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Development of a tRNA-Synthetase Microarray for Protein Analysis

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Proteins are composed of 20 different amino acids. In the translation process, each of these 20 amino acids is specifically recognized by their cognate aminoacyl-tRNA synthetase. The fidelity of this recognition system is essential if translation is to function properly. The development of an *in vitro* system based on this recognition scheme would make a powerful analytical tool with which to analyse translation, as well as providing an additional biomimetic scheme for protein analysis. Aminoacyl-tRNA synthetases microarrays could be applied to protein fingerprinting and sequence analysis. The fabrication of aminoacyl-tRNA synthetase arrays requires the use of advanced protein arraying technology that has only recently become available. In order to demonstrate the feasibility of this scheme, glutamyl-tRNA synthetase (GluRS) was immobilized on the streptavidin-based *XNA on Gold™* biochip platform. The streptavidin layer provides a simple, efficient immobilization scheme that reduces nonspecific binding and improves the biocompatibility of the surface. Here, we demonstrate that biotinylated GluRS can be successfully immobilized on *XNA on Gold™*. The immobilization efficiency was determined by double labelling GluRS with biotin and the fluorescent label Cy5. The CCD fluorescent microscopy images revealed that the GluRS was efficiently immobilized and evenly distributed over the surface. Control experiments indicate a very low degree of nonspecific binding which is essential if detection of these multicomponent, low-affinity interactions is to be realized. Furthermore, we show that immobilization does not significantly reduce the function of the enzyme. In addition to the specific aims of this study, this technology would provide valuable insights into the biomechanics of translation as well as being a tool for studying tRNA modifications and subclasses. Moreover, the implications for developing coupled transcription and translation systems should not be overlooked. Protein

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(The color pictures can be obtained from the author.)

analysis schemes based on this approach would provide an urgently needed compliment to traditional methods. Finally, these arrays might also be useful tools in our efforts to understand the regulatory functions that small RNAs, i.e., iRNA, have been shown to play.

1. Introduction

At present, over 150 complete genomes have been sequenced.[1] Sequencing a genome is but the first step in understanding an organism, and in retrospect, arguably the simplest. Current estimates put the number of genes in the human genome at about 30,000. This should correspond to 30,000 proteins and, in the case of simpler organisms, this does hold true. However, in the case of higher organisms the number of functionally unique proteins is several-fold higher than the number of genes. Thus, from a single gene, a number of functionally distinct variants can be synthesized by a variety of different mechanisms such as alternative RNA splicing, post-translational modifications, as well as through other regulatory mechanisms.[2] The genome merely provides a 'blueprint' for life, not life itself. It is the translation of this genetic information into a myriad of biomolecules that actually constitutes life. Each biomolecule, be it a nucleic acid, protein, carbohydrate or lipid, has a specific function to perform. It is this cascade of interactions that constitutes life. In order to understand this process, the involvement of each biomolecule must be understood and assigned a function. The term functional genomics has become the mantra of the post-genomic era. That is to say, we have the blueprint, now how do we go about making sense of this massive amount of genetic information?

One approach has been to simply apply the high-throughput (HTP) strategies and technologies that proved advantageous in DNA sequencing to other classes of biomolecules. In the case of proteins, the vast majority of de-novo protein sequencing has been performed using Edman degradation combined with proteolytic degradation.[3] Many HTP strategies have been successfully applied to these methods in order to increase their throughput. These have become important drug screening tools for the pharmaceutical industry since the majority of drug target are proteins. While these methods get the job done, they do have a number of drawbacks, and it is for this reason that a number of alternative protein sequencing technologies have been developed. These are almost exclusively based on mass spectrometry.[4] This approach has many advantages including good sensitivity, the ability to identify post-translational modifications, and adaptability for HTP analysis. However, the technique is limited in a functional sense since it only addresses chemical composition issues. In order to address functional issues, mass spectrometry must be combined with other techniques. At present, *in vitro* analytical techniques capable of physically and functionally characterizing proteins are sorely needed.

