Polypyrrole-Peptide Microarray for Biomolecular Interaction Analysis by SPR Imaging.

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Abstract

Nowadays, high-throughput analysis of biological events is a great challenge which could take benefit of the recent development of microarray devices. The great potential of such technology is related to the availability of a chip bearing a large set of probes, stable and easy to obtain, and suitable for ligand binding detection. Here, we described a new method based on polypyrrole chemistry and allowing the covalent immobilization of peptides in a microarray format and on a gold surface compatible with the use of Surface Plasmon Resonance. This technique is then illustrated by the detection and characterization of antibodies induced by hepatitis C virus and present in patients' serums.

Key Words: Peptide chips, pyrrole, SPR imaging, antibody, hepatitis C virus

1. Introduction

Among the miniaturized devices used for biological analysis, chips bearing an array of peptides are of special interest. Indeed, peptide arrays open many applications such as enzyme substrate interaction measurement *(1)*, epitope mapping *(2, 3)*... (for reviews, see *(4, 5)*). Among them, the screening of antibodies in biological samples is an important topic related to the detection of infectious diseases and the monitoring of the evolution of the disease *(6-8)*. Beyond this application side, peptide arrays are very attractive in a chemical point of view since peptides are much shorter and more stable than proteins, suitable for the production of stable microarrays. Nevertheless, the stability of the device is dependent on a strong linkage between the peptides and the surface of the microarray, allowing the chip to be easily regenerated to perform high-throughput assays. Screening analyses require a detection method compatible with microarray format. Surface Plasmon Resonance imaging (SPRi) is a good alternative as it is an accurate one-step method for real-time measurement of ligand binding without labelling or previous purification *(9)*. This optical detection allows the monitoring of biological recognitions occurring on the surface of a biochip bearing spots containing different biomolecules. This is performed by following the change in the reflected light intensity induced by ligand binding (**Fig 1**). Moreover, the SPRi signal is correlated with the amount of ligand present in the injected solution *(10)*.

We describe in this chapter an approach combining the use of peptide chips and the SPRi. In order to be compatible with the gold layer used because of its optical properties, we have developed an electrochemical based process allowing the fast grafting of biomolecules into a matrix of polypyrrole: the biomolecule is firstly modified with a pyrrole moiety and then copolymerized with free pyrrole *(11)*. In the case of peptide chip, the pyrrole moiety is added on the N-extremity of each peptide to be arrayed before its copolymerization. The peptide chip is constructed on a glass prism covered with a thin gold layer and an application

example is given, dealing with the screening of serums from patients infected with hepatitis C Virus (HCV) for their content in anti-HCV antibodies.

2. Materials

2.1. Synthesis of N-(2-mercaptoethyl)-6-(1H-pyrrole-1-yl) hexanamide (= pyrrole-SH)

1. 2, 5-Dimethoxytetrahydrofuran, mixture of cis and trans isomers 98% (Sigma-Aldrich).

2. 6-aminocaproic acid 99+ % (Acros Organics).

3. Cysteamine 98% (Sigma-Aldrich).

4. 1, 4 dioxane anhydrous 99.8 % (Sigma-Aldrich).

5. Acetic acid puriss (Fluka).

6. N-Hydroxysuccinimide 98 % (Sigma-Aldrich).

7. N, N'-Dicyclohexylcarbodiimide (Fluka).

8. N, N-Dimethylformamide (DMF) for analysis (Carlo Erba).

9. Dichloromethane (CH_2Cl_2) puran (SdS).

10. Ethyl alcohol anhydrous (EtOH)), for analysis (Carlo Erba).

11. Chloroform-d, 99.9 % D, stabilized with 0.5 wt silver foil (Sigma-Aldrich).

12. Silicagel PF_{254} containing $CaSO_4$ for preparative layer chromatography

2.2. Preparation of pyrrolylated peptides

1. Dimethylsulfoxide (DMSO) analytical reagent (Prolabo)

2. Peptides modified with a maleimide group at their NH2 terminus (Neosystem, Strasbourg, France)

3. Reacting buffer: 50mM NaH_2PO_4, 50mM NaCl, 10% (p/v) glycerol, final pH adjusted at 6.8 with a NaOH solution. Store at 4°C (-20°C for a conservation longer than 2 weeks).

