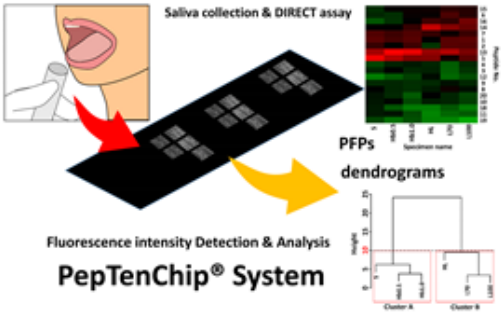
Graphical Abstract

Applications of a novel biodetection system to saliva using protein fingerprints with data processing

Yuki Tominaga[a] , Kenji Usui[b], Akiyoshi Hirata[a], Hiro-O Ito[c] and Kiyoshi Nokihara[a]

[a] *HiPep Laboratories, Nakatsukasa-cho 486-46, Kamigyo-ku, Kyoto 602-8158, Japan*
[b] *FIRST (Faculty of Frontiers of Innovative Research in Science and Technology), Konan University, 7-1-20 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[c] *Department of Preventive Dentistry Institute of Biomedical Sciences Graduate school, Tokushima University, 3-8-15 Kuramoto-cho, Tokushima 770-8504, Japan*



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[c] *Department of Preventive Dentistry Institute of Biomedical Sciences Graduate school, Tokushima University, 3-8-15 Kuramoto-cho, Tokushima 770-8504, Japan*

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ABSTRACT

A fundamental method has been developed focusing on a facile and rapid examination of periodontal disease. Periodontal disease is an oral disease thought to affect 80% of adults, and early detection with treatment is desirable for the improvement of the quality of life. Unfortunately conventional methods are not consistent as the disease is caused by a number of bacteria and detection relies on the skills of the dentist. Thus an objective detection system is required. We have performed an experiment on saliva using a novel biodetection system, designated PepTenChip®. A disease model for saliva was prepared using a specimen from a healthy subject and a mixture of hemoglobin (f-Hb) and lactate dehydrogenase (LDH), which is used as a periodontal disease marker protein with healthy saliva. PepTenChip® is a peptide microarray in which fluorescent labelled structured peptides are immobilized on a novel amorphous carbon substrate. Since the peptides used as capture molecules are fluorescently labelled, labeling of analytes is not necessary. The fluorescence intensity change before and after application of analytes are detected rather than the ON/OFF detection common to conventional microarrays using a set of antigen-antibody. The fluorescence intensity value changes according to the concentration of captured protein allowing the generation of protein fingerprint (PFP) and dendrograms. The present method does not rely on a "one to one" interaction, unlike conventional biodetection, and advantages can be envisaged in the case of an undefined or unknown cause of disease. The statistical analyses, such as multivariate analyses, allow classification of the type of proteins added in saliva as mimetics of disease. PepTenChip® system is useful and convenient for examination of periodontal disease in health care.

1. Introduction

Periodontal disease is a common gum disorder thought to affect about 80% of the population in many countries.[1] It is an inflammatory disease caused by various species of dental plaque bacteria and symptoms vary from mild gingivitis to severe alveolar pyorrhea. Recent studies indicated that the progress of the disease cause a systemic illness through blood vessels infected by bacteria from periodontal tissues. [2] Consequently, early detection and treatments are required for improvements in the quality of life (QOL). However examination methods for periodontal disease have not been established. Currently diagnosis for this disease relies predominantly on the judgment of a dentist through examination of periodontal pocket and confirmation of bleeding from periodontal tissue. Current methods are not entirely reliable as it depends on the skill of the dentists, hence an objective and facile examination method is necessary. Conventional biodetection involves the discovery of distinct targets responsible for the disease and mostly involve applications of “antigen-antibody” interactions.[3-5] Such detection of known substances is based on "one to one" recognition, while detection of disease-related substances, unknown targets and/or surrogate markers is difficult. During the last decade considerable efforts have been devoted to the development of a novel biodetection system using labelled structured peptides, arrayed on a chip surface. In fact proteins consist of different amino acids with post-translational modifications and exhibit structures containing α-helices, β-sheets and β-loops. Protein-protein interactions can be mimicked by protein-peptides in which the peptides have the appropriate structures. The structural changes of the protein as an analyte are reflected in the fluorescent-intensity changes of peptides as capture molecules in

