Integrated Method of *in Situ* Cell Free Synthesis of a Protein Array on Gold Surface and Real Time Kinetic Monitoring by SPR Imaging

Javier Batista Pérez, Amita Nand, and Zhu Jinsong

***Abstract*—Protein microarrays represent a promising alternative in proteomics and in the biomedical industry. The widespread use of this technology has been limited, largely due to the labor intensive protein production, the quality of proteins expressed in different systems, and the shelf life of these arrays. The novel method Nucleic Acid Programmable Protein Array (NAPPA) overcomes these limitations by synthesizing the proteins *in situ*. NAPPA entails spotting plasmid DNA encoding the relevant proteins, which are then simultaneously transcribed and translated by a cell free system. NAPPA assays are based on fluorescence detection, which need a protein labeled, often *in-situ* or by fluorescent tag, and can't give real time information or kinetics. An attractive alternative to traditional fluorescence-based microarrays detection methods is the technique of surface plasmon resonance imaging (SPRi). SPRi brings a significant advantage in the analysis of biological samples where labeling multiple biomarker with fluorophores or nanoparticles is not practical. A new surface chemistry is developed to carry out an *in-situ* and cell free synthesis of an SPR compatible protein array. The DNA which encodes the P53-GST-(e-coil) fusion protein is arrayed on the gold sensor surface and through the expression with cell lysate extract the corresponding protein array is obtained. Both the expression process and the posterior characterization are implemented on the flow cell covering the sensor chip. The kinetic interaction of the fusion proteins with the specific antibody anti-P53 was analyzed.**

***Index Terms*—Cell free, *in-situ*, protein array, SPRi**

I. Introduction

Microarray biosensors have become a valuable tool for the rapid, multiplexed detection of surface bioaffinity interactions. Many researchers are interested in developing protein microarrays with applications in the areas of proteomics [1]-[3] and drug discovery [4], [5]. In addition, the detection and the profiling of multiple protein biomarkers in biological fluids (e.g. blood, serum, urine) by antibody microarrays is a potentially powerful method for the diagnosis of diseases and monitoring subsequent therapeutic treatments [6], [7]. In order to build protein microarrays, one needs the content to be spotted on the array and an appropriate surface chemistry to capture the protein. These components must be optimized to produce and provide proteins of good integrity and stability. The goal is to preserve the functionality of the protein in order to minimize false negatives. The widespread use of functional protein microarrays has remained limited, largely due to the intensive striving requisite to synthesize the proteins, to the quality of the synthesized proteins in heterogeneous systems, and the stability of the proteins during storage. A novel method for producing protein microarrays, called Nucleic Acid Programmable Protein Array (NAPPA) [8], [9], overcomes these limitations by synthesizing proteins *in situ*. NAPPA entails spotting plasmid DNA encoding the relevant proteins, which then are simultaneously transcribed and translated by a cell free system. The expressed proteins are captured and oriented at the location of the expression by a capture reagent that targets a fusion protein towards either the N- or C-terminus of the protein. The traditional detection system used for NAPPA has been based on the fluorescence detection, which needs labeled binders and cannot give real time information or kinetics. An attractive alternative to this methodology is the surface-sensitive optical technique of Surface Plasmon Resonance imaging (SPRi) [10]-[12]. This research aims at obtaining cell free *in-situ* synthesized protein arrays compatible with SPRi. To achieve this goal, it is necessary to redesign the NAPPA chemistry from glass based chips to gold based chips. When we use SPRi, it is possible to make a serial binding experiment with a single protein, which allows us, for instance, to study the interaction between the proteins arrayed on a single chip with different samples. It would be convenient that the capturing agent is covalently attached to the gold surface, so that it can stand regeneration cycles. The strategy is to use a coiled peptide k-coil covalently attached to the gold surface which is playing a dual role: to fix the DNA molecules [13] and to capture the *in-situ* synthesized fusion protein which contains a complementary peptide e-coil that has a specific and tight interaction with the k-coil [14].

II. Materials

The k-coil peptide (KVSALKEKVSALKEKVSALKEKVSALKEKVSALKEnLGGGC) was purchased at SBS (Beijing, China). The p53-GST-[e-coil (EVSALEKEVSALEKEVSALEKEVSALEKEVSALEKnLGGGC)] Plasmid DNA (Fig. 1) was kindly donated by Joshua Labear from Harvard Institute of Proteomics, Biotinylated p53-GST-(e-coil) plasmid DNA (bDNA): prepared at our lab using the original p53-GST-(e-coil) plasmid DNA as described [15]-[18]. DTSSP, Streptavadin

Manuscript received Jun 12, 2012; revised July 23, 2012.

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(S.A.) and NaOH were purchased from Sigma Aldrich. Methionine and rabbit cell lysate were purchased from Promega. RNAase free H_2O was purchased from Takara (Japan). Polyclonal anti-GST and GST protein were purchased from at GE Healthcare. The monoclonal anti-p53 antibody was purchased from RayBiotech, Inc. PBS was purchased from Solarbio (Beijing, China).

