ÉCOLE CENTRALE LYON

Thèse de l'Université de Lyon délivrée par l'Ecole Centrale de Lyon

Soutenue publiquement le 28/09/2015

Par

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Préparée à l'Institut des Nanotechnologies de Lyon

Elaboration of protein microarray for rapid screening and quantification of breast cancer biomarkers

Ecole Doctorale d'Electronique, Electrotechnique, Automatique
Spécialité: Micro et Nanotechnologies

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Acknowledgements

It was a great pleasure for me to make a thesis in the team of chemistry and nanobiotechnology in Lyon Institute of Nanotechnology (INL), and I want to thank a lot of people for these three years.

Firstly, I want to thank my supervisor Dr Eliane Souteyrand and my co-supervisor Dr Emmanuelle Laurenceau. They helped me defining the structure of my thesis as well as giving me a lot of support and encouragement during last three years. Furthermore, I learned a lot from my bosses on how to organize time, how to resolve the problems, how to be a good researcher. All these are invaluable experience for me and make me benefit all my life.

Then I want to thank all the colleagues in my team. Thanks Dr. Yann Chevolot for revising my publications and for discussing the problems that I have confronted during my thesis. Thanks Dr. Thomas Gehin for spotting, it's fundamental because I cannot do anything without it. I also much appreciate Dr Virginie Monnier, Dr Magali Phaner-Goutorbe, Dr Jean-Pierre Cloarec, Isabel Nabeth and Maryline Diserio, we passed excellent coffee time almost every day. Also, great thanks to all doctors (Alice Goudot, Amandine Cornillon, Salome Ansanay-Alex and Francisco Palazon) and future doctors (Lucie Dupin, Francesca Zuttion, Maxime Boksebeld, Mathieu Caillau and Feixiong Chen). It's amazing that we had created such a good and relaxed working environment.

I would also appreciate the collaboration with the Montpellier Research Cancer Institute (IRCM). Thanks Dr. Jérôme Solassol and Dr. Alain Mangé for giving us samples and helping us analyzing data.

Of course I greatly appreciate the PhD fellowship from China Scholarship Council (CSC) which gave me financial support to do my thesis. Also, I want to thank all my Chinese friends: Xianqing Meng, Huanhuan Liu, Tianli Huang, Kai Zhang, He Ding, Yu Bai, Zhen Lei, Xuchen Liu, Xingrong Huang, Yu Fan, Changwei Zhou, Xiangkun Sun, Wei Chen and Bingxue Zhang. I can not cite all but I keep all of you in my mind.

Finally, I want to thank my family and my boyfriend. Thank you.

Abstract

Breast cancer becomes the most common cancer among women. In order to improve women's chances of survival and life quality, to be diagnosed at an early stage and to receive correct treatment are the most promising ways. In this context, we aim at developing an antigen microarray for screening serological biomarkers to diagnose breast cancer patients as early as possible. Among numerous potential biomarkers, recent researches showed that antibodies against heat shock proteins (HSPs) are associated with tumor genesis and would be good diagnostic and prognostic biomarkers for breast cancer. Therefore, we used customized antigen microarray to screen anti-HSP antibodies in 50 breast cancer patients and 26 healthy controls. Our results indicated clearly that combining multiplex detection of anti-HSPs antibodies could discriminate breast cancer patients from healthy controls with sensitivity 86% and specificity 100%. Then, we elaborated an antibody microarray to detect the concentration of urokinase type plasminogen activator (uPA) in 16 cytosolic extracts of breast tummor tissue. uPA is good prognostic and predictive biomarker for breast cancer, low levels of uPA (≤3 ng/mg of protein) is associated with low risk of recurrence and no benefit of chemotherapy for breast cancer patients, and vice versa. Our results showed that the results obtained from our antibody microarray were surface dependent compared with the results obtained from ELISA. Furthermore, the use of our antibody microarray requires 25 times less sample volume compared with ELISA kit, thus solving the main limitations of ELISA. Finally, we determined and optimized the parameters which affected the performances of protein microarray, e.g. microarray surface chemistry, experimental duration, the concentration of solutions, etc. Furthermore, we studied the storage conditions for both chemically functionalized microarray surface as well as printed protein microarray. Results showed that our protein microarrays retain efficient biological activity for at least 3 month of storage.

