centrifuged using an ultrafiltration filter (cutoff molecular weight: 100 kDa, Ultra free-0.5; Millipore Co.) for 40 min at 10,000 rpm, 15°C. Immediately after this procdure, 75 μL acetate buffer (4 mM, pH 5) without triton X-100 is added. The two samples are combined in a microtube to get 150 μL of stratum corneum sample solution (total sample volume).

Principle of test strip

To analyze the CatL using the test strip, a 100 μL sample solution is dropped on the sample pad. The impurities with large molecular weights are filtered by the sample pad. Immediately after, the sample solution dissolves the colloidal gold-antibody conjugate in it from the conjugate pad. The sample solution moves in a lateral direction on the membrane by capillary action and reaches a test line (Fig. 1b). The target molecule (CatL, antigen) in the sample solution is immobilized with the colloidal gold-antibody conjugate by the antigen–antibody complex reaction. If the colloidal gold agglutinates over a fixed density, the test line turns red in color (Fig. 1c). The reflectance is inversely proportion to the CatL.

The qualitative result can be judged visibly by the appearance of two reddish purple lines in the control and test lines (Fig. 2). Both control and test lines will appear when catepsin L is included in the sample. A negative result is evaluated by the appearance of only a single line, that is, the control line. Only one line in the test region or no line present on the test strip was considered to be an invalid test.

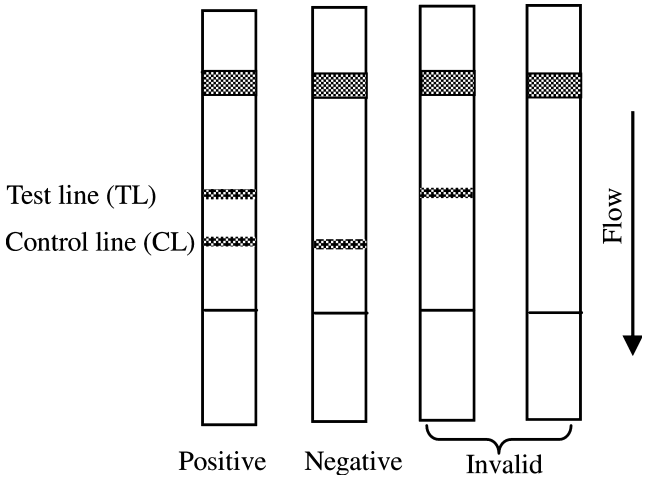

Fig. 2. Qualitative judgement of cathepsin L using two lines.

Fabrication of reader

A desktop-sized reader (25.5 × 26.5 × 14.5 cm^3, 6 kg) was fabricated to measure the reflectance of the test strip (Fig. 3a). The fabricated reader consisted of two light-emitting diodes (OSTCXBTHC1S; OptoSupply Co., Ltd, Hong Kong, China) and a charge coupled device image sensor unit (CCD unit: ARTCAM-098II-OP, Artray Co., Ltd, Japan) to measure the reflectance (Fig. 3b), a holder of the test strip with a peltier device (FPH1-7102NC, Fujitaka Co., Ltd, Kyoto, Japan) and a personal computer (Latitude D531, Dell Inc., TX, USA) to display and record the data. When a test strip was put on the holder, the CCD illuminated vertically the test strip (Fig. 3c).

The reflectance of the test line is measured by the CCD unit. The temperature of the test strip was controlled at 20°C by the peltier device. Prior to the measurement, the standard reflectance of the test strip was measured when a 0 mU/mL buffer solution was dropped on it to cancel out the influence of irregular reflection of the water surface. The amount of light received from the white plate was noted as S_w (white color level) and the amount of light received from the test strip when 0 mU/mL buffer solution was dropped on it was note as S_0. The standard reflectance, R_0, was calculated using the following equation:

$$R_0 = \frac{S_o}{S_w} = 0.817 \quad (2)$$

When the sample solution was dropped on the test strip, the amount of light received from the test strip after the reaction was noted as S_t. The reflectance, R_t, was calculated using the following equation:

$$R_t = \frac{S_t}{S_w} \quad (3)$$

Finally, the relative reflectance, r, can be calculated using the following equation:

$$r = \frac{R_t}{R_0} \quad (4)$$

The optical density, OD, is obtained from this relative reflectance by the equation:

$$OD = -\log r \quad (5)$$

Sensitivity of biosensor

The sensitivity of the biosensor, which is the system consisting of the test strip and the reader, was evaluated using CatL standard solutions between 95 and 950 μU/mL prepared by 4 mM acetic acid buffer solution (pH 5).

