Peptide Array-Based Peptide-Cell Interaction Analysis

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Abstract: Peptide arrays represent a powerful research tool, especially for the development of medical applications to screen peptides for the replacement of animal-derived proteins. This minireview focuses on the applicability of cellulose-bound peptide arrays to assay peptide-cell interactions. Moreover, the successful combination of peptide array and bioinformatics to overcome the limitation of library size in array-type screenings is reviewed.

Keywords: Cell assay, cell adhesion, cell proliferation, cell specificity, bioinformatics.

I. INTRODUCTION

Interest in the use of peptides as an ideal synthetic biological material that interacts with cells has increased recently in medical and cellular biology. There are increasing applications of peptides reported as: (a) a stimulating factor for cellular events [1], (b) a scaffold for cell culture [2], and (c) a tag molecule for specific targeting [1,3]. For medical applications and clinical use, peptides can more easily penetrate the plasma membrane when compared to large proteins such as antibodies, and moreover, they can also escape from severe immune responses compared to large proteins [4]. The assured safety of synthesized peptides is also an important advantage to replace of animal-derived proteins, which is strongly required for clinical applications in the advancing regenerative medicine.

Although peptide arrays have long been applied to assay various biological targets [5], there are still few reports on peptide-cell (animal cell) interaction assays. To the best of our knowledge, the work of Otvos *et al.* [6] is the only publication other than our work that clearly indicates the SPOT peptide arrays' applicability to cell-stimulation assays. Their pioneering work indicated the applicability of a cellulose-support-type peptide array for direct stimulation of animal T helper cells. However, most animal cells are anchorage-dependent and require an adhesive surface to be cultured. Kato *et al.* have developed a peptide array-based interaction assay of solid-bound peptides and anchorage-dependent cells (PIASPAC) method and applied it to various types of cells for assaying (a) cell-adhesion peptides [7-10], (b) cell-specific peptides, and (c) tumor-inhibition peptides [11-13]. In this chapter, such applicability and performance of peptide-cell interaction assays on SPOT-type arrays is reviewed. Additionally, the successful application of bioinformatics for peptide-cell interaction assays [8] to overcome the problem that lies in the peptide array-based cell assay is explained.

II. PEPTIDE ARRAY-BASED INTERACTION ASSAY OF SOLID-BOUND PEPTIDES AND ANCHORAGE-DEPENDENT CELLS (PIASPAC)

The schematic diagram of PIASPAC, which is the basic strategy of the peptide-cell interaction assays in this chapter, is illustrated in Fig. (**1**).

The peptide array was synthesized with conventional SPOT synthesis protocols [5]. The design was customized as follows. The peptide array was designed to consist of triplicate copies of each peptide in one array. Each spot was designed to be randomly placed within each array to reduce positional bias. The positive peptide (RGDS peptide) and negative peptide (linker only, no peptide) were placed in each array for normalization. On the β-alanine modified cellulose membrane (grade 542; Whatman, Maidstone, UK), two repeated couplings of Fmoc-11-aminoundecanoic acid (Watanabe Chemical Industries, Ltd., Hiroshima, Japan) were used as an optimized cell-assay spacer. Activated Fmoc amino acids (0.5 M) were spotted with a peptide auto-spotter (ASP222; INTAVIS Bioanalytical Instruments AG, Köln, Germany). The synthesized array was then thoroughly washed with diethyl ether, methanol, phosphate-buffered saline (PBS; pH 7.4) before the assay.