2.3. Immobilization of the peptides on the chip

1. 1M Pyrrole in acetonitril (Tokyo Kasei, Japan). Store at -20°C, in a dark bottle to minimize pyrrol oxydation.

2. 2mM Pyrrole-peptide conjugate (prepared as described in 3.2)

3. Reacting buffer: 50mM NaH_2PO_4, 50mM NaCl, 10% (p/v) glycerol, final pH adjusted at 6.8 with a NaOH solution. Store at 4°C (-20°C for a conservation longer than 2 weeks).

4. The chips consist in glass prisms coated with a 50nm-thick layer of gold (Genoptics, Orsay, France).

5. The microarrayer required for immobilizing the peptides on the chip is available commercially (Genoptics, Orsay, France). It mainly consists in an inox needle (260nm internal diameter) which is filled with the solution (6nL) containing the peptide to be grafted and which can move to a precise location on the chip. An electrical pulse between the needle (counter electrode) and the gold surface of the chip (working electrode) induces the synthesis of the polypyrrole film and its deposition on the gold surface.

2.4. SPRi interaction monitoring

1. Injection and washing buffer (PBS): 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl, 138mM NaCl, pH 7.2. Store at 4°C.

2. Saturation buffer: Bovin Serum Albumin (BSA, Sigma-Aldrich) 1% (w/v) in PBS.

3. Regeneration buffers:

    a) 0.1M Glycine, final pH adjusted at 2.3 with a HCl solution. Store at 4°C.

    b) 1 % (w/v) Sodium Dodecyl Sulfate (SDS, Sigma-Aldrich) in water. Store at room temperature.

4. The SPRi apparatus is a SPRiPlex (Genoptics, Orsay, France). The prism is inserted in the apparatus and a flow cell (6µL) inside the instrument is hermetically sealed over the prism. A light source (660nm) illuminates the prism and a CCD camera monitors the ligand-probe interactions. Data are analysed with dedicated software (SPRi-view).

3. Methods

3.1. Synthesis of N-(2-mercaptoethyl)-6-(1H-pyrrole-1-yl) hexanamide (= pyrrole-SH)

1. A mixture of 2, 5- dimethoxytetrahydrofuran (0.49mol, 65ml), 6-aminocaproic acid (0.43mol, 56g), acetic acid (450ml), and dioxane (600ml) is heated under reflux for 4h and stirred at room temperature overnight *(12)*.

2. Remove the volatiles under reduced pressure. The residue is coevaporated in ethanol (2x100ml) to eliminate acetic acid.

3. Dissolve the residue in 500ml of dichloromethane. This organic solution is washed with 2x250ml of water and evaporated to dryness.

    Product 1 (**Fig 2**) is obtained with a yield of 82% after purification by flash chromatography on a silicagel (600g) column with a discontinuous gradient of ethanol in CH_2Cl_2. After deposition in the minimum amount of CH_2Cl_2, the elution starts with CH_2Cl_2 (500ml), then CH_2Cl_2/EtOH: 98/2 (300ml), CH_2Cl_2/EtOH: 95/5 (400ml) and finally CH_2Cl_2/EtOH: 90/10 to elute the product. Product 1 is obtained after evaporation of the solvents as a brownish oil.

    M. S. product 1 (m/z) = 182.1 (M+).

1H-NMR product 1 (200MHz ;CDCl$_3$) δ(ppm) :1.72 (m, 6H, -CH$_2$-(CH$_2$)$_3$-CH$_2$-); 2.34 (t, 2H, -CH$_2$-COOH); 3.87 (t, 2H, CH$_2$-N); 6.13 (dd, 2H, 3-H and 4-H pyrrole); 6.64 (dd, 2H, 2-H and 5-H pyrrole).

4. Product 1 (144mmol, 26.05g), N-hydroxysuccinimide (144mmol, 16.56g) and N, N'-Dicyclohexylcarbodiimide (159mmol, 32.75g) are dissolved in DMF (1l) and stirred overnight at room temperature.

5. Eliminate the white precipitate of N,N'-dicyclohexylurea by filtration on a sinter glass.

6. Remove the solvent by evaporation on a rotavapor. Product 2 can be used without further purification. Alternatively it can be purified on a silicagel column in the same way than product 1 (elution CH$_2$Cl$_2$/EtOH 95/5).