Key words: protein microarray, autoantibodies, breast cancer diagnosis, predictive biomarkers, storage

Abbreviations

AAbs	Autoantibodies
APDMES	(3-aminopropyl) dimethylethoxysilane
ASCO	American society of clinical oncology
AUC	Area under receiver operating characteristic curve
BC	Breast cancer
BSA	Bovine serum albumin
Car	Sodium carbonate buffer
CMD	Carboxymethyl dextran
COOH	Carboxylic acid surface
Cy3	Cyanine 3
Cy5	Cyanine 5
DCIS	Ductal carcinoma in situ
DIC	N, N'-diisopropylcarbodiimide
DI water	Deionized water
EGTM	European Group on Tumor Markers
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FI	Fluorescence intensity
FLISA	Fluorescence-linked immunosorbent assay
GRP	Glucose-regulated protein
HC	Healthy controls
HER-2	Human epidermal growth factor receptor 2
HSPs	Heat shock proteins
IHC	Immunohistochemical
MAMVE	Maleic anhydride-alt-methyl vinyl ether
MES	2-(N-morpholino) ethanesulfonic
MRI	Magnetic image resonance
NHS	N-hydroxysuccinimide
ODN	Oligonucleotide
OFAT	One-factor-at-a-time
p53	Tumor protein 53
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PMT	Photomultiplier tubes
PR	Progesterone receptor
ROC	Receiver operating characteristic curve
SD	Standard deviation
SNR	Signal-to-noise ratio
Strep	Streptavidin
TAA	Tumor associated antigen
TDSUM	Tert-butyl-11-(dimethylamino)silylundecanoate
uPA	Urokinase plasminogen activator

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General introduction

Breast cancer remains a major public health problem in the world. According to World Health Organization, in 2012 there were 1.7 million women who were diagnosed with breast cancer and the incidence has increased by more than 20% since 2008. Early diagnosis and monitoring disease development represent promising approaches to reduce the growing cancer burden. Conventional diagnostic methods include mammography, clinical breast examination, breast self-examination and magnetic resonance imaging, etc. However, the use of these procedures has limitations including false positive, high cost, unnecessary biopsy, over diagnosis and undue anxiety etc.

Recent researches showed that screening tumor biomarkers could aid the diagnosis of breast cancer, monitoring tumor progression and response to certain therapy. Protein microarrays have already demonstrated their great potential as screening tool. However, efficient protein microarray still remains a challenge due to protein variability. Various factors influence the performance of protein microarray including surface chemistry, spotting buffer, spotting concentrations, etc. So our purpose is to develop efficient protein microarray to screen biomarkers in breast cancer patients, thus providing diagnostic, prognostic and predictive value for each patient.

In chapter I, we introduced the worldwide situation of breast cancer. The most efficient way to reduce the heavy burden is to diagnose the patient at an early stage and provide correct therapy to each cancer patient. Several serum and tissue biomarkers have been already used in clinic for these two purposes. However, current serum biomarkers used lack sensitivity and specificity for diagnosis and new biomarkers are needed to be developed. Recent reviews described more 1200 proteins as potential new biomarkers, so the current challenge is not to find one more biomarker candidate but to evaluate and validate their clinical relevance in order to perform an efficient test. So, Chapter 1 deals with an overview of state of the art about serum and tissue biomarkers in view to select only those that are of high interest. Recent studies showed that autoantibodies could be good diagnostic and prognostic biomarkers because they are stable and relatively easy to be detected in serum **on one hand. On the other hand,** tissue biomarkers including urokinase type plasminogen activator (uPA) and its main inhibitor plasminogen activator inhibitor 1 (PAI-1) are advantageously used in clinic. In the two cases, tests are limited by the detection method-ELISA. In contrast, protein microarray is an efficient tool for screening both serum and tissue biomarkers by consuming

small sample volume. The challenges of protein microarray will be also introduced in this chapter.

The purpose of the chapter II is using customized protein microarray to profil anti-heat shock proteins (HSPs) antibodies in breast cancer serum. As protein microarray is influenced by various factors, so we firstly optimized key parameters including surface chemistry, spotting concentration; then we detected the autoantibodies against hsps in breast cancer serum under the optimal conditions. In total, 50 breast cancer patients and 26 healthy controls were tested. Our results showed that combining multiplex detection of anti-HSPs antibodies could discriminate breast cancer patients from healthy controls with sensitivity of 86% and specificity of 100%.

Chapter III reports the fabrication and the use of antibody microarray to quantify the concentration of urokinase type plasminogen activator (uPA) and its main inhibitor plasminogen activator inhibitor 1 (PAI-1) in breast tumor tissue. We firstly optimized the various conditions for the immobilization of antibodies. Then the best conditions were chosen to detect the concentration of uPA in 16 cytosolic extracts of breast tumor tissue. Results showed that the results obtained from our antibody microarray were surface dependent compared with the results obtained from ELISA.

Chapter IV aims to optimize the parameters that influence the performance of protein microarray. These factors concern experimental duration, the concentration of solutions, storage conditions, etc. For instance, the study of protein microarray storage conditions showed that printed proteins could retain their biological activity for at least 3 months.