* Corresponding author. Tel.: +01-75-813-2101; fax: +01-75-801-0280; e-mail: noki@hipep.jp

a dose dependent manner and are converted to a color bar-code, designated a "PFP" for visualization. [6-9] There are four major fundamental technologies which have been completed recently: those are (1) capture molecules, fluorescent labelled structured peptides libraries has been constructed, [7, 8] (2) chip plates, a novel material made from amorphous carbon, [10] (3) arraying technology (deposition of a few pico-litres), several methods including printing by piezo elements have been employed which showed low throughput. Pin-deposition was the most reliable with regard to cost, reproducibility and throughput, and (4) fluorescent detection, small and maintenance free device suitable for use in biosafety level 3 and 4 rooms has been constructed. The present biodetection chip, designated PepTenChip®, allows minimization of the amounts of both analytes (nano gram) and capture molecules (several femto mole). [9, 11] In the present study these four technologies have been applied to saliva as analyte. Proteins, f-Hb and LDH, were used as a blood marker and a cell damage marker, respectively. The present analyses were carried out using labelled structured peptides as capture molecules and analytes, which served as a disease model of saliva containing with or without hemoglobin (f-Hb) and lactate dehydrogenase (LDH).[12] Additionally data handling by the statistical methods are also described.

2. Materials and Methods

2.1. General

The major reagents for peptide syntheses, amino acid derivatives, solid supports (TentaGel® S RAM, Rapp Polymere GmbH, Tuebingen, Germany), (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and tetramethylrhodamine (TAMRA) were from HiPep Laboratories (Kyoto, Japan). Other reagents and solvents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and used as received. Water was prepared by a Milli-Q apparatus (Millipore, Tokyo, Japan). On-line LC-MS was used for quality assessment of peptides used in this study. Peptide solutions were prepared in a 384-well microtiter plate (Thermo Fisher Scientific K.K., Kanagawa, Japan). Marker protein for periodontal disease was purchased from Sigma-Ardrich Japan K.K. (Tokyo, Japan).

2.2. Synthesis of designed fluorescence labelled peptide for capture molecules

Structured peptide libraries used in the present study have been previously reported.[7, 8] Briefly the assembly was performed using automated synthesizers, PSSM-8 (Shimadzu Corp., Kyoto, Japan) and PetiSyzer®s (HiPep Laboratories) by the Fmoc solid-phase strategy and cleaved from resin. Each peptide has been characterized by LC (LC20A, Shimadzu Corp.)-ITMS (HCTultra, Bruker Japan K.K., Kanagawa, Japan) system on HiPep-Cadenza (3.0 i.d. × 150 mm, HiPep Laboratories) after purification on preparative scale columns (50 mm i.d. x 250 mm, HiPep Laboratories). Several peptides having difficult sequences could be obtained in higher yield by coupling at elevated temperature.[13] The peptides for arraying were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Nacalai Tesque inc.) and stored at -80°C until arraying. The sequence of designed peptides are listed in Electronical Supplemental Information Table S1.

2.3. Derivatization of amorphous carbon substrate

A carboxyl PepTenChip® (PTC-CA-NN-02, HiPep Laboratories) [14] was derivatized with 6-maleimide caproic acid (EMCA, HiPep Laboratories) by the following procedures. Firstly, the substrate was placed in a reaction vessel for chips (Pap jars, Evergreen Scientific, CA, USA) and 10 mM N,N'-diisopropylcarbodiimide (DIPCDI, Sigma-Aldrich Japan K.K.) and 10 mM N-hydroxysuccinimide (NHS) dissolved in DMF were added and incubated at room temperature for 1 h. To the resulting mixture 10 mM hexamethylene diamine (Nacalai Tesque Inc.) dissolved in DMF was added and gently stirred at room temp for 1 h, washed 5 times with methanol and dried *in vacuo*. The chip-surface was thus aminated. A solution of 20 mM EMCA and 10 mM DIPCDI in DCM was prepared in LibraTube® RT5M (HiPep Laboratories) and shaken at room temperature for 1 h. After removal of white precipitates by filtration the liquor was added to the above Pap jar, diluted with DCM (30 mL) and pyridine was added (10 mM). The resulted mixture was shaken at room temperature for 1 h, washed with 5 times with methanol and dried *in vacuo*.