M. S. product 2 (m/z) = 279.3 (**Fig. 2**)

1H-NMR product 2 (200MHz;CDCl$_3$) δ(ppm):1.72 (m, 6H, -CH$_2$-(CH$_2$)3-CH$_2$-); 2.33 (t, 2H, -CH$_2$-CO); 2.88 (tt, 4H, CH$_2$ of NHS); 3.87 (t, 2H, CH$_2$-N); 6.13 (dd, 2H, 3-H and 4-H pyrrole); 6.64 (dd, 2H, 2-H and 5-H pyrrole).

7. Product 2 (5mmol, 1.89g) and cysteamine (6mmol, 463mg) are dissolved in DMF and stirred overnight at room temperature.

8. Evaporate the solvent and dissolve the residue in 50ml of dichloromethane. This organic solution is washed by 2x50 ml of water and evaporated to dryness. The oily residue is taken off in 5 ml of dichloromethane and filtered on a small column of silicagel. After elution and evaporation of the solvent the product is obtained as pale yellowish oil with a yield of 76%.

M. S. product 3 (m/z) = 266.1 (M+) (**Fig.2**).

1H-NMR product 3 (200MHz ;CDCl$_3$) δ(ppm) :1.72 (m, 6H, -CH$_2$-(CH$_2$)$_3$-CH$_2$-); 2.17 (t, 2H, -CH$_2$-CONH spacer); 2.68 (t,2H, CH$_2$-SH cysteamine) ; 3.53 (t,2H, CH$_2$-N

cysteamine); 3.87 (t, 2H, **CH₂**-N spacer); 6.13 (dd, 2H, 3-**H** and 4-**H** pyrrole); 6.64 (dd, 2H, 2-**H** and 5-**H** pyrrole).

3.2. Preparation of pyrrolylated peptides (Fig.3A) (*see* **note 1**)

1. Each peptide-maleimide are solubilized in DMSO at 5mM concentration.

2. Prepare the reacting solution by mixing 40µl of 5mM peptide-maleimide with 10µl of 0.2M pyrrole-SH and complete to 100µL final volume with reacting buffer. These conditions lead to a 2mM final concentration of peptide-maleimide with a molar ratio peptide / pyrrole-SH = 10 (*see* **note 2**).

3. Incubate 2h at room temperature, under stirring and in the dark to minimize pyrrole oxidation. Store at -20°C.

3.3. Immobilization of the peptides on the chip ("electrospotting")

All solutions are prepared extemporaneously.

1. Prepare a 40mM pyrrole solution by diluting 20µL of the 1M stock solution with 480µL of reacting buffer.

2. Prepare a 200µM pyrrolylated peptide solution by diluting 5µL of the 2mM solution with 45µL of reacting buffer.

3. Prepare the spotting solution in a 96 U well-plate by mixing 15µL of the 200µM pyrrolylated peptide solution with 15µl of the 40mM pyrrole solution. Final concentration for pyrrole-peptide and free pyrrole are 100µM and 20mM respectively (*see* **note 3**), in a 30µl final volume (*see* **note 4**). Be careful to ensure a good homogeneisation of the spotting solution without introducing air bubbles (*see* **note 5**).

4. Immediately put the plate containing the spotting solutions in the microarrayer and carry out the spotting on the chip. The spotting conditions consist in a 2V electrical pulse for 100ms (*see* **note 6**) on defined areas of the gold prism (**Fig. 4**).

5. When all peptide spots have been realized (*see* **note 7 and 8**), the chip is removed from the microarrayer, rinsed with water and gently dried with an argon or air jet. Chip is kept dry, at room temperature.

3.4. SPRi interaction monitoring

1. Insert the chip into the SPRiPlex and start the apparatus. The conditions are the following: flow rate = 37.5µL/min, room temperature, injection loop = 500µL, working buffer = PBS. Be careful to avoid air bubbles which can be eliminated by flushing PBS through the circuit (*see* **note 9**).

2. Saturate the chip by injecting PBS-BSA saturation buffer during 10min (*see* **note 10**).

3. Rinse the chip with PBS during 10min.

4. Inject regeneration buffer during 5min to stabilize and condition the chip according to the following tests (*see* **note 11**).