Each spot corresponding to different peptides was punched out as disks using a disposable 6-mm-diameter biopsy punch (Kai Industries Co. Ltd., Gifu, Japan) and embedded in a 96-well plate using thin tweezers, with the same surface in the same direction [Fig. (**1**)**A**]. After soaking the punched disks with the appropriate cell culture medium, cells were directly seeded on the disk with the optimized cell density for each cell. Cells could be seeded with a serum-containing medium or serum-free medium according to the assay objectives. Serum is commonly used in our assays and was found to have no inhibitory effect on finding objective peptides. After the cells adhered to the disk (approximately 30 min or more depending on the cells), cells were washed by pipetting with the appropriate culture medium for the cell-adhesion assay. Later, the cells were cultured on the disk without washing and were washed once more after the culture time determined for the cell proliferation assay. Culture and washing times must be optimized for assay purposes. Most of our incubation conditions were set to 1 h for the adhesion assay and 48–72 h for the cell-proliferation assay. After washing the nonspecifically adherent cells away, Calcein AM (Molecular Probes, Leiden, The Netherlands) was added to each well, and the viable cells on the disk were measured with a fluorescent plate reader. The Calcein AM assay should be optimized for each cell type before the screening. The values of more than three peptide disks were averaged. The cells on the membrane could be observed with a fluorescent microscope, a confocal microscope, or a scanning electron microscope (SEM) [Fig. (**1**)**B**, Fig. (**2**)**A-F**]. For SEM observations, the cells were fixed with 4% glutaraldehyde solution for 12 h at 4°C, washed with ultrapure water and fixed with 1% osmium tetroxide for 30 min at room temperature. After the steps of dehydration (ethanol 70%, 80%, 90%, 95%, 100%, and repeated 100% treatment for 5 min) the buffer was exchanged to ethanol:*t*-butylalcohol = 1:1 for 1 min, and then exchanged to *t*-butylalcohol by three times incubation for 10 min. The sample was lyophilized under vacuum for 2 h, and then plasma-coated with osmium and observed based on a conventional SEM protocol. For fluorescence or confocal microscopy observations, the cells on the membrane were fixed with 4% paraformaldehyde solution for 12 h at 4°C and stained in accordance with conventional immunohistochemistry protocols [Fig. (**1**)**B**, Fig. (**2**)**G-H**].

Following types of cells were examined in the previous works: NIH/3T3, Jurkat (T lymphocyte), A3 (T lymphocyte with con-

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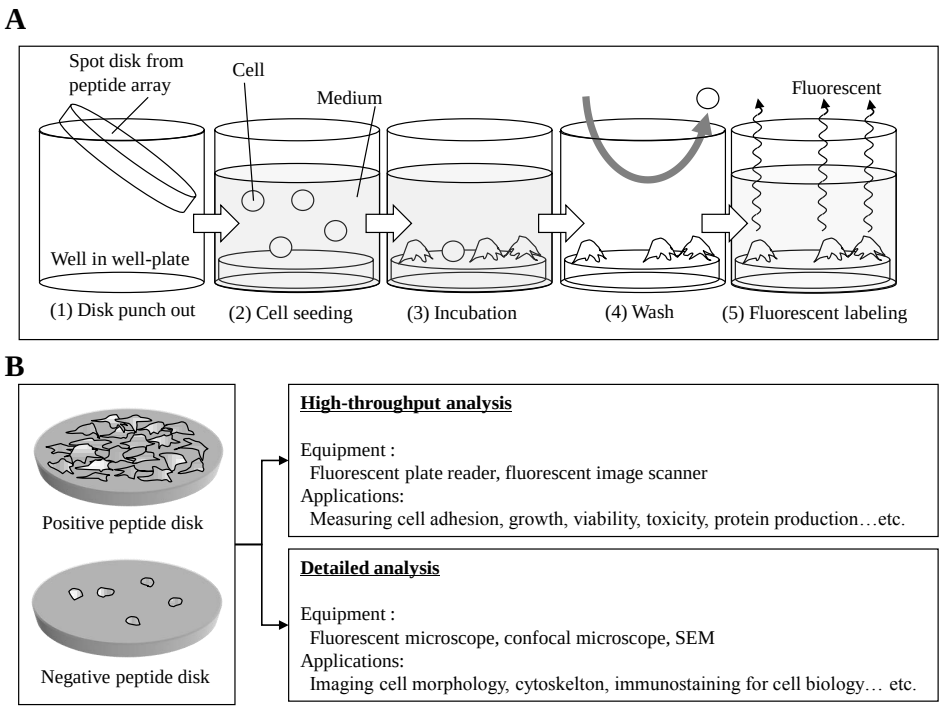

Fig. (1). Schematic diagram of PIASPAC. (**A**) Illustration of PIASPAC experimental scheme in well-plates (ref. [7]). (**B**) PIASPAC screening image and its applications.