Chapter 1

State of the Art

1.1 Breast cancer: Key data and detection techniques

Breast cancer remains a major public health problem in the world. According to World Health Organization, in 2012 there were 1.7 million women who were diagnosed with breast cancer and the incidence has increased by more than 20% since 2008 [1]. It is the most frequently diagnosed cancer among women in both more and less developed regions and now it represents 25% of all cancers in women, as shown in Figure 1. Compared to incidence rate, the mortality rate of breast cancer is much lesser, probably reflecting early diagnosis as well as improved treatment options [2]. The development of early diagnosis or/and disease monitoring represents promising ways to reduce the growing cancer burden [3]. Therefore, in the following, conventional diagnostic and disease monitoring methods for breast cancer will be discussed.

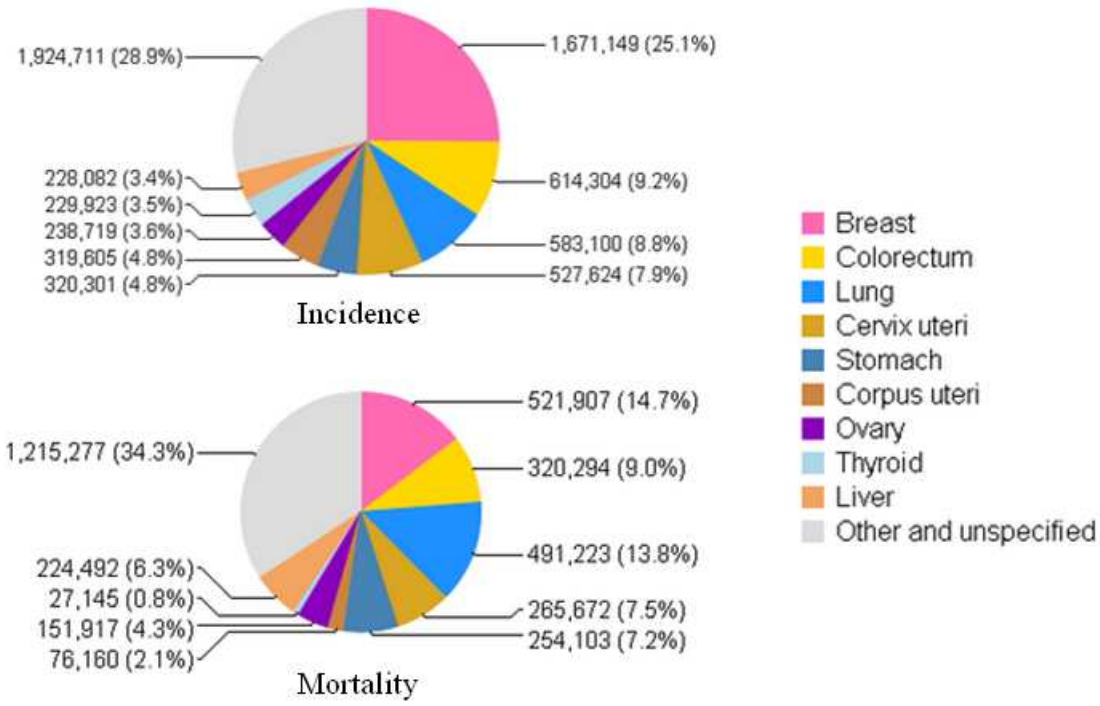


Figure 1 *Estimated Incidence, Mortality rate of breast cancer worldwide in 2012 [2]*

Early diagnosis is one of the most effective and affordable approaches to improve women's chances of survival, which could facilitate treatment of breast cancer patients in their pre-invasive state prior to metastasis. It is reported that five-year survival of women with breast cancer is highly associated with tumor stage. For example, the 5-year survival of very early stage tumor (stages 0 and I) is approximately 98%, it decreases to 85% for stage II tumors, 60% to stage III tumors and only 20% for stage IV tumors [4]. Conventional diagnostic

techniques of breast cancers include mammography, clinical breast examination, breast self-examination and magnetic resonance imaging. However, the use of these procedures has potential harms including false positive, high cost, unnecessary biopsy, over diagnosis and undue anxiety [5, 6].

Mammography is the best-studied breast cancer screening modality and the only recommended imaging tool for screening the general population of women. Guidelines for breast cancer screening vary within and among countries [7]. In the United States, the US Preventive Services Task Force recommends that women aged 50 to 74 years undergo a screening mammogram every 2 years. For women who are younger than 50 years old, examination should be based on the individual women's context, including her values regarding the benefits and risks [8]. In contrast, the American Cancer Society recommends that women should begin annual screening at age 40 [9]. Screening women 40 to 49 years of age is more controversial than older ages, with less evidence available to determine the risk–benefit balance.