2.4. Preparation of Peptide microarray

A solution of synthetic peptides (100 μM) for capturing in HFIP with 1% acetic acid was dispensed on to a 384 well microtiter plate and arrayed using a microarrayer (NanoPrint(TM) LM-60) with a printing pin14 (946MP4, spot diameter 135 μm) manufactured by Arrayit Corp., CA, USA). After arraying, the chip-plate was washed three times with 2-mercaptoethanol (10 mM) and ultrapure water. After spin drying, the substrate was analyzed with a fluorescence detector (PTC-FD 11, HiPep Laboratories) to confirm desired array quality. The resulting PepTenChip®s allow continuous detection of fluorescence intensity changes of the chip surface.

2.5. Detection of saliva sample

Saliva from healthy and disease models, which are the mixture of disease marker proteins, were applied on the substrates. Hence 10 μL of PBS (Phosphate Buffered Saline, pH 7.0) was applied as blank. The drop of samples were spread using cover glass (18 x 18 mm, Matsunami Glass Ind., Ltd., Osaka, Japan) and incubated at ambient temperature for 30 min in the dark. After 30 min the fluorescence intensity of the microarray was measured using a fluorescence detection device (PTC-FD 11) and it was defined as I0. Fluorescence images obtained in the assay were quantified using the image analysis software Array-pro(TM) Analyzer (Nippon Roper K. K., Tokyo, Japan). After measuring I0, the cover glass was removed from the substrate, washed three times with 2-mercaptoethanol solution and ultrapure water, spin-dried, and assayed for the specimens. The assay of the specimen was performed in the same manner as the detection of PBS, and the resulting fluorescence intensity was set to I1. After assays, the fluorescence intensity changes were calculated according to the following equation. The fluorescence intensity changes were subjected to various statistical analyses.

Fluorescent intensity change= $(I_1 - I_0) / I_0$

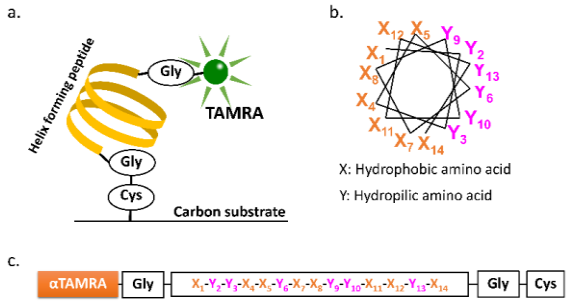
2.6. Statistical Analysis

For statistical analyses, peptide-probes which showed fluorescence intensity dependent on concentration of marker proteins were selected by the RSQ function of Microsoft® Excel®2013. In this procedure, a total of 20 peptides were selected from 500 peptides as these showed significant intensity changes. PFP was prepared using the program for statistical analysis, R ver. 3.2.1. Furthermore, dendrogram were created by clustering Euclidean squared distances and Ward's method from the results of PFP.

3. Results and Discussions

3.1. Peptide Microarray

Labelled peptide libraries having α-helical structures used as capture molecules have been constructed by solid phase synthesis, purified and their secondary structures have been confirmed by CD spectra. [4, 5] Peptide design is illustrated in Fig. 1 where the fluorescent dye was TAMRA, Gly was used as a spacer and the Cys residue was for immobilization on to chip surface using EMCA. Five hundred different peptides were arrayed on an amorphous carbon plates using a micro-arrayer with a pin-system, 946MP4, as described in the experimental section. The spot diameter was 135 μm and amounts of applied peptide solution was *ca.* 1.1 nL/spot (Fig. 2). The microarrays have been prepared as described in the experimental section. Peptides which change their fluorescent intensity depending on the concentration of proteins in the analyte have been selected from 500 structured peptides in the library, that is, those selected peptides interacted with proteins (f-Hb and LDH). Table 1 shows the list of specimens and concentration of proteins used in this assay. Saliva specimen assays were performed at n=3 using PepTenChip®.