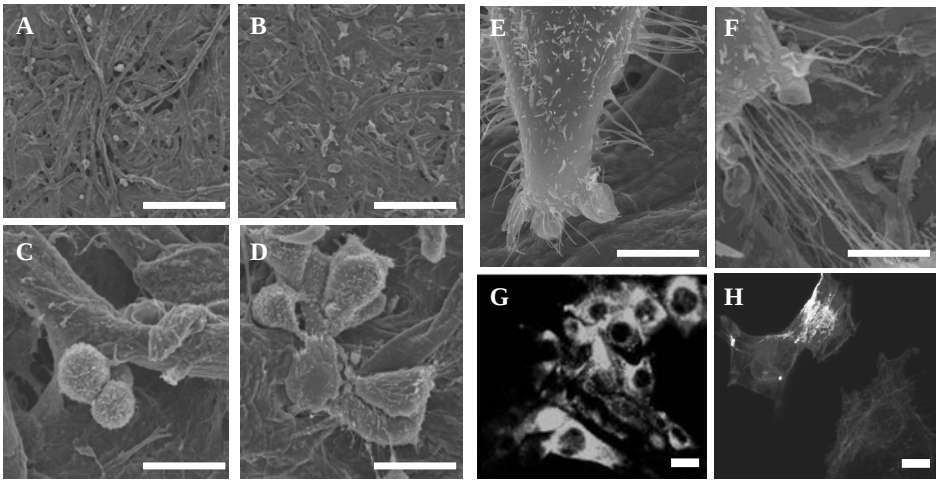

Fig. (2). Detailed images of NIH/3T3 cell adhesion (1h) on peptide disks. SEM image of cells on negative disks with no peptide (**A**, **C**) and positive disks with RGDS peptide (**B**, **D**). SEM image of filopodias from cell surface on RGDS peptide disk (**E**, **F**) (ref. [7]). Immunohistochemistry staining with anti-vitronectin (**G**) and anti-actin (**H**). White bars indicate a scale of 300 μm (**A**, **B**), 30 μm (**C**, **D**), 10 μm (**E**, **G**, **H**) and 6 μm (**F**).

firmed functional apoptosis cascade), I9.2 (T lymphocyte with confirmed defective apoptosis cascade due to caspase 8 mutation), K562 (T lymphocyte), RPMI1788 (B lymphocyte), HeLa (human epithelial adenocarcinoma), HUVEC (normal human umbilical vein endothelial cells), HAEC (normal human aorta epithelial cells), SMC (normal human smooth muscle cells), and NHDF (normal human dermal fibroblast cells). No cytotoxic effect from the assay was observed for any of these cell lines.

III. CELL-ADHESION PEPTIDE SCREENING

In eukaryotes, most anchorage-dependent cells are known to recognize and functionally react with adhesive extracellular matrices (ECMs). In regenerative medicine and tissue engineering, regulation of growth, activation, and differentiation of anchorage-dependent cells is an essential technique to prepare cells for treatment. There are well-known short peptides, such as RGDS [14], and PHSRN [15]. However, massive efforts had been required to screen most of these effective peptides. To provide a high-throughput screening technique to obtain such peptides PIASPAC method has been developed [7].

In the PIASPAC method, cells are directly seeded onto the peptide array spot [Fig. (**1**)]. By SEM observation, the number of adherent cells on the negative control (peptide disk with linker only) [Fig. (**2**)**A**] was significantly lower than that on the RGDS peptide disk [Fig. (**2**)**B**]. The difference could be averaged within a well (i.e., spot) by fluorescent indicators for high-throughput screening [Fig. (**1**)**C**]. Cell morphology was found to change drastically [negative control, Fig. (**2**)**C**; RGDS, Fig. (**2**)**D**]. NIH/3T3 fibroblast cells expanded widely on the cellulose fiber and were found to produce more filopodias and ECM fibers [Fig. (**2**)**E-F**]. The PIASPAC assay was compatible with conventional immunohistochemistry protocols [Fig. (**2**)**G-H**]. These results indicated that the PIASPAC