A recent study published in 2014 has almost totally denied the benefit of the mammography. In this study, they divided women aged to 50 to 59 years into two groups, one group receiving both mammography and clinical breast examination while another receiving only clinical breast examination. Then they studied the 25-years cumulative mortality from these two groups. Results showed that the mortality was essentially equivalent between women who received mammography and clinical breast examination versus women who received only clinical breast examination [10]. Once the article has been published, it provoked heated debate in academic community. Some people defended that the clinical breast examinations were performed by well-trained clinicians, while community clinicians may not perform such high-quality clinical breast examinations, thus limiting the applicability of these results to general practice [5]. Others declaimed that the whole experiment design was questionable and the consequent results were null [11]. Evidently, controversy exists on the benefits of mammography as a routine screening test for women; therefore, it needs time to prove the real benefits of mammography for women.

MRI is a useful and sensitive tool for evaluating abnormalities and diagnosing breast cancer. While no studies have shown a mortality benefit for the general population from this screening, it is therefore not recommended as screening modalities for the general population. However, it is recommended by both US Preventive Services Task Force and American

Cancer Society to be used as a supplemental screening in special high-risk populations such as women with BRCA1 and BRCA2 mutations [12, 13].

Other methods like Physical Examinations (regular breast self-examination and routine clinical breast examination) are not recommended. These Examinations could be harmful and they couldn't reduce breast cancer mortality, but double the number of biopsies. Methods like thermography and ultrasound are neither recommended because their benefits are unknown [5, 6].

Currently, screening of tumor markers represents another approach for cancer diagnosis and receives considerable interest. Tumor markers are associated with tumor genesis; therefore, screening these biomarkers could aid early diagnosis as well as better management of breast cancer.

In parallel, the concept of personalized medicine is more and more accepted by academic as well as clinic research. According to US President's Council of Advisors on Science and Technology, personalized medicine refers to the tailoring of medical treatment to the specific characteristics of each patient. The goal of personalized medicine is to reduce the burden of disease by targeting prevention or treatment more effectively [14]. Ideally, personalized medicine delivers the right care to the right cancer patient at the right time and results in measurable improvements in outcomes and a reduction on health care costs [15]. In the case of cancer, considering that each solid tumor in each person is unique in cause, in rate of progression and in responsiveness to certain therapy, personalized medicine is particularly well adapted and necessary. The essence of personalized medicine lies in the use of biomarkers. Screening tissue or serum markers could provide prognostic value and predictive value. Beyond a help for diagnose, prognostic markers and predictive markers can estimate recurrence risk and predict therapy efficacy respectively. Classifying patients with low or high risk for recurrence and administering optimal therapies could avoid overtreatment for breast cancer patients. Currently, screening biomarkers are replacing the traditional "one size fits all" medicine. Personalized medicine based on biomarkers is already having a remarkable impact [16, 17].

So in the following, the routine used tumor biomarkers in breast cancer will be introduced.

1.2 Tumor biomarkers

During tumor genesis, cancer or other cells of the body will produce substances in response to cancer conditions. These substances are called tumor markers and they are produced at much higher levels in cancerous conditions. Tumor markers can be found in blood, urine, tumor tissue, or other tissues or bodily fluids of cancer patients. Screening these markers could aid early detection, risk stratification, prediction and disease prognosis of breast cancer.

A number of researchers found that tumor markers could be detected several months prior to clinical detection of breast cancer, thus screening these biomarkers could let the patients to be diagnosed at a more earlier stage [4]. Prognostic markers were defined to be markers that have an association with some clinical outcomes, such as overall survival or recurrence-free survival. They enable distinguish the clinical outcomes of patients in the absence of therapy. Moreover, prognostic markers give support to evaluate the efficiency of certain therapy. For example, increasing levels of several prognostic markers are associated with failure of certain therapy.

Predictive markers are generally used to make more specific choices between treatment options. They serve as indicators of the likely benefit to a specific patient of a specific treatment. A predictive marker might indicate that a patient expressing the marker will benefit more from a new treatment than from standard treatment, whereas a patient not expressing the marker will derive little or no benefit from the new treatment [18, 19].

Among all kind of biomarkers, protein biomarkers represent one of the ultimate levels of cellular function and thus give a picture of cell health. Protein biomarkers could be antigens as well as antibodies produced by immune and hormonal responses. Today, we do not suffer from a lack of candidate protein biomarkers. More than 1200 protein biomarker candidates for cancer have been described in the scientific literature; however, only 9 have been approved as tumor associated antigens by US Food and Drug Administration (FDA) [20]. The rate of introduction of new protein biomarkers approved by FDA has remained flat over the past 15 years, with an average of 1.5 new proteins cleared per year (median of 1 per year) for all diseases [21].