Here, we describe our initial efforts to develop a biomimetic sensor based on arrays of immobilized aminoacyl-tRNA synthatases (aaRS). The scheme essentially attempts to mimic a small part of the translation process, much akin to the way PCR mimics DNA replication. The universality of aaRS recognition in the translation process makes it reasonable to assume that the interactions involved are sufficiently robust to allow the

development of an *in vitro* system. As the first step in this process, glutamyl-tRNA synthetase (GluRS) was immobilized. Immobilization and detection of protein was tested using the *XNA on Gold*™ microarray platform.[5] The unique microarray platform employs a streptavidin-based immobilization scheme, which provides a fast and simple way of coupling GluRS. The GluRS was coimmobilized with its cognate tRNA on the array. The presence of the immobilized GluRS was verified by labelling with the fluorescent dye Cy5.

2. Concept

The immobilization and sensing scheme is shown in Fig. 1. In translation, proteins are synthesized using 20 amino acids. Each amino acid has one specific synthetase and one or more isoaccepting tRNAs. Each aminoacyl-tRNA synthetase catalyses the addition of a specific amino acid to a specific tRNA to form aminoacyl-tRNA with a high degree of accuracy. Generally, aminoacylation is carried out in two steps.[6] In the first step, the aaRS activates the amino acid using ATP and Mg^{2+} to yield the aminoacyl-adenylate (aa-AMP). In the second step, the aminoacyl moiety is transferred from the aa-AMP to the 3'-terminal adenosine of tRNA to form the aminoacyl-tRNA. The overall reaction scheme is as follows.

$$AA + tRNA + ATP + MG^{2+} \xleftrightarrow{aaRS} AA - tRNA + AMP + Ppi \quad (1)$$

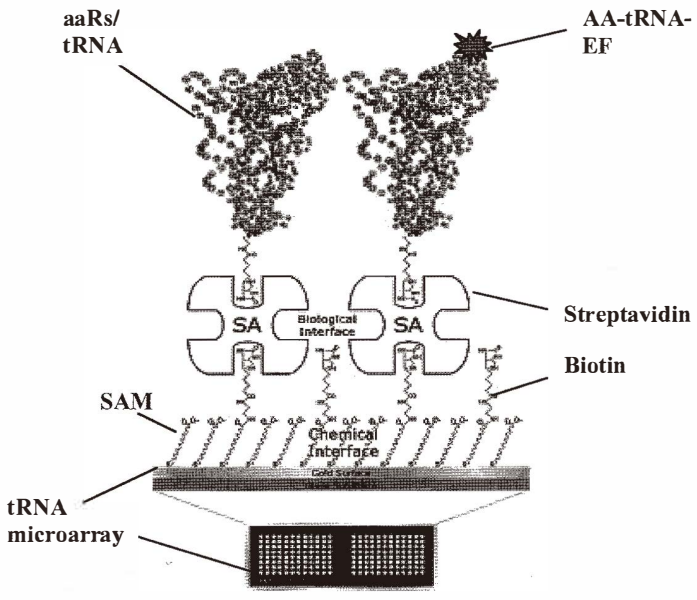


Fig. 1. Schematic of *XNA* on *Gold*™ immobilization and tRNA sensing strategy.

From studies of the translation process, it is known that elongation factor (EF) specifically binds to all amino-acid-charged tRNAs with high affinity and very poorly to uncharged tRNAs. On the basis of this interaction, it should be possible to develop a ligand assay capable of monitoring the aminoacylation reactions catalyzed by the synthetases. This would make it possible to identify an amino acid in a simple ligand binding assay. Using arrays of immobilized synthetases and mixtures of EF, tRNAs and the unknown amino acid, it should be possible to perform massively paralleled amino acid analysis. The high specificity of the interaction could lead to dramatic improvements in sensitivity and may be less sensitive to contaminants than traditional techniques.

3. Materials and Methods

3.1 *Materials*

The *E.coli* bacterial strain over-expressing GluRS was kindly provided by BMC, Uppsala University, Sweden. The monoQ HR5/5 column, Dextran T500 and Sephadex G-75(fine) separation materials were purchased from Amersham Biosciences, Uppsala, Sweden. EDTA, Tris-aminomethane, and glycerol were from Merck, Germany. PMSF was obtained from Boehringer Mannheim, Germany. DTT was bought from Sigma-Aldrich, St. Louis, USA. Bradford reagent and standard BSA were Bio-rad products. EZ-linkPEO-maleimide-activated biotin was bought from Pierce. The NANA sep 30K OMEGA was from Pall. The *XNA on Gold*™ biochips were kindly provided by BT Biomedical Technology AB, Sweden. All other reagents used were of analytical reagent grade. Milli-Q water (18MΩ.cm) was used.