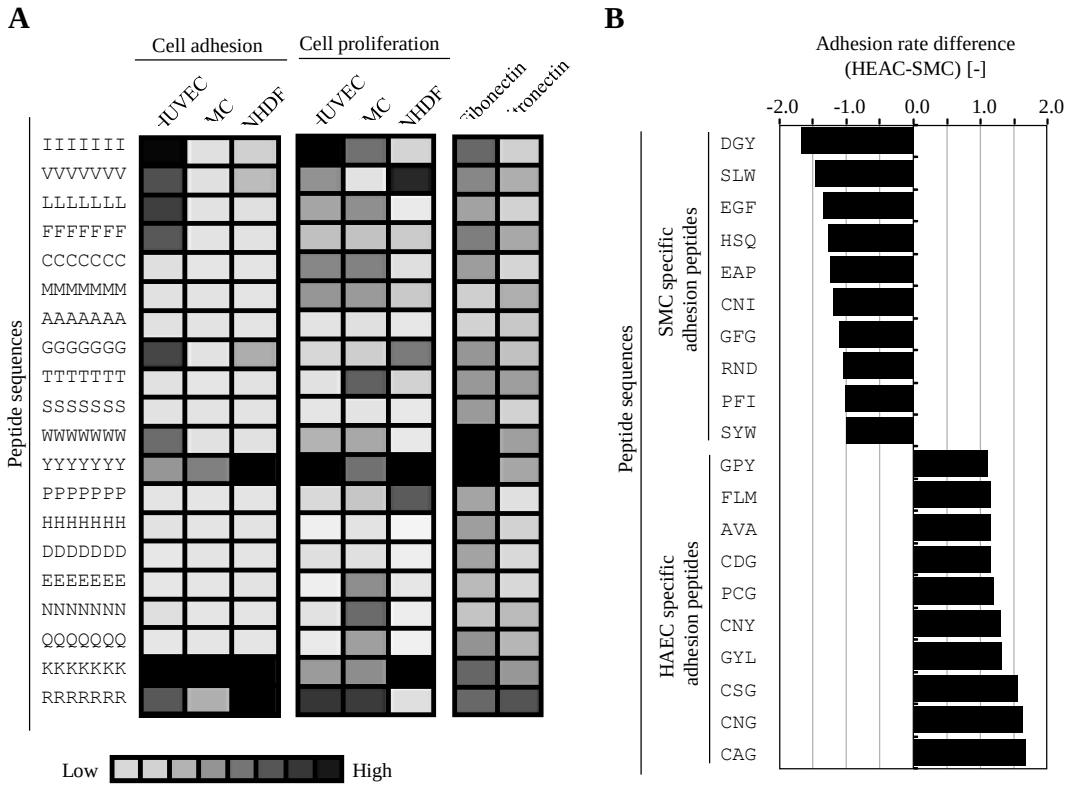

Fig. (3). Heat map image of the cell-specific preference of 7-mer peptides. (**A**) Cell adhesion (left panel) and cell proliferation (middle panel). All data were scaled by standard normalization (mean = 0, SD = 1) and colored by maple tree software (freely available at http://mapletree.sourceforge.net/). The right panel indicates the conventional antibody detection on peptide array after 1 h incubation with a cell culture medium. (**B**) Top 10 cell-specific adhesion peptides obtained from collagen type IV screening. Colors in the heat map indicates normalized values; dark (+1.5, adhesive), light (-1.5, non-adhesive).

method could be applied not only to high-throughput screening that measures groups of cells as an average rate but also to analyze the detailed effect of peptides based on cellular events.

In our previous work [7], each residue within the RGDS peptide was substituted by the other common 19 L-amino acids on a peptide array. Results indicated that the RGDS sequence was strictly recognized by cells. Such results also excluded the nonspecific adhesion of cells to various peptides or the weak interaction with similar sequences. Therefore, the advantageous feature of the peptide array, i.e., the combinatorial examination of sequence, could be applied for cell adhesion peptide screening with the current setup.

IV. CELL-SPECIFIC PETIDE SCREENING

For the development of medical implant devices (such as artificial heart, valve, vessel, etc.) that are sustained in the human body, the effective surface coating has been a long-standing, critical issue in the research field of artificial organs and biomaterials. The critical risks that may end a patient's life after the implantation are frequently caused by the medical devices for the circulatory organs [16]. The most abundant failures that occur with such devices are thrombosis and stenosis, which are mainly caused by the nonspecific surface adhesion of unwanted blood-derived cells and proteins, and also with the overgrowth of unwanted cells from the surroundings. In nature, injuries of our body are healed by correctly organized cell positioning, adhesion, and growth of appropriate cells. However, medical device implantation severely disrupts such cell organizations. The most effective solution developed recently to compensate for such disruption is coating the device with ECMs that helps to repair the proper cellular organization. For clinical application, peptides, non-animal derived material, that assist cell-specific organization are highly required [17]. In this chapter, the unpublished results of the PIASPAC method to identify such cell-specific peptides are indicated.

To examine the cellular preferences for adhesion on a peptide-coated surface, 20 different 7-mer peptides that contained repeated single amino acids were assayed for their cell-adhesion and cell-proliferation effects by PIASPAC, as described in the previous section. Three normal human cells (HUVECs, SMCs, and NHDFs) were selected to mimic the cellular population in cardiovascular tissues. The results indicated that there were cell-specific preferences of cell adhesion and cell proliferation even with simple 7-mer tandem amino acids [Fig. (**3**), left and middle panels]. The accumulation of ECM proteins (fibronectin and vitronectin) that are known to affect cellular behavior on peptides were also investigated [Fig. (**3**)**A**, right panel] by conventional antibody hybridization with a SPOT array after 1 h of incubation with a cell culture medium (EGM-2 with EGM bullet kit, Cambrex, Inc., Charles City, IA, USA). It was found that hydrophobic amino acids are preferred in providing endothelial cell-specific adhesion and proliferation. The highly charged amino acids were confirmed to enhance cellular adhesion by ECM accumulation. However, results indicated that they are negative factors for cell-specific organization.