Thus, we will focus on the serum and tissue biomarkers that have been approved by American Society of Clinical Oncology (ASCO) or European Group on Tumor Markers Recommendations (EGTM) for routine usage. But as performances of biomarkers are

evaluated through various indicators such as true positive, true negative, false positive, false negative, sensitivity, specificity, positive predictive value and negative predictive value, receiver operating characteristic (ROC) curve, the area under the ROC curve (AUC) and p value, we will firstly define these indicators.

1.2.1 Indicators of biomarker value and data analysis

Methods for calculating the true positive, true negative, false positive, false negative, sensitivity, specificity, positive predictive value and negative predictive value were based on the methodology provided in Epidemiology [22].

As shown in Table 1, cell ′a′ represents the test which correctly diagnosed the disease, which are the true positives (TP). Cell ′b′ is those who have positive results for the test but do not have disease; the test has wrongly diagnosed the non-disease and it is false positives (FP). Cell ′c′ represents those who have disease but have negative results with the test. The test has wrongly labeled a diseased person as ′normal′, which means false negatives. Cell ′d′ is those who have no disease as determined negative with the test and it represents true negatives (TN). Sensitivity is the ability of a test to correctly classify an individual as ′diseased′, which equals a/(a+c). The ability of a test to correctly classify an individual as disease- free is called the test's specificity (Specificity = d/(b+d)). Positive predictive value (PPV) is the percentage of patients with a positive test who actually have the disease (PPV = a/(a+b)). Likewise, negative predictive value (NPV) is the percentage of patients with a negative test who do not have the disease (NPV = d/(c+d)) [23].

Table 1 *Calculation for sensitivity, specificity, positive predictive value and negative predictive value*

Test	Disease	Non disease	
Positive	True positives (TP) a	False positives (FP) b	Total test positive a+b
Negative	False negatives (FN) c	True negatives (TN) d	Total test negative c+d
	Total disease a+c	Total non-disease b+d	Total a+b+c+d

ROC

The receiver operating characteristic (ROC) curve is commonly used in medical decision making. ROC graphs are two-dimensional graphs in which true positive (TP) rate is plotted on the Y axis and false positive (FP) rate is plotted on the X axis. Since TP rate is equivalent to sensitivity and FP rate is equal to 1 − specificity, the ROC graph is also called the sensitivity vs. (1 − specificity) plot. Figure 2 shows an ROC graph with five classifiers labeled A through D [24].

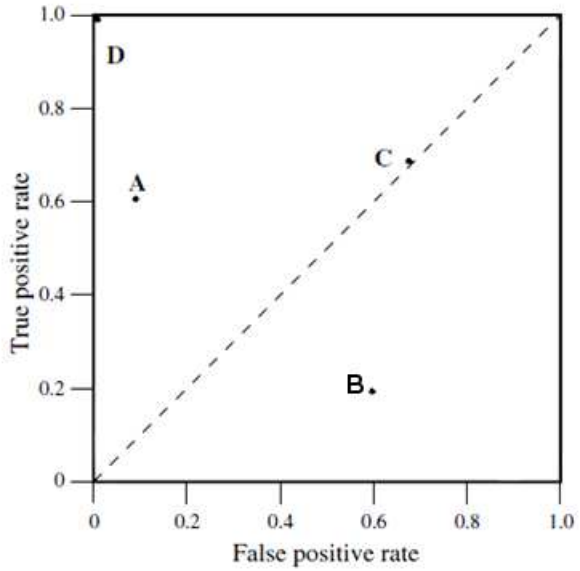


Figure 2 *A basic ROC graph showing five discrete classifiers [24].*

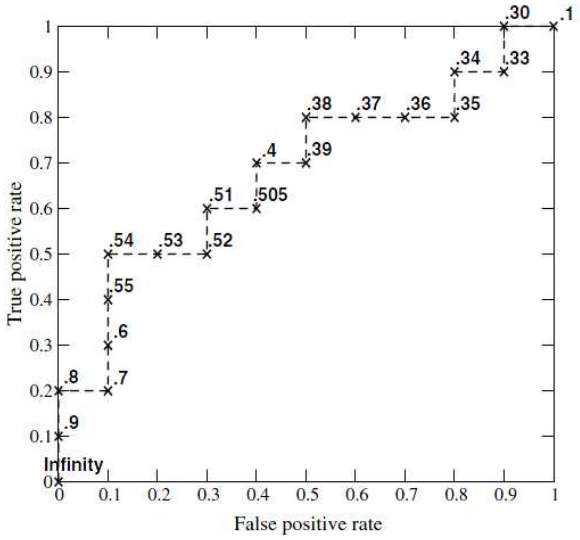
Several points in ROC space are important to note. The lower left point (0, 0) represents the strategy of never issuing a positive classification; such a classifier commits no false positive errors but also gains no true positives. The opposite strategy (1, 1) means unconditionally issuing positive classifications, which commits maximal true positives as well as maximal false positives. The point D (0, 1) represents perfect classification. The diagonal line y = x represents the strategy of randomly guessing a test. Point C is virtually random. Any classifier that appears in the lower right triangle performs worse than random guessing. In Figure 2, point B performs much worse than random. In contrast, the point in the top left corner of ROC space is better, e.g. point A, which means that TP rate is higher while FP rate is lower [24].