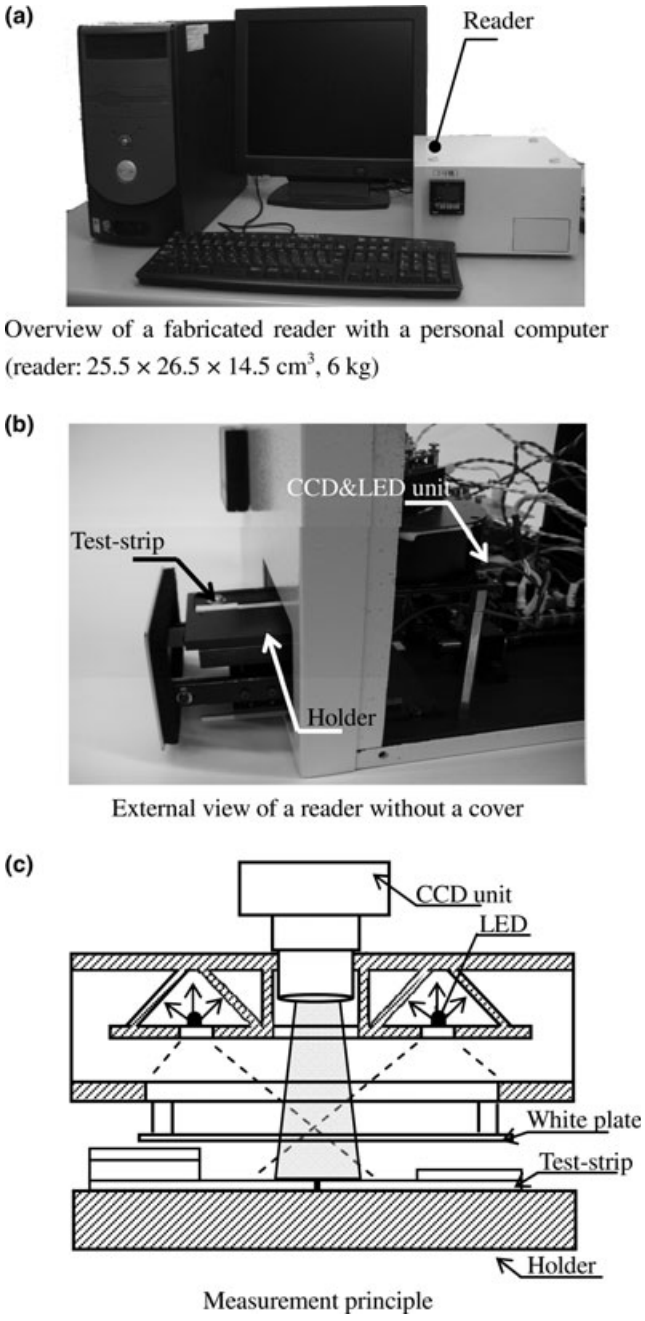

Fig. 3. External view and the measurement principle of a desktop-size reader used for the measurement of optical density. (a) Overview of a fabricated reader with a personal computer (reader: 25.5 × 26.5 × 14.5 cm^3, 6 kg). (b) External view of a reader without a cover. (c) Measurement principle.

Detection of human CatL

As a feasibility study of the biosensor, CatL levels were analyzed as a comparison of chronic

photo-stress using both 10 healthy male young adults in their early 20s (20–22 years, 21.0 ± 1.1 years) and 10 male elder adults (61–69 years, 64.8 ± 3.1 years). The stratum corneum samples were collected from the upper cheek as a sun-exposed area. Prior to the experiment, medical doctors diagnosed that the subjects had no skin diseases. The study protocol was approved by the Ethical Committee of the Institutional Review Board of both Iwate University and the University of Toyama. Signed informed consent was obtained from each subject who enrolled in the study.

Statistical analysis

Within-group comparisons were performed using the Wilcoxon signed-ranks test. *P*-values $P < 0.01$ were taken to represent statistical significance. Unless otherwise stated, all data are expressed as the mean ± standard deviation (SD).

Results and Discussion

Wavelength characteristics of colloidal gold

The wavelength characteristics of the colloidal gold solution fluctuated wildly between 500 and 600 nm at all of the concentrations (Fig. 4). It was revealed that the optimal wavelength for detecting the colloidal conjugate is 524 nm. The transmittance values of each concentration were 0.46 (0.5×10^{11} particles/mL), 0.22 (1.0×10^{11} particles/mL), and 0.06 (2.0×10^{11} particles/mL). The wavelength used for the measurement of the reflectance by the reader was set at 520 nm.