in this study, (b) Arrangement of arrayed peptides.Fig. 1 Design of synthetic peptides. (a) A schematic diagram of the fluorescent labelled peptide on a substrate, (b) Design of peptides with α-helix structure. A random amphipathic peptide library was constructed by adding hydrophobic amino acids to X and hydrophilic amino acids to Y, (c)

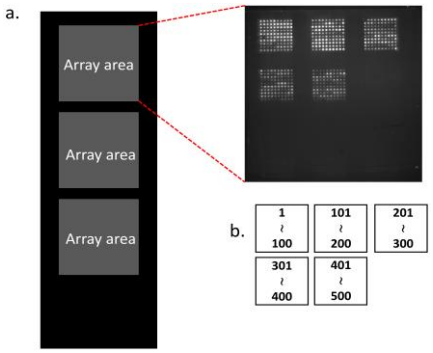


Fig. 2. The microarray prepared in the present study. An amino-amorphous carbon plate (25 x 75 mm) has three blocks of derivatized area and 500 labelled peptides were immobilized. (a) Design of microarray constructed

Analysis of Variance (ANOVA) was performed in order to test whether there was a significant difference in data obtained by the assay. ANOVA results are indicated in Table 3. Comparing the F value (F, 8.367) calculated in this ANOVA analysis with the F value (F0.01, 8.3967, Electronical Supplemental Information Table S4) in the F distribution table, F0.01 <F. Therefore, we concluded that there was a difference in the fluorescence intensity change value at the 1% significance level. In addition, the P value (P value in Table 3) was $7.34e^{-5}$, which was a very small value. From these two results, it was judged that the data obtained in this study was significantly different at P = 0.01. In f-Hb data, peptides with strongly changing fluorescence intensity were found when f-Hb concentration reaches 100 μg/mL (Fig. 3a). In particular, peptide No. 202, 203, 204, 205, 213, 215 show marked changes and are considered to be useful capture peptides which interact with hemoglobin. In the case of LDH, peptides were found in which the fluorescence intensity varied with the protein concentration (Fig. 3b). In particular, peptide No. 203, 204, 279, 319 generate remarkable fluorescence intensity change by LDH concentration, and are judged to be useful for detection of LDH. Hence peptide No. 203 and 204 were also selected from f-Hb data, thus they interact with both f-Hb and LDH.

Table 1. The list of specimens using this study.

Sample name	Sample content
S	Saliva only
Hb0.5	Saliva + f-Hb 0.5 μg/mL
Hb1.0	Saliva + f-Hb 1.0 μg/mL
Hb10	Saliva + f-Hb 10 μg/mL
Hb100	Saliva + f-Hb 100 μg/mL
L10	Saliva + LDH 10 mU/mL
L70	Saliva + LDH 70 mU/mL
L100	Saliva + LDH 100 mU/mL
L1000	Saliva + LDH 1000 mU/mL
HL	Saliva + f-Hb 1.0 μg/mL + LDH 100 mU/mL

[a]The sample name Hb1.0, Hb10, L10, L100, and L1000 are used for selection of peptides in which protein concentration dependent fluorescence intensity change. Hb0.5, Hb1.0, L70, L100 and HL are concentrations actually used for saliva analyses in dental clinic. (n=3 results). Concentration of f-Hb and LDH are conventionally used in the field of dental diagnosis to discriminate positive and negative in periodontal disease.[12]

Table 2. Comparison of properties of peptides classified by PFP.
[a]I. P. is Isoelectric Point, and H. I. is Hydrophobicity Index.