5. Rinse the chip with PBS during 10min. The chip is ready for sample injections. Each analysis includes the following steps:

   - injection of the sample diluted in PBS (*see* **note 12**) during 10min

   - washing with PBS during 10min

   - regeneration with 0.1M Glycine pH2.3 during 5min

   - washing with PBS during 10min

6. After a large number of analyses, depending on the complexity of the samples (*see* **note 13**), it is possible that the chip becomes less responsive. In this case, it could be useful to inject 1 % SDS in water during 5min, followed by PBS during 15min.

3.5. Application to antibody detection

Detection and analysis (quantitative and qualitative) of antibodies in serum are crucial not only to diagnose a disease, but also to evaluate its developmental stage and its evolution. SPRi technology allows high-throughput analyses in real time using a label-free detection process suitable for screening a large number of analytes. This technology requires the construction of biochips. The approach described here is based on electropolymerization of pyrrole and presents several advantages: easy and rapid preparation, versatility, high reproducibility, control of the density of immobilized probes. Moreover, the immobilization process used to prepare the peptide chips leads to a low background, making them suitable for the analysis of complex biological samples such as serums.

Recently, this technology has been used to analyse the humoral response induced by hepatitis C virus (HCV). A set of 20 aa-peptides (62) has been grafted in triplicates on two chips as described above and 45 serums from patients with HCV infection were injected (1/50 dilution, in PBS). Representative sensorgrams are shown in **Fig.5**. The histograms in **Fig.6** show the corresponding final results, and represent the specific SPRi signal obtained for each tested serum. Thus, this method enabled us to sketch an antibody profile for each of the patients, which is of great importance in the clinical field.

Other applications can take benefit from this technology, from epitope mapping to analysis of enzyme-substrate interaction, making it a powerful tool for both basic research and clinical applications such as diagnosis and pharmacological studies.

4. Notes

1. A more convenient but more expensive way to obtain peptide-pyrrole conjugates consists in getting it already synthesized by the manufacturer (Altergen, Bischheim,

France)(**Fig.3B**). The great advantage is that 100% of the peptide put in the spotting solution are pyrrolylated instead of 20-40% for home made conjugates, depending on the yield of the reaction between peptide-maleimide and pyrrole-SH.

2. An initial molar ratio (IMR) peptide/pyrrole-SH of 10 leads to a good coupling reaction between the two molecules. Nevertheless, it is recommended to test several IMR (5, 10, 20, 40) to optimize the coupling conditions.

3. Different final peptide concentrations (50, 100, 200, 500µM) have to be prepared to determine the optimum leading to the best reactivity with the tested ligands. This point is important as the coupling yield (usually between 20-40%) could differ from one peptide to another. In all cases, a 20mM final concentration of free pyrrole is required.

4. The final volume is not crucial as the volume required for the electrodeposition is in the nL range. Nevertheless, as the samples could remain a long time in the plate before being electrodeposited, depending on the number of the spots to be realised, a significant evaporation must be feared if sample volume is too small.

5. The presence of air bubbles in the sampling needle introduces a defect in the electropolymerization (interruption of the electrical current).

6. These conditions lead to a 2-5nm thick polypyrrole film. Different pulse durations can be tested (50, 100, 200ms) to optimize the electrodeposition. It is worth to notice that a too long pulse and/or a too important voltage could lead to a thicker polypyrrole film. Moreover, it could induce a depletion of pyrrole molecules as well as an hyperoxydation of the pyrrole and consequently, a degradation of the film.

7. It is recommended to realize the spots in triplicates to improve the quality of the results. In this case, it is better to set the three identical spots perpendicularly to the flow direction in the SPRiPlex, to avoid a potential ligand depletion between the first and the third spot, as it could occurs if the three spots are parallel to the flux.

8. To quantify the background level, control spots including irrelevant peptides and/or polypyrrole alone have to be performed.