Optimization of colloidal gold-antibody conjugate

The reflectances of the test line were 0.85 ± 0.03, 0.65 ± 0.03, and 0.64 ± 0.04 (mean ± SD) when the concentrations of colloidal gold on the conjugate pad were 0.5×10^{11}, 1.0×10^{11}, and 2.0×10^{11} particles/mL, respectively (Fig. 5). The reflectance showed saturation at 1.0×10^{11} particles/mL. Thus, it was revealed that 1.0×10^{11} particles/mL of colloidal gold is enough to analyze CatL in the immuno-chromatographic test strip.

Sensitivity of biosensor

Based on optimal assay conditions, CatL standard solutions were analyzed using both the

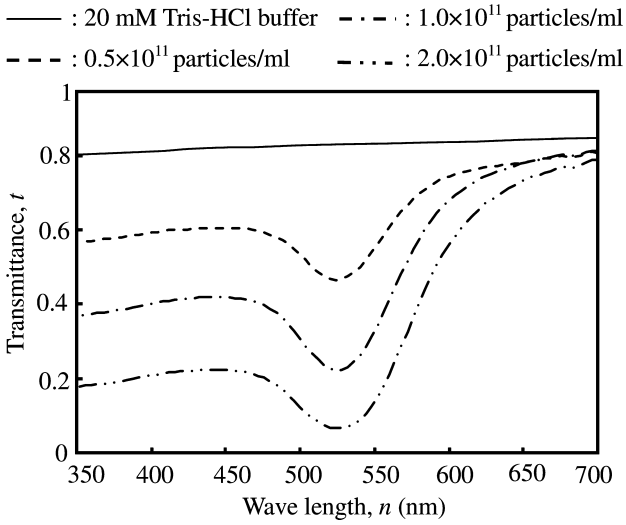

Fig. 4. Wavelength characteristics of colloidal gold solution.

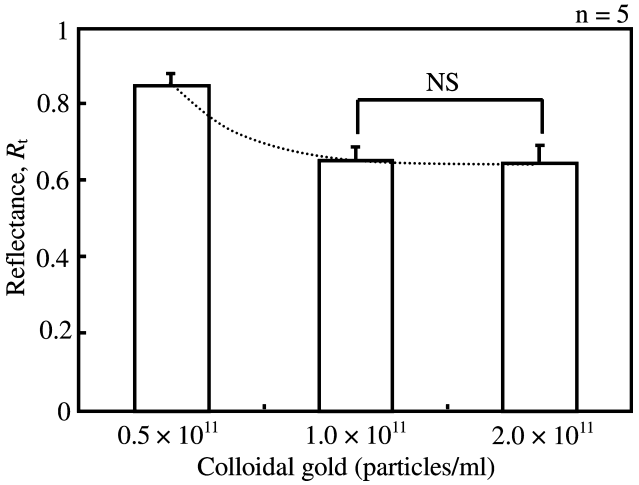

Fig. 5. Influence of different concentrations of colloidal gold on conjugate pad reflectance.

test strip and the optical analyzer. After dropping the sample, it took 90 s for the solution to reach the test line on the test strip. Ten minutes after dropping the control solution (negative), only the control line was visible. On the other hand, 10 min after dropping CatL standard solution (positive), two reddish purple colored lines, the control and test lines, appeared (Fig. 6).

To evaluate the sensitivity of the biosensor, the optical density, OD, of the test line was measured by the reader. The OD values of CatL at 95, 285, 475, 665, and 950 μU/mL were 0.05 ± 0.007, 0.078 ± 0.011, 0.112 ± 0.008, 0.125 ± 0.01, and 0.175 ± 0.016, respectively (mean ± SD, Fig. 7). The coefficient of variation (CV) was 15%. With regard to the calibration curve of the biosensor for CatL analysis, the R^2 value was 0.94; the relationship between the optical