Collagen type IV is known to be the main ECM in the basement membrane, the layer which divides and organizes the area of endothelial and smooth muscle cells in the blood vessels [18]. Collagen type IV-derived peptides were scanned by the PIASPAC method and found several trimer peptides by comparing the cell-adhesion rate between 2 cell types [Fig. (**3**)**B**]. The RGD peptide, known as a strong cell-adhesion peptide, displayed a low cell specificity [adhesion rate difference (HAEC-SMC) = 0.79], whereas the screened peptide was higher than 1.0 or lower than -1.0. These results indicate that cell-specific peptides, as a candidate biological material for coating medical devices, could be examined by PIASPAC. It should also be noted that the function of such cell-specific peptides found by PIASPAC is assured by the solid-bound form; therefore, it is feasible to transfer the peptide effect onto different surfaces by conjugation via the C-terminus of the peptide.

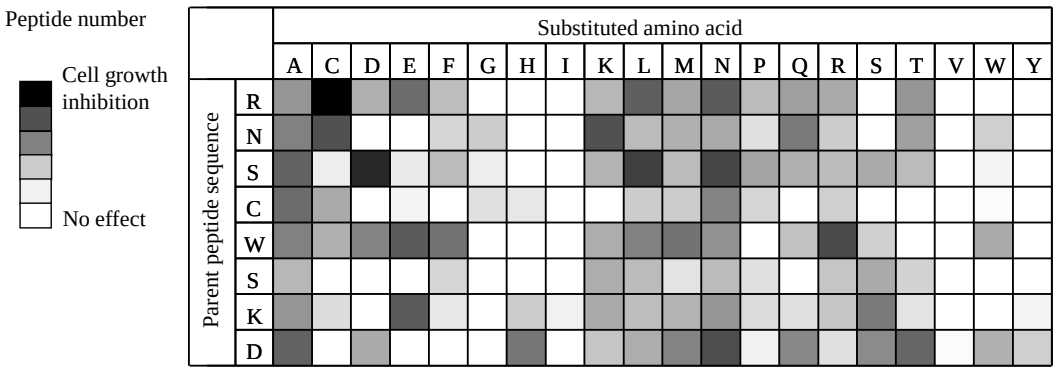

Fig. (4). Lymphoblast inhibitory peptide evolution from the cell growth inhibitory domain (RNSCWSKD) found in TRAIL by sequence substitution (ref. 13). The relative cell adhesion rate was adjusted to the control disk (linker only) set to 1.0. The values in cells are colored by excel2007 with following settings; maximum darkness (<0.5, growth inhibition), maximum lighness (>1.25, normal growth), in between values are colored with gradient.

V. TUMOR-INHIBITORY PEPTIDE SCREENING

Death receptors that are highly expressed in tumor cells are promising as anticancer molecular targets. Death receptors regulate cellular homeostasis by inducing apoptotic cell death through stimulation from death-inducing ligands, such as the Fas ligand (FasL) [19] and the tumor necrosis factor-related apoptosis-inducting ligand (TRAIL) [20]. These death ligands may involve functional peptide domains that could be isolated from the mature proteins for use as a drug candidate. The following study describes a functional peptide domain screening from death-inducing ligands by PIASPAC [11-13].

In our previous work, the extracellular domain of the human FasL was scanned by PIASPAC by assaying cell growth inhibition. The tetramer CNNLP peptide was found to significantly inhibit lymphoblast cell growth [11]. The tumor growth inhibitory effect of CNNLP was universal towards 5 different tumor cell lines, however it was not effective on normal human dermal fibroblasts. The effect of the CNNLP peptide somehow differed from the original FasL, however it was confirmed that a cellular-stimulating peptide, which is active in a solid-supported form, could be identified by PI-ASPAC.

The extracellular domain of the human TRAIL protein was scanned for lymphoblast growth inhibitory domains by PIASPAC [12], and the octamer RNSCWSKD peptide was found to be most effective in induction of apoptosis. The peptide was further engineered to evolve by PIASPAC [Fig. (**4**)], and an arginine substitution to cysteine at the N-terminus resulted in a higher tumor-growth inhibitory effect by apoptosis [13]. These results show that cell-interacting peptide screening by PIASPAC can decipher functional peptide domains that might serve as candidate peptide drugs in soluble form. However, readers should be aware that the screened effect with the solid-bound form may be lost by cleaving peptides from the support in order to generate soluble peptides.