3.2 *Expression and purification of E.coli GluRS*

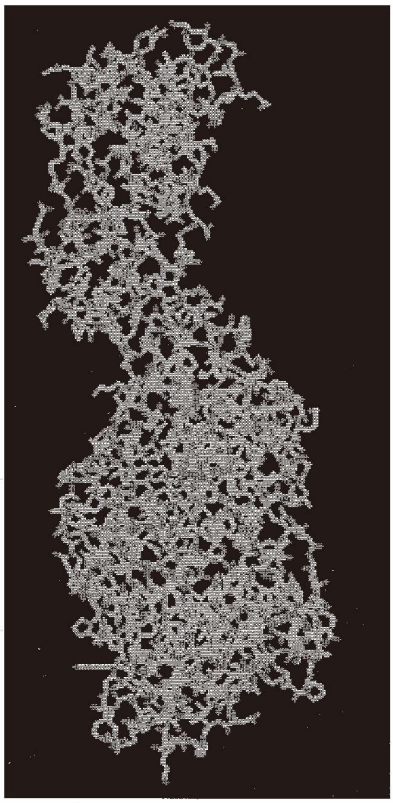
Mono-colonies of *E.coli* GluRS were inoculated into 200 ml of LB medium containing ampicillin (100 μg/L). The culture was grown under agitation at 30°C until an optical density (600 nm) of 0.4 was attained. The culture was used to inoculate 2 liters of medium containing ampicillin (200 μg/L) and was incubated under agitation until an optical density of 0.7 at 600 nm was reached. The culture was incubated at 42°C for an additional 4 h and then harvested.

GluRS was purified as described in the literature.[7] Briefly, after centrifugation, the bacterial pellet was resuspended in PBS buffer containing EDTA, PMSF and DTT. The cells were disrupted by repeated sonication. The supernatant-containing GluRS was removed after centrifugation and fractionated in a polyethylene glycol 8000-dextran T500 two-phase system. After mixing for one hour, the two phases were separated by centrifugation at 12,000 g for 15 min and the top phase containing the GluRS was removed. The fourfold diluted top phase then was subjected to anion-exchange chromatography on a MonoQ HR5/5 column. Ultimately, GluRS was eluted at 15% concentration of NaCl from 0 to 30% NaCl gradient. After comparison with a protein standard, GluRS was confirmed.

3.3 *Structure simulation of GluRS from E.coli*

In order to maintain the bioactivity of GluRS after immobilization on the XNA surface, verification of the available sites for the specific immobilization of the enzyme is a critical step for the rest of the studies.

In this experiment, since GluRS was prepared from *E.coli* but not from *Thermus thermophilus*, it is necessary to analyze the 3D structure of GluRS in *E.coli* using a method of indirect simulation. Because the 3D structure data of GluRS from *Thermus thermophilus* is available from the protein data bank,[8] and the amino acid sequence of GluRS is also available from a public database,[9] the stucture can be constructed with Rasmol 2.6 software (see Fig. 2). By analysis and comparison between amino acid sequences of GluRS from *Thermus thermophilus* and *E.coli* with Clustal X 1.8 software, high similarity in their amino acid sequences has been confirmed. Therefore, using Jackal software, the 3D structure of GluRS from *Thermus thermophilus* could be used as a reference model to simulate the 3D structure of GluRS from *E.coli*. By means of this comparative study, the simulated 3D structure of GluRS from *E.coli* was established and could be reconstructed with Rasmol 2.6 software, as shown in Fig. 3. In the simulated result of the 3D structure of GluRS from *E.coli*, cystines containing a thiol group and lysines containing a primary amion group were colored green and orange, respectively, thereby being highlighted on the 3D structure. From the analysis of Fig. 3, a pocket comprising four cystines could be found. Since the four cystines are located very close to each other, the possibility of