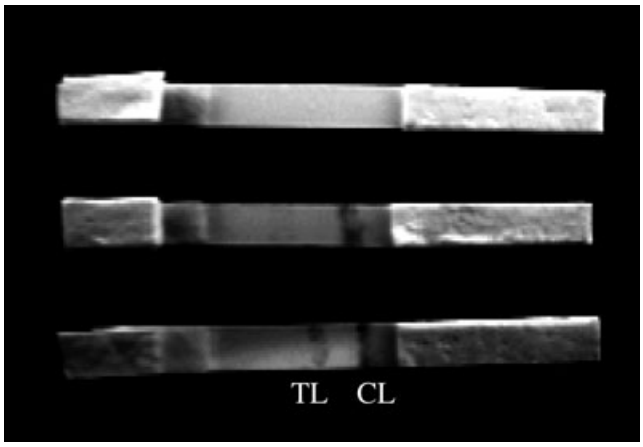

Fig. 6. External view of the test strips before; upper, and 10 min after application of the control solution; middle, and the cathepsin L solution; lower.

density and the CatL was $OD = 1.4 \times 10^{-4}$ CatL + 0.03739. The optical density was linearly proportion to the CatL. When the critical level was defined by Currie solution (3.29 × SD) (15,16), the detection limit was 172.2 μU/mL. A significant difference was observed between both 285 and 475 μU/mL, and 665 and 950 μU/mL, respectively ($P < 0.01$).In addition, a significant difference was not observed between 475 and 665 μU/mL. Therefore, the measured results were divided into a series of three different ranges such as low range: CatL ≤ 300 μU/mL, middle range: 300 μU/mL < CatL ≤ 1,000 μU/mL, and high range: 1,000 μU/mL < CatL to evaluate the possibility of a semi-qualitative assessment.

Detection of human CatL

All of the CatL values of the young subjects were classified into the low range, while all of the CatL values of the elder subjects were classified into the high range (Fig. 5). A significant difference was observed between the two groups ($P < 0.01$), and the elder subjects showed higher values. This tendency agreed well with previous studies which found higher levels of CatL in sun-exposed areas of skin (12,17).

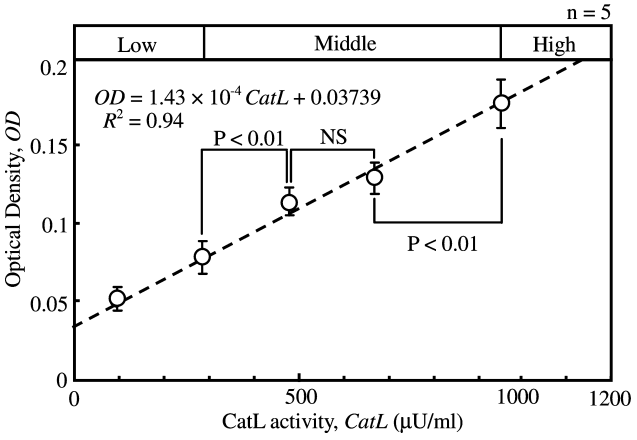

Fig. 7. Calibration curve of the biosensor for cathepsin L analysis.

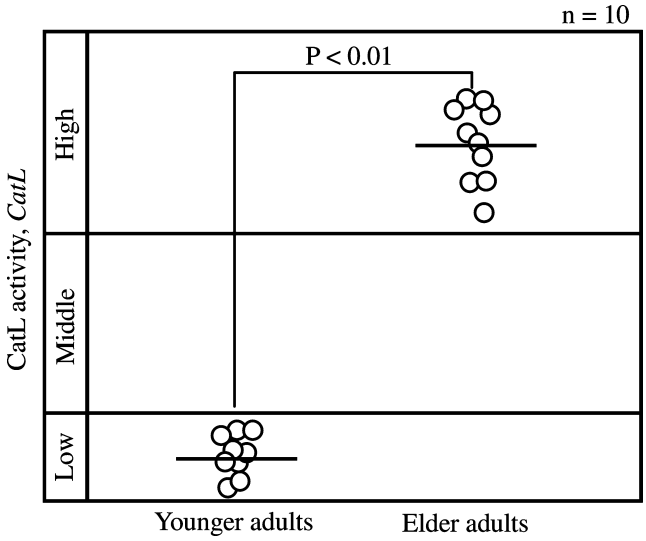

Fig. 8. Comparison of cathepsin L between younger and elder adults.

It was suggested that the newly developed biosensor of CatL can be used for the evaluation of human CatL in the stratum corneum semi-quantitatively. In addition, the truncated sampling-reporting cycle (<15 min) allows speedy reporting of CatL levels.

Conclusion

To realize an on-site testing technique of CatL in stratum corneum, we fabricated an immunochromatographic type biosensor using colloidal gold. It was suggested that the biosensor can be used for the semi-quantitative analysis of CatL in the stratum corneum to monitor the presence or absence of a skin biomarker, which might be useful for the evaluation of skin conditions non-invasively and easily.

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