Generally, a diagnostic test contains several samples and each sample has a tested value. In order to construct a ROC curve, we rank all the tested value and produce a discrete classifier with a threshold, which means that if the tested value is higher than the threshold, the

classifier produces a Yes, otherwise a No. Each threshold value produces a different point in ROC space. Figure 3 shows an example of ROC curve on a test set of 20 instances.

No.	Class	Threshold Value	No.	Class	Threshold Value
1	P	0.9	11	P	0.4
2	P	0.8	12	N	0.39
3	N	0.7	13	P	0.38
4	P	0.6	14	N	0.37
5	P	0.55	15	N	0.36
6	P	0.54	16	N	0.35
7	N	0.53	17	P	0.34
8	N	0.52	18	N	0.33
9	P	0.51	19	P	0.30
10	N	0.505	20	N	0.1

(a)



(b)

Figure 3 *ROC ‘‘curve’’ created by threshold a test set. (a) The Table shows the values of 20 samples obtained from one test and ranked from 0.9 to 0.1; “P” means positive, “N” means negative. (b) The graph shows the corresponding ROC curve with each point labeled by the threshold that produces it [24].*

The test contains 20 samples (10 positives and 10 negatives) and the samples were ranked by their tested values, from 0.9 to 0.1, as shown in Figure 3a. Threshold is the tested value of each sample and according to each threshold value, it produces a different point in ROC space (Figure 3b). For example, a threshold of +1 produces the point (0, 0). As we lower the threshold to 0.9, the first positive sample is classified positive, thus the sensitivity is 10%

(1/10); also no negative is classified positive, thus there is no false positive and the specificity is 100%, yielding (0, 0.1). As the threshold is further reduced to 0.7, two positive samples are classified positive, thus the sensitivity is 20%; also one negative is classified positive, thus the false positive rate is 10%, yield (0.2, 0.1). Similarly, as the threshold is further reduced, the curve climbs up and to the right, ending up at (1, 1) with a threshold of 0.1 [24].

One point in ROC space is better than another if it is close to the top left which means that TP rate is higher while FP rate is lower, e.g. Figure 2 point D. Likewise, if the ROC curve is more close to top left, it means that the classifier is good at identifying likely positives as well as likely negatives (high sensitivity and high specificity).

AUC

AUC is the area under ROC curve. The ideal test would have an AUC of 1, indicating a perfect situation with 100% sensitive and 100% specific; a random guess would have an AUC of 0.5, represented by the diagonal line from the lower left corner to the upper right, as shown in Figure 2. In general, ROC curves with an AUC≤0.75 are not clinically useful and an AUC≥0.97 has a very high clinical value [25].

P-value

Chi-square $\chi 2$ test was used to determine whether the frequency of biomarker in cancer serum was significantly higher than healthy controls. Two significant levels ($P < 0.05$ and $P < 0.01$) are commonly used [26].

1.2.2 Serum biomarkers used in clinic

In recent years the discovery of cancer biomarkers has become a major focus of cancer research. When we compared the clinical practice guidelines published by American Society of Clinical Oncology (ASCO) from 1999 to 2013, it is evident to find that more emphasis was given on cancer biomarkers for breast cancer management in last 20 years [27-29].

Serum tumor markers are soluble molecules in blood that can be detected by monoclonal antibodies. They are released into the blood by tumor cells or by other cells in response to tumor cells. Serum markers have appealing features. Based on the circulatory nature of blood through almost every part of the human body, the measurements of blood components could

reflect the dynamic evolution of the disease. Furthermore, obtaining blood samples is poorly-invasive, so their levels can be easily repeated when required [30, 31]. Currently, serum biomarkers used in clinic for breast cancer patients include certain members of mucin glycoproteins family (MUC-1) and Carcino Embryonic Antigen (CEA), which are recommended by ASCO and European Group on Tumor Markers Recommendations (EGTM). So in the following part, we will focus on these biomarkers in breast cancer.

1.2.2.1 MUC-1 and CEA

MUC- 1 is involved in tumor genesis through complex pathways, e.g. promoting receptor tyrosine kinase signaling, constitutive activation of growth and survival pathways, and down regulation of stress-induced death pathways. Soluble form of MUC-1 family include cancer antigen CA 15-3, CA 27-29, CA 549, among which CA 15-3 and CA 27-29 are widely used in breast cancer. Because of their similar diagnostic sensitivities and specificities, the use of one MUC-1 marker is enough [33].