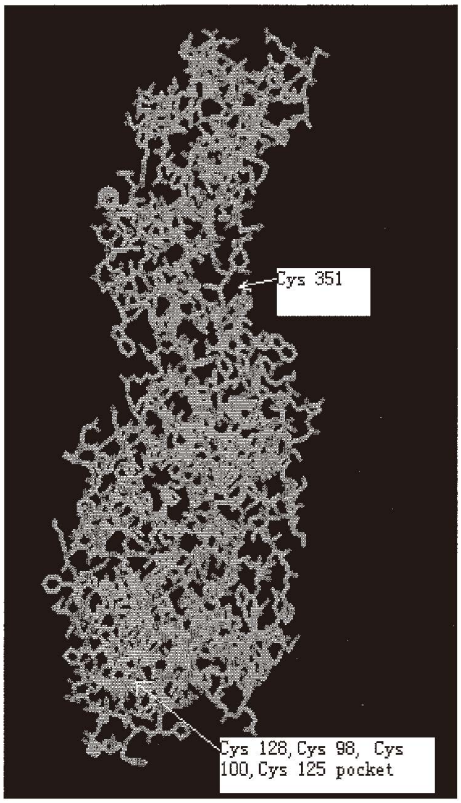


Fig. 2 (left). 3D structure of GluRS from *Thermus thermophilus*, visualized with Rasmol 2.6 software.

Fig. 3 (right). Predicted 3D structure of GluRS from *E.cloi* obtained using Jackal simulation software and visualized with Rasmol 2.6 software.

forming disulfidebond could be very high. Meanwhile, cystine 351 is on the surface of the GluRS molecule and far from other cystines, therefore the cystine 351 containing a thiol group could be a good candidate for the labeling of biotin through maleimide activation.

From the analysis of GluRS from *E.coli* with Rasmol 2.6 software, 24 lysines exist in the enzyme and most of them can be clearly observed on the enzyme surface, which ensures that the GluRS molecules from *E.coli* could further be labeled with NHS-activated Cy5.

On the basis of the evaluation of the computer simulation of GluRS from *E.coli,* experiments to demonstrate GluRS immobilization and labeling with maleimide-activated biotin and NHS-activated Cy5 were carried out and are described in the following sections. The labeled and immobilized GluRS chips were observed using a CCD fluorescent microscope.

3.4 *BSA biotinylation and purification*

Standard BSA solution (1 mg/ml dissolved in PBS) was labeled with EZ-linkPEO-maleimide-activated biotin according to the Pierce instruction.[10] Briefly, 900 μl of BSA solution was mixed with 100 μl of PEO-maleimide-activated biotin solution and reacted at 4°C for 2 h. The biotin-labeled BSA molecules were separated from the free biotin by elution through a G-75 desalting column. After the fractionation, the concentration of biotinylated BSA was measured using Bradford reagent.

3.5 *GluRS biotinylation and purification*

The fraction of GluRS eluted from the HPLC column was concentrated with NANO sep 30 K at 4°C, 4500×g for 10 min, and rinsed with PBS twice under the same conditions. Afterwards, the condensed GluRS solution was diluted tenfold in volume with cold PBS. The GluRS molecules were labeled with EZ-linkPEO-maleimide-activated biotin according to procedure similar to that for BSA in instructions from Pierce. Briefly, 900 μl of GluRS solution was mixed with 100 μl of PEO-maleimide-activated biotin solution and reacted at 4°C for two hours. The biotin-labeled GluRS molecules were separated from free biotin molecules by elution through a G-75 desalting column. After the fractionation, the concentration of biotinylated GluRS was measured with Bradford reagent.

3.6 *Biotinylated GluRS fluorescensylation and purification*

After applying 1 ml of eluted biotin-labeled GluRS to one Cy5 vial, it was thoroughly mixed and reacted at 4°C for one hour with additional mixing every 15 min. The Cy5-labeled GluRS molecules were separated from excess Cy5 by G-75 chromatography, its final concentration was again measured with Bradford reagent, and it was stored in a dark container at 4°C for later immobilization on the XNA chip and fluorescence scanning.