Peptide#	Seq. (α-TAMRA-G-XXXX-GC-NH_2)	Mwt.	I.P.	H.I.
202	FQQ FFK FFQ QFF KF	1964.3	10.3	0.04
203	LQQ FFK FLQ QFF KF	1896.3	10.31	0.19
204	LQQ LFK FLQ QLF KF	1828.2	10.31	0.33
205	LQQ LLK FLQ QLL KF	1760.2	10.31	0.47
206	FKK FFS FFK KFF SF	1882.3	10.3	0.37
213	LSS LLK FLK KLL SF	1637.1	10.6	1.02
214	FSS FFK FFS SFF KF	1800.1	10.3	0.81
215	LSS FFK FLS SFF KF	1732.1	10.31	0.96
237	LQQ LLR FLR RLL QF	1844.3	12.8	0.31
279	LSS LLK VLK KLL SV	1541	10.6	1.22
287	LEE LLR VLE ELL RV	1724	4.52	0.59
319	AQQ AAK IAK KAA QI	1439.7	10.6	-0.17
332	AEE AIR IAE EAI RI	1583.8	4.52	0.16
352	AKK FFR FAK KFF RF	1868.3	12.52	-0.3
354	AKK AAR FAK KAA RF	1563.9	12.52	-0.59
380	AKK AFS FAK KAF SF	1577.9	10.3	0.09
400	AQQ FFR FAR RFF QF	1896.2	12.8	-0.26
449	ARR AVQ VAR RAV QV	1579.9	12.98	-0.07
452	AQQ AAR VAR RAA QV	1495.7	12.8	-0.34
498	IQQ IFR FIR RIF QF	1912.3	12.8	0.37

3.2. Selection of peptides that interact with proteins

The correlation coefficient was calculated from the fluorescence intensity change obtained by the assay for each protein using the RSQ function of Microsoft(R) Excel(R) 2013 (in Electronical Supplemental Information Tables S2, S3). The 20-peptides were selected (Table 2) in descending order of the correlation coefficients calculated by RSQ function (in Electronical Supplemental Information Table S3, S4) and the change in fluorescence intensity was compared (Fig. 3).