CEA is an oncofetal glycoprotein and is also widely used in breast cancer. CEA levels are less commonly elevated than the levels of MUC-1. However, CEA measurement can provide additional complementary information with MUC-1. Therefore, the combination of one MUC-1 marker and CEA is a good serum marker panel for monitoring patients with breast cancer [34].

1.2.2.2 Recommendations by ASCO and EGTM

For screening and diagnosis

The panel of MUC-1 (CA 15-3 or CA 27-29) and CEA is not recommended for screening and early diagnosis of breast cancer due to their low sensitivity [34, 35]. The soluble form of MUC-1 was identified as a more specific marker with respect to CEA. However, MUC-1 disclosed low sensitivity and specificity for the early diagnosis of breast cancer, since its sensitivity is 10–15%, 20–25% and 30–35% for stages I, II, and III, respectively [36]. Therefore, lack of sensitivity for early-stage disease combined with a lack of specificity precludes the use of these two serum markers for the early diagnosis of breast cancer.

For early detection of recurrence

Two well-designed studies have shown that after primary breast cancer therapy, elevated levels of MUC-1 and CEA in patients are associated with distant recurrence. It can predict recurrence with an average of 5 to 6 months before other symptoms or test [37, 38]. However, both ASCO and EGTM are vigilant to recommend them for detecting recurrence for several reasons. Evidence was insufficient to demonstrate whether early detection of metastases leads to good outcomes like disease-free survival, overall survival, quality of life or toxicity. Furthermore, intensive screening may induce extra expenses and anxiety [35, 34]. Therefore, it is not recommended by ASCO [29], while EGTM [35] recommended for the follow-up of asymptomatic women, this panel should be determined every 2–4 months during the initial 5 years after diagnosis, then every 6 months during the next 3 years and at yearly intervals thereafter.

For therapy monitoring

According to ASCO, present data are insufficient to recommend the use of MUC-1 or CEA alone for monitoring response to treatment. However, in the absence of readily measurable disease, increasing levels of this panel may be used to indicate treatment failure. It should be noted that a spurious rising level of MUC-1 and CEA was observed during the first 4-6 weeks of a new therapy [34].

1.2.2.3 Conclusions

Numerous serum biomarkers candidates in breast cancer have been reported, while only few of them have been approved by FDA. Approved serum markers are useful for determining recurrence, predicting therapeutic response, maintaining surveillance after primary surgery, and monitoring therapy in patients with advanced disease. However, at present, none of them are available for an early diagnosis and screening of breast cancer because of their low sensitivity and specificity.

In addition to screening novel biomarkers, validating candidate markers is also important. Furthermore, in order to reduce the gap between discovery and validation in the biomarker development pipeline, several points need to keep in mind. 1) Biological samples should be carefully chosen based on well-established guidelines for both patients and matched controls.

2) Objectives should be clear and methods of reporting results should be critical [39]. 3) Large scale and effective methods were needed to measure biomarkers in a high throughput manner. Compared with widely used single immunoassays, multiple immunoassays show several advantages including increased efficiency at a reduced expense, greater output per sample volume ratios and higher throughput. Among multiple immunoassays, planar immunoassays e.g. protein microarrays, are relatively inexpensive and would be simple and efficient to conduct large-scale population screening [40]. Therefore, in order to validate more candidate biomarkers, large-scale validation and a transition of methods is necessary. Only these requirements were satisfied, biomarker research can become more efficient and have the chance to translate into clinical evaluation.

1.2.3 Tissue biomarkers used in clinic

In this part, we will focus on already tissue biomarkers used in clinic for breast cancers. These biomarkers include estrogen receptor (ER), progesterone receptor (PR), human epidermal receptor 2 (HER-2), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1). Table 2 summarizes the detailed information of these tissue biomarkers.

Table 2 *An introduction of clinic used tissue biomarkers*

Tissue markers	Commercial products	Determined methods	Tissue requirements	Amount needed	Clinical validation	Approximate cost
ER/PR	N.A	IHC	FFPE	~ 4 slides	Yes	N.A
HER2	N.A	IHC	FFPE	~ 4 slides	Yes	N.A
uPA and PAI-1	Femtelle® from Sekisui Diagnostics	ELISA	Fresh/Frozen	At least 300mg tissue	Yes	~ 275 €

N.A: Not available; IHC: immunohistochemistry; FFPE: formalin-fixed, paraffin-embedded.

1.2.3.1 Estrogen receptor (ER) and progesterone receptor (PR)

ER and PR are transcriptional factors which mediate the actions of estrogens and progesterone, respectively. Both receptors are now known to exist in two different isoforms.