3.7 *Biotinylated BSA and biotinylated ,fluorescenced GluRS immobilization on XNA Chip*

All operations were carried out in a dust-free hood. The XNA chip was thoroughly rinsed to remove dust from the chip surface by bath sonication for 10 min immediately before immobilization. First, the biotinylated BSA and the biotinylated, Cy5-labeled GluRS were diluted ten-fold from 45.58 pM to 0.4558 fM and from 498.3 fM to 0.4983 fM, respectively. The diluted solutions were applied individually to the corresponding dots on the XNA chip in 3 μl per dot. The dots for the control were deposited from solutions of

label-free biotinylated BSA and GluRS. The biotinylated BSA and the biotinylated, Cy5-labeled GluRS were allowed to immobilize on the chip at 4°C for 30 min, after which they were thoroughly washed three times with PBS (pH 7.4) in order to remove the excess biotinylated BSA and biotinylated, Cy5-labeled GluRS. The labeled chips were stored in dark containers at 4°C for measurements.

4. Results and Discussion

4.1 *Expression and purification of E.coli GluRS*

In order to verify whether the GluRS was heat-induced to over-expression and purified, two additional experiments were carried out as follows. In the first experiment, one GluRS-plasmid containing bacterial solution was heat-induced to express GluRS and the second was not induced as a control. Both solutions were treated using the same harvest and purification procedures. As shown in Fig. 4, the two elution curves presented peaks with similar numbers and profiles except for a strong contrast between the two peaks eluted at 15% NaCl, in which the salt concentration was responsible for eluting GluRS from the ion exchange column according to results obtained from other studies.[5] In conclusion, a strong peak from the heat-induced solution (Fig. 4(B)) vs a weak peak from the non-heat-induced solution (Fig. 4(A)) could indicate that GluRS had been induced to over-expression.

In the second experiment , the fraction from the strong peak in the dilution curve was added to SDS PAGE to determine its MW. On SDS-PAGE, we found that the MW of the protein assumed to be GluRS was slightly less than the standard at 55.4 KD (see Fig. 5). In fact, the MW of *E.coli* GluRS was more than 53 KD. Therefore, we confirmed that the over-expressed protein was *E.coli* GluRS. The purity of the over-expressed GluRS in the fraction was nearly 90%.

4.2 *Biotinylated BSA immobilization onto streptavidin-modified microarray for AFM measurement.*

According to AFM images shown in Fig. 6, aggregated BSA molecules could be visualized on the chip in a concentration range from 0.4558 fM to 455.8 fM. Moreover, as compared with the images obtained with BSA in concentrations of 455.8 fM and 45.58 fM,

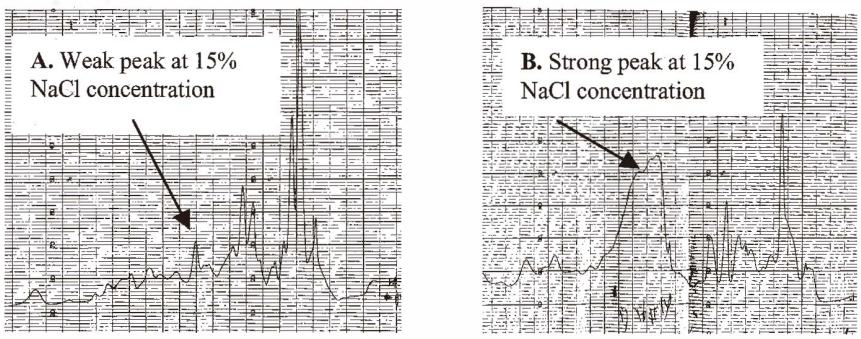


Fig. 4. (A) Elution curve of top phase of fluid from non-heat-induced bacteria over-expressing GluRS. (B) Elution curve of top phase of fluid from heat-induced bacteria over-expressing GluRS.