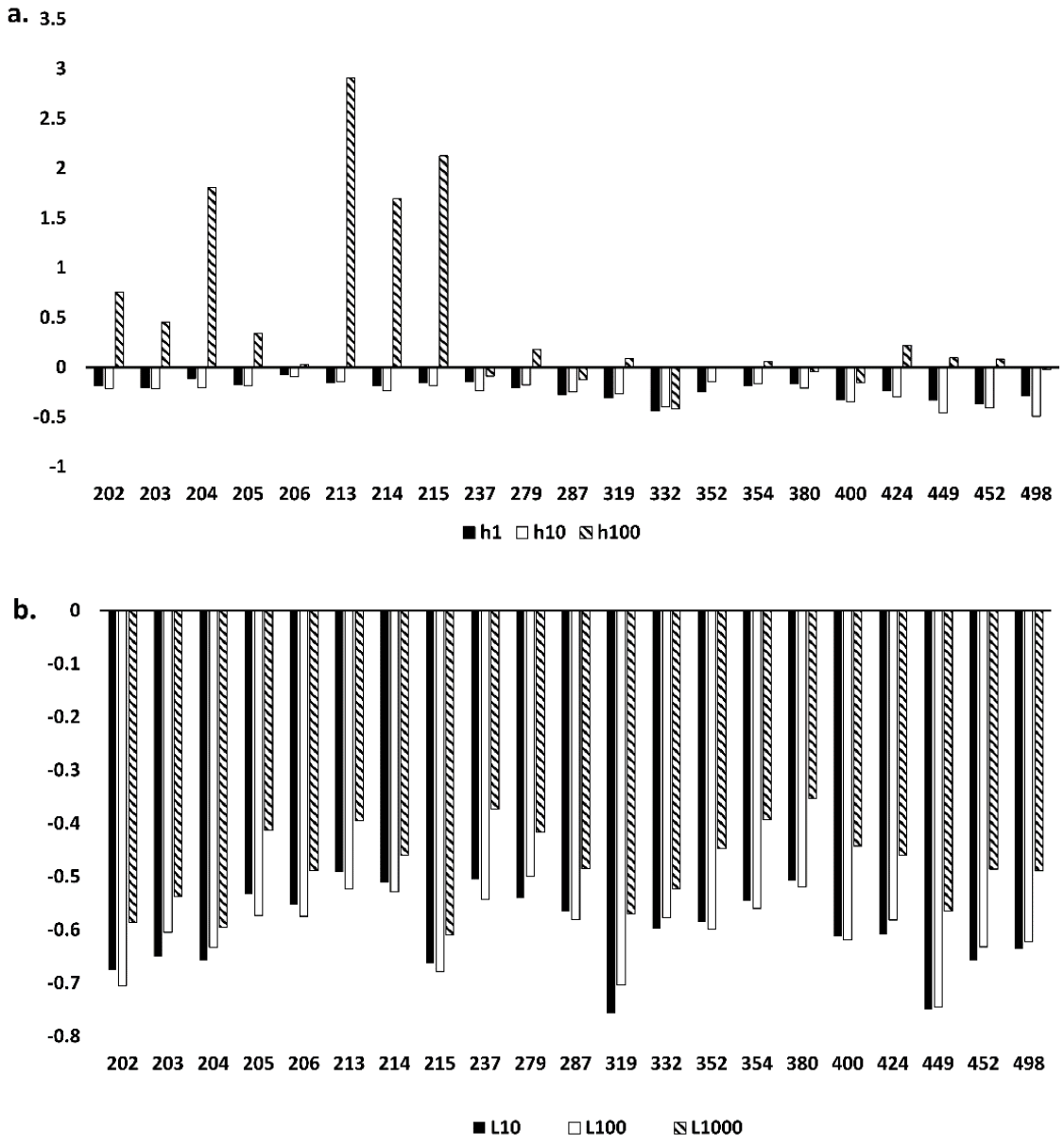


Fig. 3 Comparison of changes in fluorescence intensity with respect to protein concentration. Fluorescence intensity change, (a): specimen is f-Hb; (b): specimen is LDH.

Table 3. Outline of Analysis of Variance conducted on fluorescence intensity change.

	Degree of freedom	Sum of squares	Mean square	F value	P value
Between groups	5	1.013	0.20268	5.866	7.34e-5
Within group	114	3.939	0.03455		

[a]Degree of freedom (between groups) = number of groups – 1 = 6 – 1, Degree of freedom (within group) = (data number within group – 1) x number of groups = (20 – 1) x 6.

3.3. PFPs and Dendrogram

Fig. 4 shows PFP prepared from values in the fluorescence intensity changes. The red and green color indicate higher and lower value of the changes, respectively. Hence LDH increases intensity with many peptides. The above selected 20 peptides can be classified into two groups by the PFP analysis with the value of fluorescence intensity change. The peptides were classified into two groups and their sequences are compared (Table 2). The average Hydrophobicity Index (H.I.) of the Peptide of Group 1 was 0.15 and the average H.I. of the peptide of Group 2 was 0.35, and the peptides in Group 2 were slightly more hydrophobic. Also, fluorescence intensity change of peptide 206 and 352 indicate marked increments in the assay with specimens. Both peptides consist of a lot of Lys- and Phe-residues, thus these amino acids are considered to play a key role for interaction. Phe-rich peptides classified in Group 1 increase fluorescence intensity change (Fig. 4), suggesting that Phe-residue plays an important role for interaction with proteins. A further dendrogram was created based on the results of the PFP. In the dendrogram, the distance between each specimen is close, that is, the specimens are similar in height and connection of each branch. For the dendrogram obtained in this study, dividing a branch with a height of 20 can be classified into two clusters (Fig. 5). Cluster A in Fig. 5 contains LDH and another cluster B in Fig. 5 contains f-Hb. Since the cluster A contains HL (mixture of f-Hb and LDH), LDH has stronger influences.

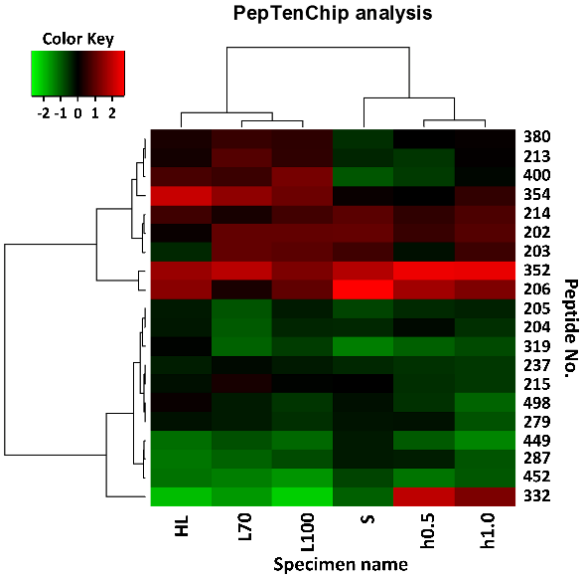


Fig. 4 PFP prepared from fluorescence intensity change of selected 20 fluorescent labelled peptides. Red indicates increase in fluorescence intensity and green indicates decrease in fluorescence intensity.