VI. SCREENING ENHANCEMENT BY BIOINFORMATICS

The largest disadvantage with PIASPAC is the library size limitation, since the number of spots is limited by the scale of conventional multi-well plates. However, Kaga *et al.* has reported that bioinformatics-assisted screens could compensate for such a disadvantage [8, 21].

Bioinformatics has commonly been used to analyze genome-related data, such as microarray gene expression data. The multi-parametric analysis strategy in bioinformatics is applicable to any type of data, including those derived from peptide array assays. Fortunately, peptide array data, which consist of both positive and negative data from one assay, is more suited to informatics analysis compared to the data from phage displays. Data that consist of both positive and negative quantitative data are known to build better analysis/prediction models than data with only positive results.

Kaga *et al.* has previously described the application of the PI-ASPAC method in combination with bioinformatics [8]. In this study, further explanation and additional details are described with illustration in Fig. (**5**). The strategy consists of 4 major steps to be repeated as cycles: (Step 1) Peptide array-based library screening, (Step 2) Construction of informatics models, (Step 3) Interpretation of modeling results, and (Step 4) Design of the peptide array.

In step 1, the first peptide array is designed from any type of peptide. Since step 1 is the same conventional peptide array screening strategy, the obtained positive sequences could be regarded as the candidate peptides for further investigation.

Step 2 links the interaction results to each peptide sequence from step 1. The data should be cleansed to eliminate medium-range peptides to consist of clearly positive or negative peptides. The informatics model automatically identifies the best numerical equations to predict peptide activity only from the peptide sequence information. In such a calculation, the comparative datasets (positives and negatives) are thoroughly examined to find the hidden rules that distinguish the positive peptides from negative peptides in the reaction. For example, if an N-terminal Arg is commonly found in the positive dataset, and an N-terminal asparagine is commonly found in negative dataset, the charge of the N-terminus is the important rule. The model obtained in this step could be used in further *in silico* peptide screening. To construct an informatics model, one way to numerically convert the sequence is to use indices that express the physicochemical properties of amino acids [Fig. (**6**)].

In step 3, the constructed model and its results are interpreted by the user. In this step, informatics models automatically select the most important information that relates to the peptide effect or activity. For example, in Fig. (**6**), the extracted rule is the combination of "a big hydrophobic amino acid at position 1 (P1)" and "a small amino acid at position 3 (P3)" as the most important factor to enhance the peptide effect. Such an indication rule is effective in reducing the variety of the next examination library by discarding "non-effective" amino acids from the substitution candidates. This process also increases the efficacy of gaining objective peptides, compared to the simple repetition of random screens.

In step 4, the next generation peptide array is designed according to the restriction rules determined in step 3. For example, if the rule is "P1 big charge", the substitution at position 1 is limited to only 3 amino acids (Arginine, Lysine, and Histidine).

These 4 steps could be repeated until a sufficient number of objective peptides are obtained. From our data, 2 cycles could provide sufficient screening efficiency.