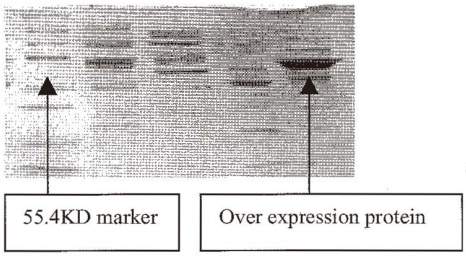
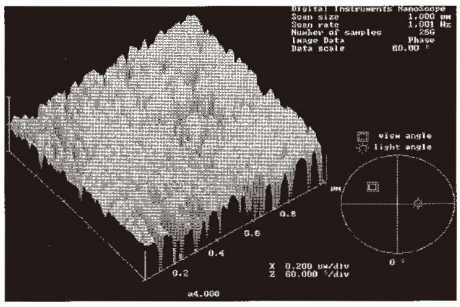
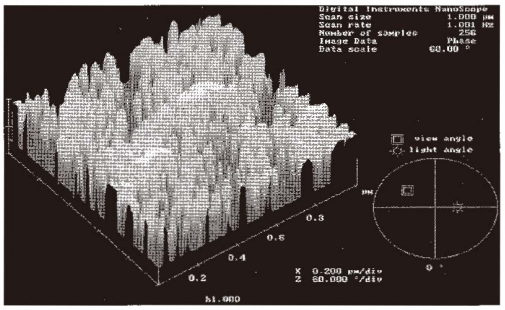


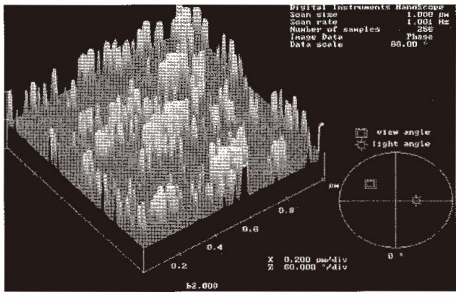
Fig. 5. Over-expressed protein on SDS-PAGE.



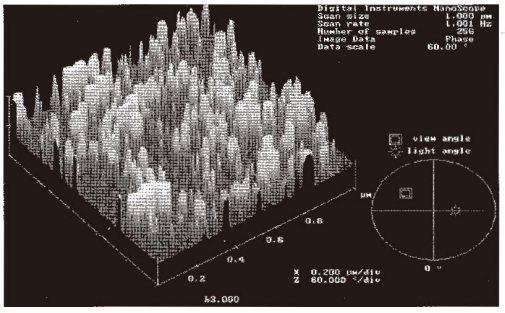
(A) 445.8 fM biotinylated BSA



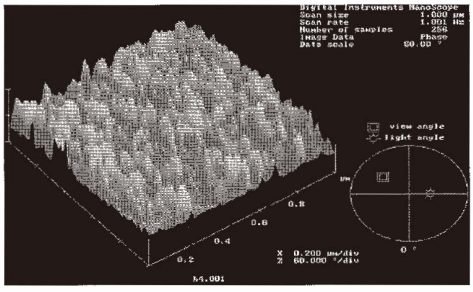
(B) 45.58 fM biotinylated BSA



(C) 4.558 fM biotinylated BSA



(D) 0.4558 fM biotinylated BSA



(E) Blank

Fig. 6. Tenfold serial diluted biotinylated BSA chips (from 445.8 fM to 0.4558 fM) and blank were scanned by AFM.

more ordered and individual dot arrays can be distinguished when the immobilized concentration is reduced to less than 4.558 fM. This can be explained by considering the fact that the streptavidin binding sites on the XNA surface were saturated when the immobilized concentration of the biotinylated BSA was more than 4.558 fM. Therefore, molecular overlapping on the surface could appear when the BSA concentration was more than 4.558 fM. Accordingly, in order to detect cognate amino acids with the fluorescent-labeled GluRS, the appropriate concentration for the immobilization should be around 1 fM.

4.3 Determination of biotinylated GluRS labeled using Cy5 with cooled fluorescent CCD microscope

According to the data provided by the AFM studies, we applied different concentrations of the biotinylated and Cy5-labeled GluRS of 0.4983 fM and 498.3 fM, respectively, for immobilization on the XNA chip. As shown in Figs.7 and 8, homogeneous fluorescence images within the immobilized dot area were obtained using a cooled CCD fluores-