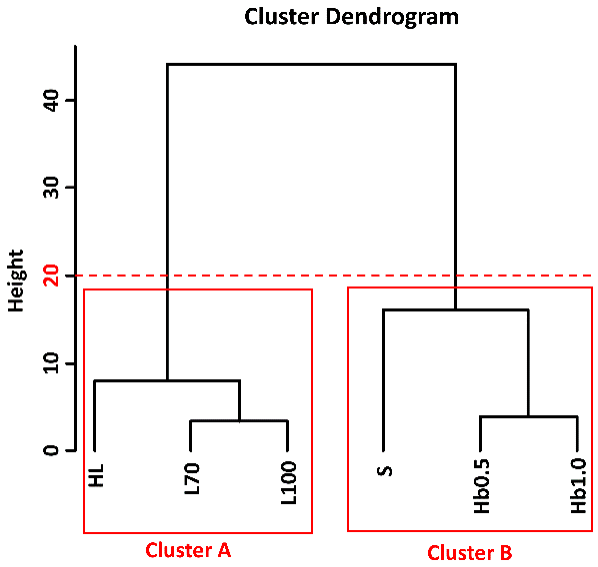


Fig. 5. A dendrogram created from PFP. When divided by height 20, the dendrogram are classified in two clusters (A, B).

3.4. Principal Component Analysis (PCA)

Principal component analysis of the selected peptides is shown in Fig. 6a. The model protein samples are classified in the first principal component axis (PC1), and the saliva specimens on the second principal component axis (PC2). In PC1 samples containing f-Hb (Hb 0.5, Hb 1.0) were negative in contrast to those containing LDH (L70, L100) which were positive. The PC2 specimen of healthy saliva (S) was clearly classified as positive. In contrast saliva specimens containing the present model proteins (Hb 0.5, Hb 1.0, L 70, L 100, HL) were negative. These results show that each specimen can be classified from fluorescence intensity change of labeled peptides as capture molecules. Fig. 6b and 6c show the main component loading amounts and characteristic amino acids in each peptide. In PC1 increasing the number of Phe residues tends to increase of principal component loading amounts (Fig. 6b). For PC2 increased charged resulted in increasing loading (Fig. 6b). Thus, hydrophobic interaction of the aromatic ring of the Phe residue and electrostatic interactions by charged amino acids have a significant influences for the detection of saliva. It is expected that PepTenChip®s arrayed with Phe- and charged amino acid- rich peptides afford a practical detection system.