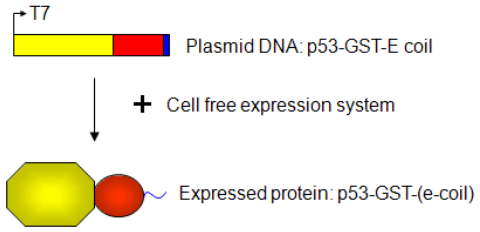


Fig. 1. Plasmid DNA and correspond fusion protein structures.

III. Chip Fabrication

Two methods were used to express the target proteins (Fig. 2): The first one employed the streptavidin-biotin interaction. DTSSP is used as a cross linker to fix the streptavidin to the gold surface the biotinylated plasmid DNA is bound to the streptavidin, and the k-coil peptide is printed in order to capture the target fusion protein when the expression process takes place [14]. The second method uses the k-coil peptide and unmodified plasmid DNA. Here the k-coil is bound to the gold surface by the thiol group of its C-terminal cysteine; the DNA binds via electrostatic interactions with the α-helical of the k-coil peptide [13]. Consequently the "free" k-coil molecule can be bound to the target fusion protein when the expression process is taking place.

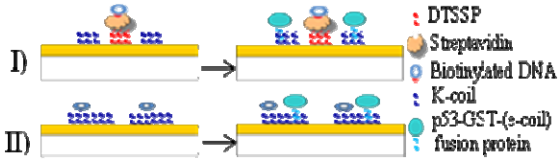


Fig. 2. Methods employed for *in-situ* protein expression.

Two printing solutions were prepared, one following the method I and the other one following the method II. Then the gold slides were washed for 30 minutes with 1x PBS, 5 minutes with ddH_2O and dried with N_2. The printing solutions are spotted manually, (approximately 0.3 µL each spot). The first slide was printed with these two printing solution (each solution in different spots). The second slide was printed with the printing solution correspond to the method II, with a positive control spot which contain GST protein and negative control spot with contain the DNA alone. After that, non fat milk 5% solution is used to block the chip overnight at 4 ^{0}C.

IV. Expression

Before the expression, both chips are washed with 1X PBS and rinsed and dried respectively with water and N_2. Then, the flow cell is assembled and washed 3 times with 100 µL of RNAase free water. At this point, the cell free expression system is added to the flow cell and the latter is placed in Plexarray^{TM} Kx5 (Plexera LLC, U.S). The temperature is set to 30 ^{0}C for 1.5 hr and then set to 15 ^{0}C for 30 min. After the expression is finished, the temperature is increasing to 25 ^{0}C and the cell free expression system is washed away from the flow cell by rinsing with 1X PBS running buffer. Then, the optimal SPR angle, in the lineal region of the SPR curve, is determined. Next, 3 regeneration steps, with NaOH 10mM, are carried out in order to remove all the unbound molecules from the sensor surface which could interfere with the subsequent specific interaction with the complementary biomolecule of interest.

V. Detection Method

At this point, a calibration is performed with 2*X* PBS, followed by a binding with the corresponding antibodies and a regeneration of the sensor surface after each binding event with NaOH 10mM. The data obtained was processed with Microsoft Office Excel 2007. First of all, the calibration factor is computed through the change in the intensity of the baseline of all the curves when the chip is in 1*X* PBS and 2*X* PBS. This change is in arbitrary units (*a.u*), based on the fact that the change between PBS 1*X* and PBS 2*X* in RIU is 0.00154 [19]. Therefore the calibration factor becomes:

$C_f=0.00154/\Delta I_{2X\text{-}1X}(a.u)$

We can then convert our data into *RIU* units using the following formula:

$I(RIU)=C_f*I(a.u)$

All the data are corrected following this procedure. Each ROI having one C_f. Once the data is already in RIU, the curve of all the references ROI is then obtained and subtracted from the other ROIs data. The final data are adjusted to Plexera SPR Data Analysis Module software input format, and their kinetic behavior is analyzed.

The fusion protein is fixed via the e-coil tag to the sensor surface via the previously fixed k-coil peptide. GST tag or the P53 protein can be detected with anti-GST and anti-p53 antibodies (Fig. 3).

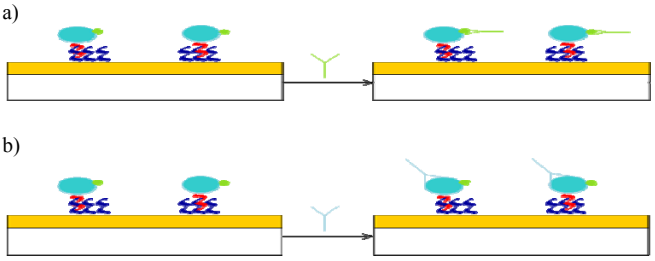


Fig. 3. Detection of the expressed protein: a) by recognition of the fusion protein GST with the anti-GST antibody and b) by recognition of the p53 protein with the anti-p53 antibody.