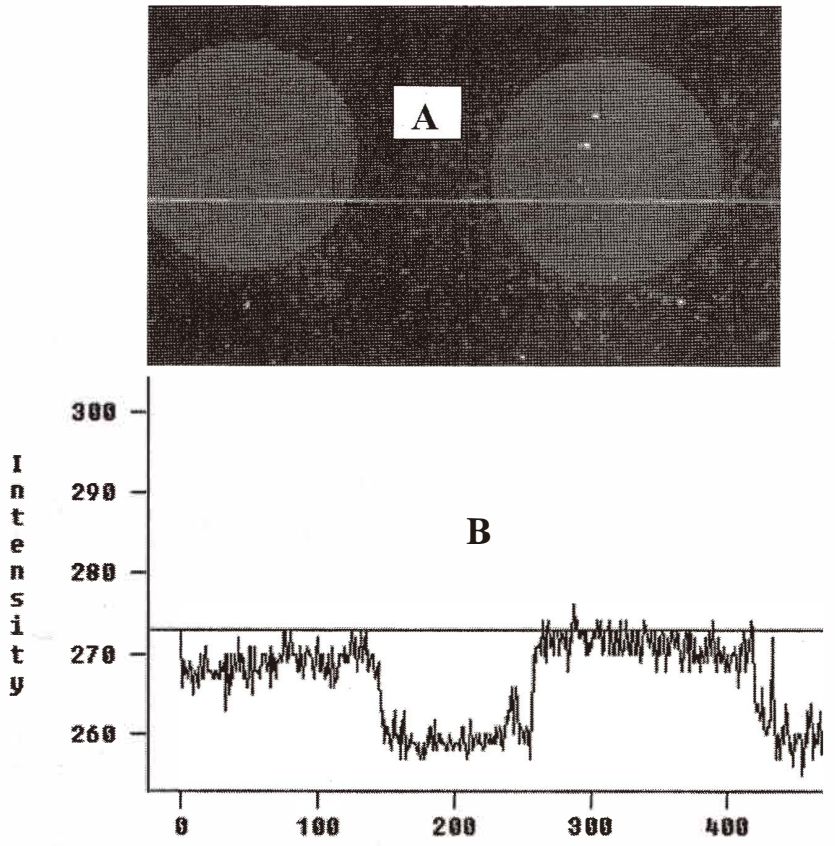


Fig. 7. CCD image for 0.4983 fM biotinylated GluRS labeled with Cy5. The circular areas show the dots of the streptavidin monolayer immobilized with biotinylated GluRS labelled with Cy5. The bright area indicates where the immobilized GluRS was even. (A) CCD image of XNA chip surface. (B) Fluorescence intensity across dotted area on which biotinylated GluRS labeled with Cy5 was immobilized.

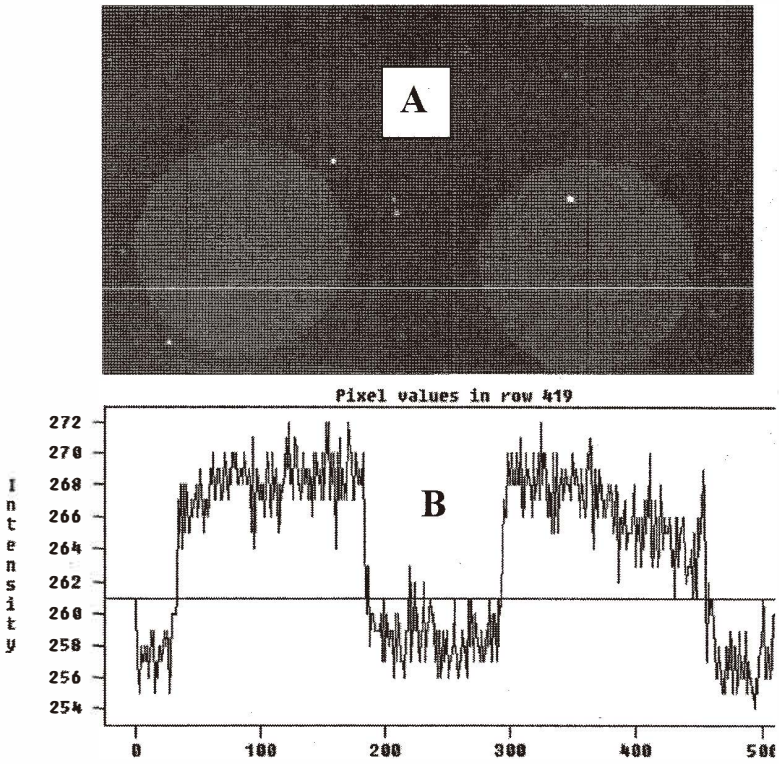


Fig. 8. Effect of fluorescent signal on Cy5-labeled GluRS concentration increased from 0.4983 fM to 498.3 fM. (A) CCD image of XNA chip surface. (B) Fluorescence intensity across dotted area on which biotinylated GluRS labeled with Cy5 was immobilized.