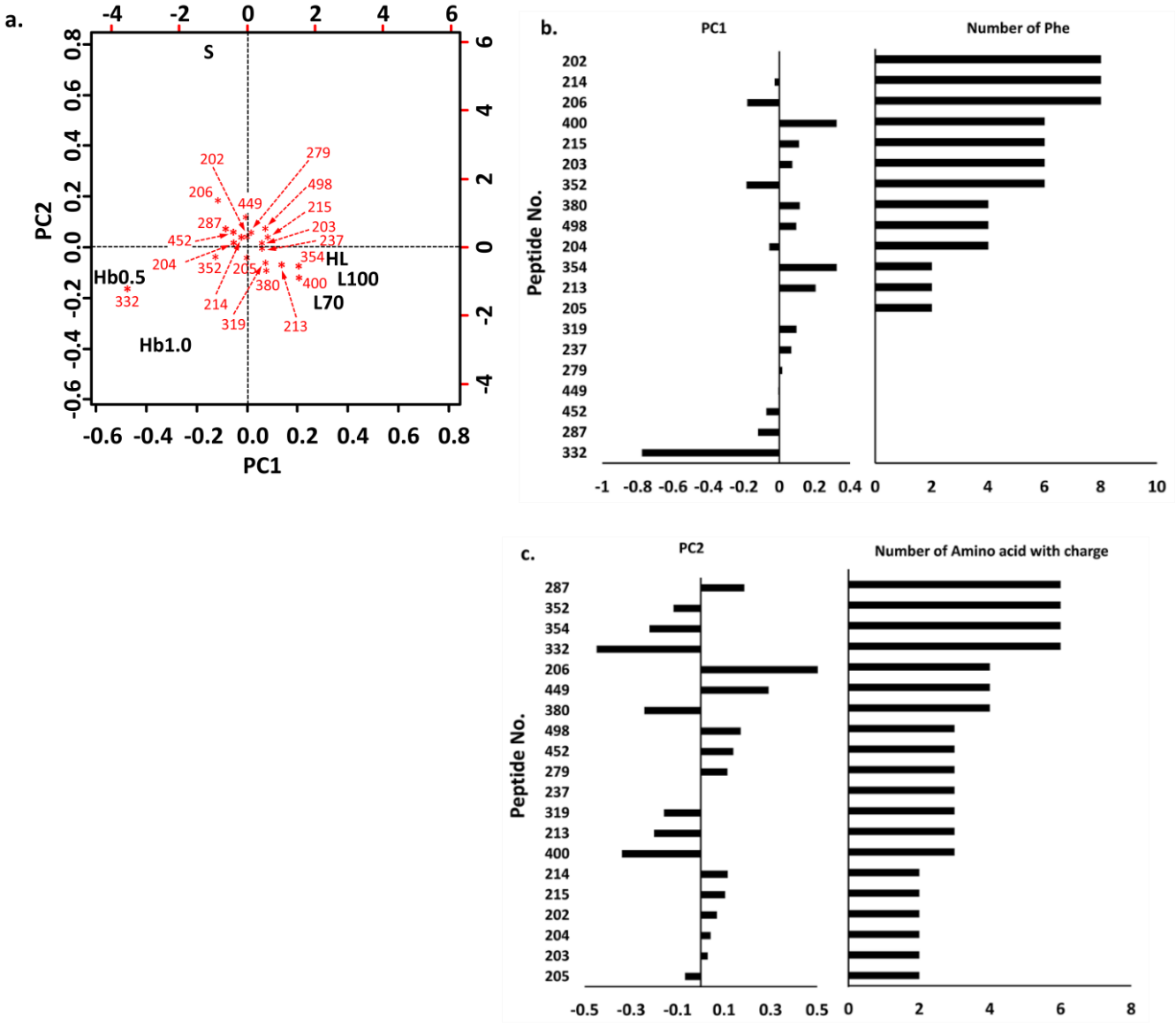


Figure 6. (a) PCA-results. Corresponding peptide location indicated with * ; (b) Principal component loading amounts in PC1 and number of Phe-residues in each peptide; (c) Main component loading amounts in PC2 and number of charged amino acid residues in each peptide

4. Conclusions

Microarrays with structured and labelled peptides in combination with statistical analysis (multivariate analysis) are very useful to discriminate specimens between healthy and disease subjects containing numerous compounds. The present study indicates that saliva can be analysed by the PepTenChip® technology using a novel concept that does not rely on "one to one" recognition. Detection of healthy saliva contained proteins used as periodontal disease markers as a model specimens have been analysed. PepTenChip® system generated PFP-patterns with fluorescent intensity change between the chip (peptide array) and the chip with analytes. In the present study 500 different designed structured peptides carrying fluorescent dye were arrayed onto derivatized substrates, PepTenChip®, made from amorphous carbon. Peptides which showed significant fluorescence intensity change depending on the concentration of model proteins in saliva were selected and multivariate analyses allowed classification of the proteins mixed with saliva and their concentration. The present microarray is useful for analyses of biological samples, in particular, biochips arrayed with a large number of different structured peptides are suitable for comprehensive analyses involving unknown markers. The array with selected interacted peptides can be used for target specific biodetection tools. [16-18] In addition, PepTenChip® is also a very powerful tool for searching for novel markers contained in the samples. The PepTenChip® can be used as a sample tray for MALDI-TOF-MS analyses to directly captured proteins on the surface, [19, 20] allowing further characterization of their molecular weight and partial amino acid sequences. Following to the present study we are planning experiments using patient saliva to provide the beneficial tool for dentists. A more accurate diagnostics may be accomplished using databases for targeted disease. Additionally different body fluids, such as blood, urine and spinal fluid will be collected in clinics under the ethical approval and applied to the present system, which is a class 1 medical device.

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