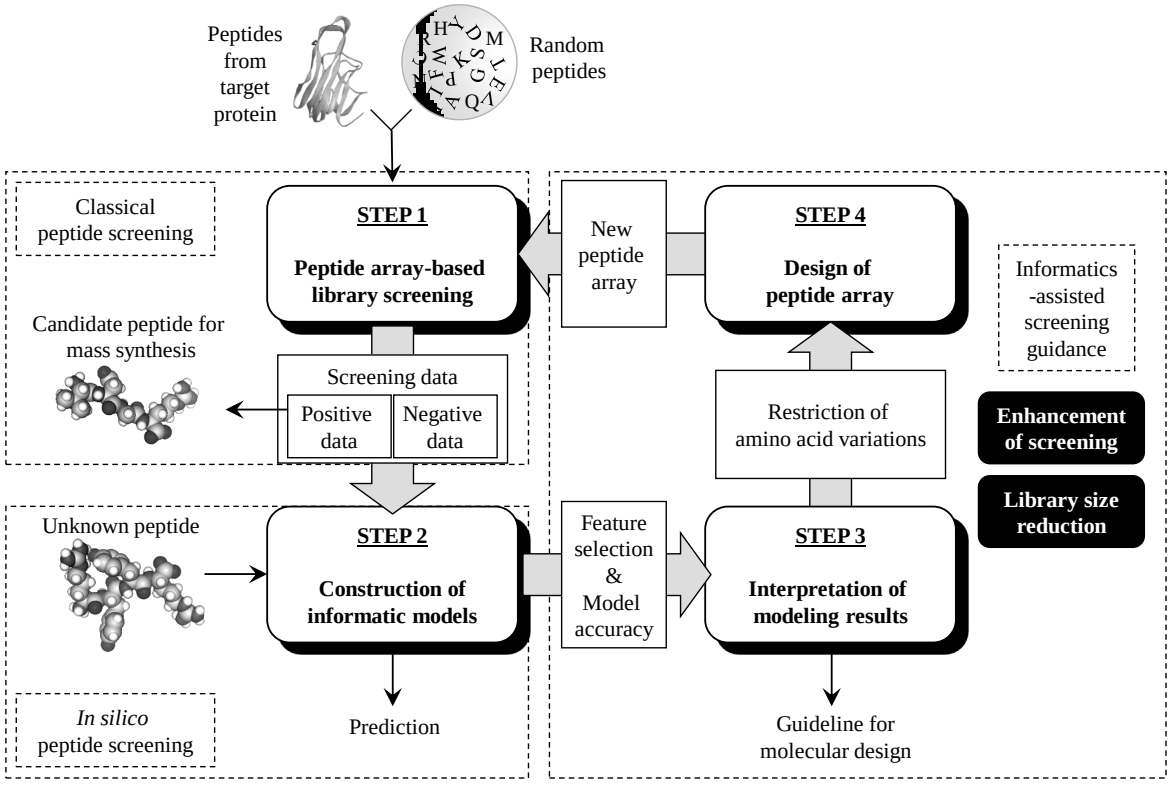

Fig. (5). Schematic diagram of informatics-assisted PIASPAC screening. The informatics assistance illustrated in steps 3 and 4 provides two advantages to the classical peptide screening with peptide arrays, the enhancement of screening efficiency and the effective reduction of the library size. By repeating the 4-step cycle, screening efficiency increases and labor decreases.

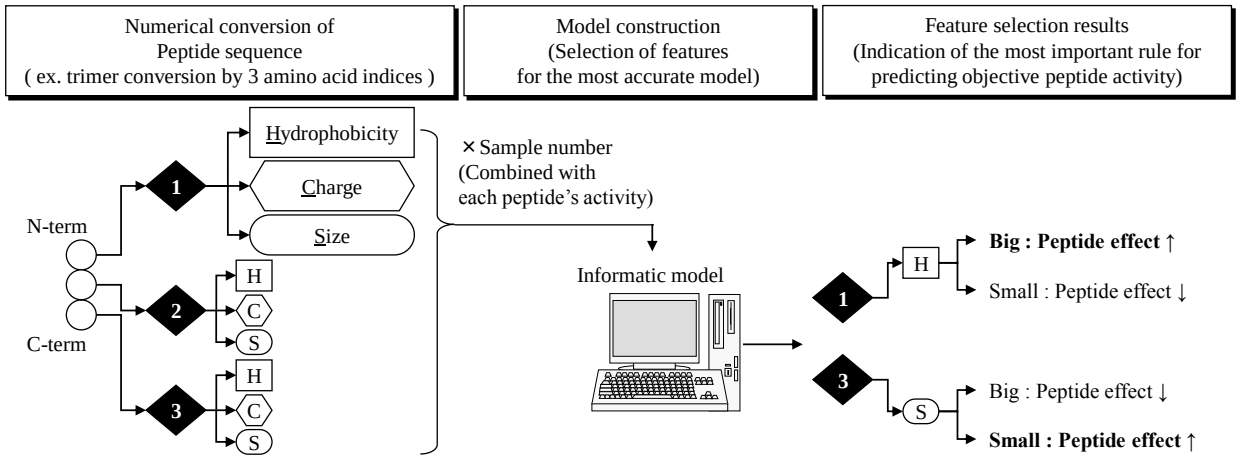

Fig. (6). Schematic diagram of the peptide sequence numerization step. The number in the black diamonds indicates the position from the N-terminus of the peptide. The numerically converted sequence information is combined with the assayed activity information to create one dataset. In the bioinformatic model construction process, the most important and commonly-found property in active peptides is selected automatically. In the example, the selected parameter combination of "P1 big hydrophobicity" and "P3 small size" is found.