cent microscope. These results indicate that GluRS could be immobilized by strong and specific binding between biotin and avidin, but not by adsorption, as shown in Fig. 9, in which there was only biotinylated BSA immobilized without Cy5 labeling. The Cy5 modification on GluRS was efficient and effected the fluorescence without any interference on GluRS binding. Comparing Figs. 7 and 8, increasing the concentration of GluRS to more than 0.4983 fM did not significantly improve the fluorescent signal, which could be due to the saturation of the streptavidin binding sites with GluRS and the formation of a monolayer of the immobilized GluRS.

5. Conclusions

Here, we have presented our initial experiments on developing a protein analysis scheme based on the ability of aminoacyl-tRNA synthetases to specifically recognize each

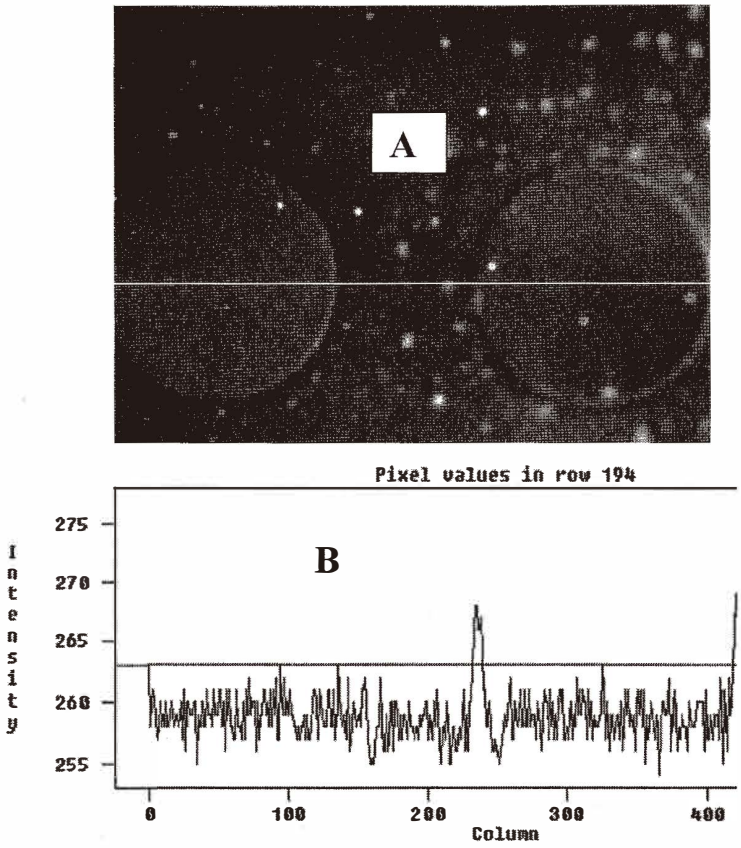


Fig. 9. BSA was labeled with biotin but not Cy5. (A) CCD image of microarray surface. (B) Fluorescence measurements taken where the line crosses the image.

of the 20 amino acids. As a first step in this process, glutamyl-tRNA synthetase was immobilized using the streptavidin-based *XNA on Gold*™ biochip platform. Our preliminary studies show that glutamyl-tRNA synthetase could be efficiently modified with biotin and further immobilized on the *XNA on Gold*™ biochip. The efficiency of the immobilization was analysed using a CCD fluorescent microscope with GluRS labelled with the fluorescent dye Cy5. The Cy5 modification did not significantly affect the immobilization efficiency. Currently, we are expanding the aminoacyl-tRNA synthetase array to further test the concept. In addition, we are developing strategies to apply this technology for the development of higher complexity 'translation on a chip' systems, for studying translation biomechanics, as well as the analysis of tRNA modifications and subclasses. In conclusion, this protein analysis scheme provides an urgently needed compliment to the traditional methods.

Acknowledgements

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