9. It could be useful to degas the PBS solution either before its use or by including a degasser in the fluid circuit.

10. The choice of the regeneration buffer depends on the interaction type between probes and ligands. Glycine is convenient for pH-dependent interactions such as antigen-antibody binding. Other solutions can be used, for example buffers containing chelator agent (EDTA, EGTA) for metallo-dependent interactions. Different regeneration solutions are proposed in *(13)*

11. If complex samples as serum or cell culture medium are injected, it is better as much as possible to saturate the chip using a control medium equivalent to the samples, but without the tested ligands (serum from healthy donors versus serum from patients, culture medium versus culture supernatant).

12. In the case of high diluted ligand, it is better to use PBS-0.1% BSA as dilution buffer to avoid non specific adsorption on the tube. In the same way, the use of silicone-treated tubes is recommended.

13. It is important to verify the stability of the chip upon the successive injection cycles and the SDS treatment. To this end, a standard solution containing the ligand for one of the grafted peptides is injected at the beginning of the experiment and then at regular intervals; the SPR signals obtained must be very similar from one control to the other.

5. References

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Figure legends

Fig. 1: Detection of ligand binding by SPRi: sensorgram obtained on two spots (control
and assay) upon ligand injection.

Fig. 2: Structures of the intermediates (products 1 and 2) and final product (3) for N-(2-mercaptoethyl)-6-(1H-pyrrole-1-yl) hexanamide synthesis (= pyrrole-SH).

Fig. 3: Pyrrole-peptide conjugates. A) Schematic representation of the coupling reaction between maleimide functional group at the N-Ter of the peptide and thiol group in the pyrrole to form a thioether linkage. B) Structure of pyrrole-peptide conjugates provided by Altergen.

Fig. 4: General scheme of the peptide addressing on a glass prism coated with gold. Different pyrrole-peptide conjugates and pyrrole monomer solutions are in the 96 well plate. The "electrospotting" is carried out on the gold surface via the needle containing the solution to be copolymerized.

Fig. 5: Sensorgrams obtained after injection of serums from 4 patients (P1-P4) and two healthy donors (H1 and H2). The serums (1/50 dilution in PBS) were injected on a chip bearing 5 HCV peptides and Ova 273-288 as a control, with a regeneration step between each injections.