To examine this informatics-assisted strategy, strong cell-adhesion peptides from total 5-mer peptide variations (20^5=32,000,000 peptides, impossible size to cover by array) were screened [8]. From the PIASPAC-based cell adhesion assay of 643 randomly selected 5-mer peptides, the first informatics model constructed from the selected best and worst 30 peptides provided the first rule [Fig. (**7**)**A**]. The colored cell in the rule matrix indicates the hidden peptide structure rule to distinguish cell-adhesion peptides from non-adhesion peptides. In the next round of screening, a second peptide array comprising 270 peptides (4-mer length, narrowed by the first rule) were assayed by PIASPAC. The second informatics model was constructed with the selected top and worst 20 peptides, and the second rule for the more affine cell-adhesion peptide was obtained [Fig. (**7**)**B**]. According to the second rule, the third array was designed to screen 50 new tetramers. To confirm the enhancement of total screening efficiency, the distribution of peptides was compared [Fig. (**7**)**C**]. The result strongly indicated that informatics-assisted screening, even if the library screening scale is decreased, drastically increased the efficiency of obtaining the objective peptide. It should be noted that the previously reported informatics model (fuzzy neural network) could be theoretically replaced by many other learning algorithms (multiple regression analysis, decision trees, etc.) that are commercially available in statistical analysis softwares (e.g., SPSS).

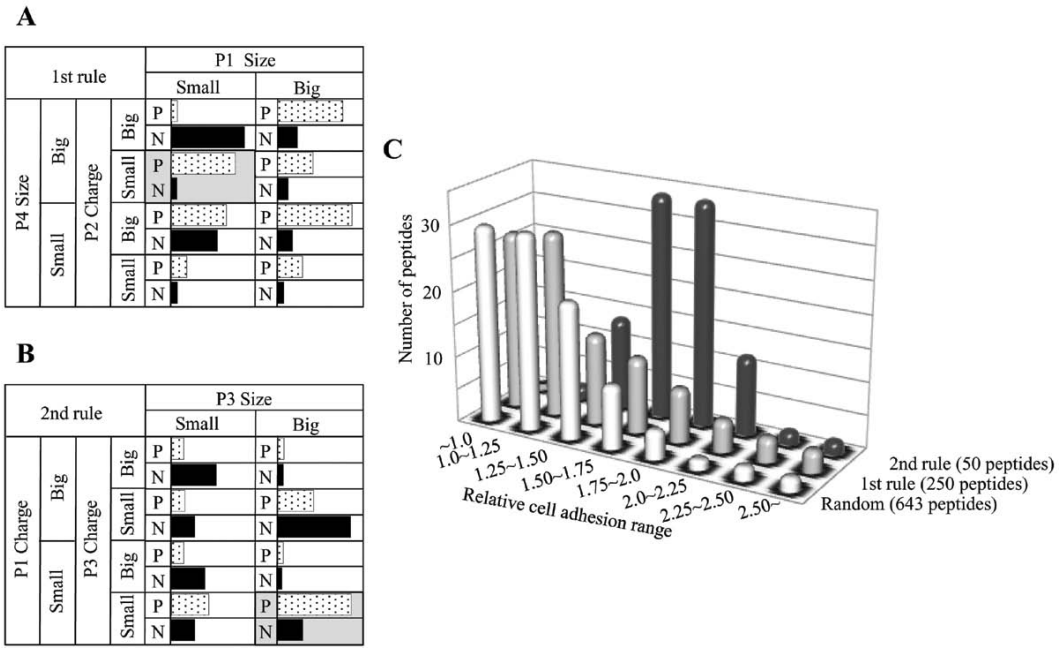

Fig. (7). Informatics-assisted cell-adhesion peptide screening from complete 5-mer variation (ref. 8). (**A**) The first rule extracted from the first model constructed by the top and worst 30 peptide data selected from a 643-peptide assay result. Each cell indicates the combination of parameters. The dotted bar in the cell indicates number of positive peptides that satisfy the rule, and the black bar indicates the number of negative peptides that satisty the rule. The gray-colored cell, which has more positives than negatives in the cell, is the best rule to be cell adhesive. (**B**) The second rule extracted from the second model constructed by the top and worst 20 peptide data selected from a 250-peptide assay result. (**C**) The screening efficacy comparison. Three peptide array results are compared by their cell-adhesion ratio distributions. White bars indicate 643 peptides randomly selected from 5-mer variations (20^5 = 32,000,000); gray bars indicate 250 peptides that followed the first rule in A; black bars indicate 50 peptides that followed the second rule in B. The distribution transferred drastically by following the obtained rule by informatics analysis.

VII. CONCLUSIONS

This chapter has outlined various applications of peptide arrays for peptide-cell interactions. With the varieties of confirmation data of PIASPAC method, this chapter also introduced bioinformatics-assisted screening strategy, which should greatly contribute to more peptide array-based works. It is expected that this review would inspire the development of further applications of peptide arrays in a biological context.

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