All process, from expression to specific recognition binding between *in-situ* synthesized proteins and its specific antibodies are monitoring by SPRi using the Plexarray^{TM} Kx5.

VI. Results and Discussion

After expression on the first slide, two kinds of spots are distinguishable (data not shown): the brightest one corresponds to the DNA+*k*-coil printing chemistry while the others correspond to the biotinylated

DNA+streptavidin+DTSSP+*k*-coil printing chemistry. It seems that the DNA+*k*-coil spots capture more amount of expressed protein, which could be explained by the fact that this chemistry is friendlier for the expression system. The spot with the DNA+*k*-coil chemistry shows a better binding than the other spots (Fig. 4). This confirms the idea that the DNA+*k*-coil chemistry is more adequate perhaps because the DTSSP cross linker can damage the DNA and reduce the efficiency of the expression.

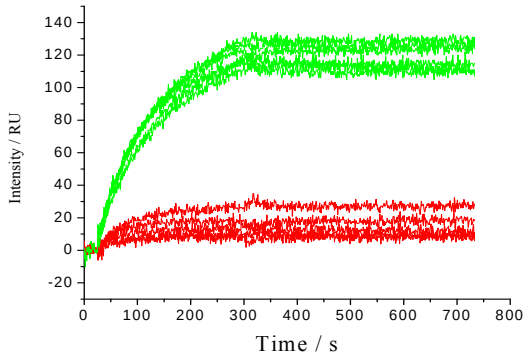
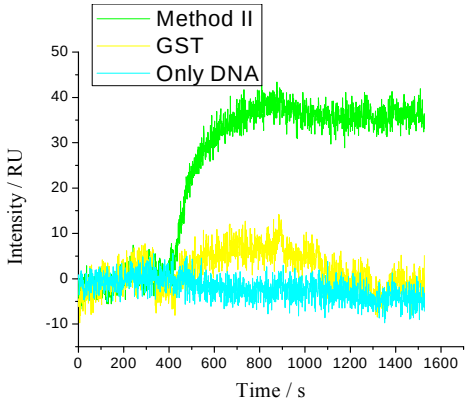


Fig. 4. Binding with the anti-GST 133nM, the red lines represent the spots which contain printing solution following method I while the green lines correspond to printing solution following method II.

As for the second slide, the same procedure is followed. When the anti-p53 (12 µg/mL) was used as analyte, all spots, except for the control spots which contain either GST protein or only DNA, showed binding (Fig. 5). On the other hand when the anti-GST antibody is used, the spots with DNA+*k*-coil also show binding (Fig. 5).

a)



b)

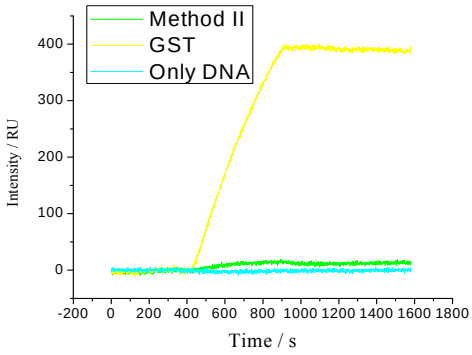


Fig. 5. Binding with anti-P53 and anti-GST antibodies, a) anti-P53 80nM, b) anti-GST 80nM.

Although these bindings are 40 times weaker than the binding shown by the positive control spot with GST, still represents a specific binding. The spot with DNA alone does not have any specific binding with neither anti-p53 nor anti-GST antibodies.

The k_a, k_d, K_D of binding with the monoclonal anti-p53 are calculate by Langmuir model for DNA+*k*-coil spots (Fig. 6), all constants are in the range of antibody-antigen interaction.

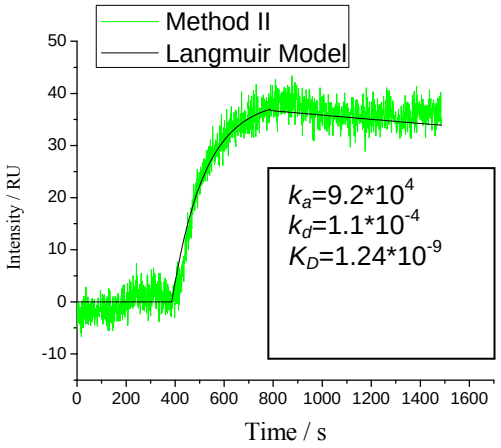


Fig. 6. Kinetic of binding with anti-p53 antibody.

VII. Summary

A new chemistry is employed to produce *in-situ* cell free synthesized protein array on gold surface. This *in-situ* chemistry is based in the fat that k-coil can capture both DNA and *in-situ* synthesized proteins. This chemistry is simpler than other reports [20], because no prior modification of the bare gold surface is required. The microarrays produced by this method can be analyzed by SPRi. The kinetics parameters of the binding between the *in-situ* synthesized proteins and the monoclonal anti-p53 antibody (which recognize the e-coil tag of this protein) were determined. The k_a, k_d, and K_D obtained are in the order of antibody-antigen interaction.

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