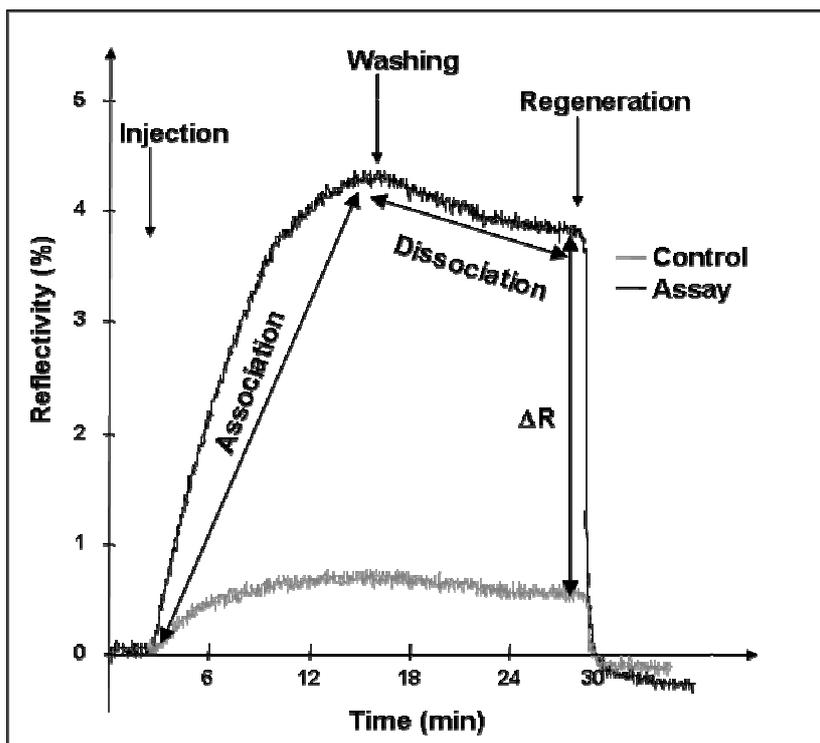Fig. 6: SPRi signal obtained for each serum in the conditions described in Fig. 5. $\square$R is determined as in Fig.1)
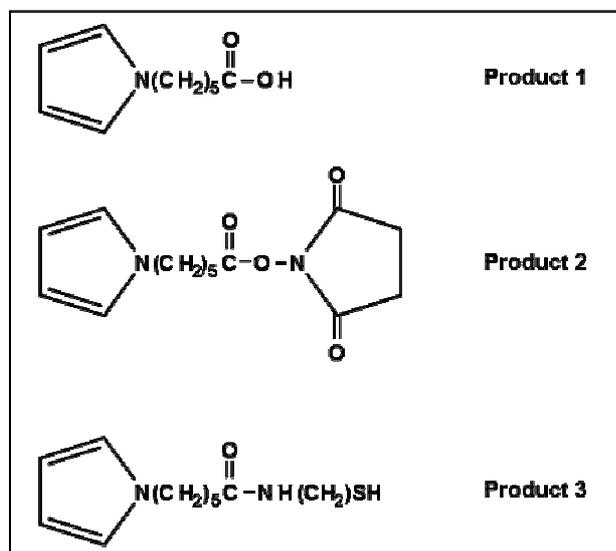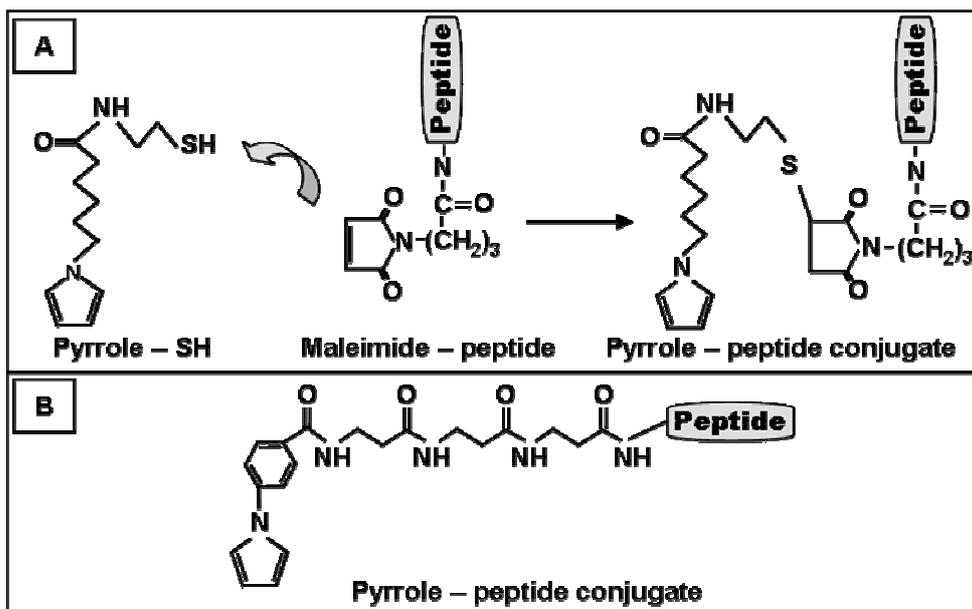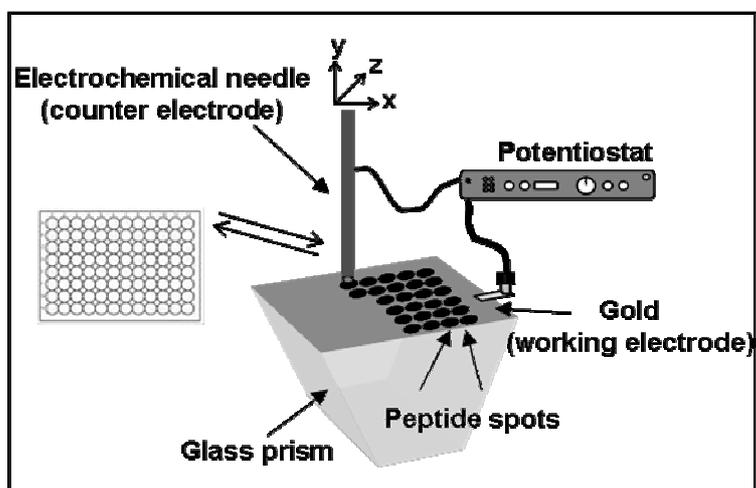
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6