For ER, these forms are known as ER-α and ER-ß; for PR the two forms are known as PRA and PRB. It appears that only ER-α is critical for mammary gland development and tumorigenesis. Currently, the determination of ER and PR is obligatory in all breast cancer patients by EGTM [35] as well as ASCO [34] guidelines. Existing assays for PR do not discriminate between the two forms [30].

Three well-established assays exist for measuring hormone receptors, namely ligand binding, ELISA and immunohistochemistry (IHC). Only IHC is recommended by EGTM to measure ER and PR levels. Compared with other two methods, IHC assays can be carried out on small tumors, including core needle biopsy material [35]. It is recommended by ASCO that for the IHC test of ER and PR in breast cancer, the levels as low as 1% positive carcinoma cells are associated with clinically significant responses to endocrine therapy. They also noticed that up to 20% of IHC determinations of ER/PR testing worldwide may be inaccurate (false positive or false negative) due to variations in pre-analytical variables, thresholds for positivity, use of relatively insensitive antibodies, and criteria for interpretation. Therefore, they proposed that specimens should be handled in a uniform manner. Furthermore, factors should be well-considered including cold ischemia time, handling of specimens obtained remotely, fixation time in neutral buffered formalin, and selection of an optimal sample for testing, etc. [41].

ER and PR could provide prognostic value. Generally, ER-positive patients have a better outcome than ER-negative patients. However, this impact only last 4-5 years, after this period, the favorable prognostic value is lost. A further limitation of ER as a prognostic factor is that it is of little value in lymph node negative patients. Patients with tumors expressing PR also tend to have a better prognosis than those lacking this receptor [42, 30]. Since both ER and PR are relatively weak prognostic factors in breast cancer, these factors should not be used alone, they could be combined with established prognostic factors in determining outcome based on EGTM recommendation [35].

In addition of prognostic value, ER and PR status is also considered to be very strong predictors of response to hormonal therapy in breast cancer patients. Both early and advanced disease, hormone receptor-positive patients have a significantly greater probability of responding to hormone therapy than patients lacking receptors. Therefore, it is recommended by EGTM [35] that patients with hormone receptor-positive tumors should be treated with some form of endocrine therapy like Tamoxifen, while receptor-negative patients should

receive an alternate form of therapy. While according to the guidelines of ASCO [34], the benefits endocrine therapy for hormone receptor-positive patients with ductal carcinoma in situ (DCIS) is not sufficient.

1.2.3.2 HER-2

Approximately 15% of breast cancers over-express oncoprotein human epidermal factor receptor (HER2) [43]. There are three methods for identifying HER-2 status, including immunohistochemistry (IHC), which measures the HER-2 full-length oncoprotein; fluorescent *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH); both methods measures the number of HER-2/neu gene copies by a fluorescent system and an enzyme-based system, respectively. Among these three methods, only IHC assay was recommended by ASCO guidelines [44, 30, 45].

Nowadays, HER2 test must be performed to all newly diagnosed breast cancer patients with metastatic tissue biopsy samples available. Tumors with HER-2 over-expression are associated with higher grade and worse prognosis. However, its prognostic value is weak, therefore, it is not recommended to use alone for determining prognosis [34]. However, notice that in presence of mutated BRCA1 gene, HER2 over-expression is negative.

All patients with positive HER-2 receptors have to be treated by immunotherapy with Herceptin® (trastuzumab). Herceptin® is a humanized monoclonal antibody that binds with high affinity to the extracellular domain of HER-2, thereby blocking its role in signal transduction. Herceptin® is now widely used for the treatment of HER-2-positive tumor patients with breast cancer [30].

1.2.3.3 uPA and PAI-1

Urokinase plasminogen activator (uPA) is an extracellular matrix-degrading protease involved in cancer invasion and metastasis. uPA interacts with its plasminogen activator inhibitor-1 (PAI-1). Both PAI-1 and uPA promote tumor progression and metastasis [46].

Consistent with the causative role of uPA and PAI-1 in cancer dissemination, several retrospective and prospective studies have shown that uPA and PAI-1 are good prognostic and predictive biomarkers.

1.2.3.3.1 Prognostic and predictive value

One large scale study involving 8377 breast cancer patients demonstrated that uPA and PAI-1 are strong and independent prognostic markers in primary breast cancer. In both lymph node-positive and lymph node-negative patients, high uPA and PAI-1 values were independently associated with poor relapse free survival and poor overall survival [47].

In addition to provide prognostic value, uPA and PAI-1 could also provide predictive value to chemotherapy. A large-scale study including 556 patients was conducted by Jänicke F et al. Results showed that for patients with high levels of uPA and PAI-1 (uPA > 3 ng/mg of protein and PAI-1 > 14 ng/mg of protein), those receiving chemotherapy displayed 43.8% lower probability of disease recurrence at 3 years than observation group [48]. This predictive impact of uPA/PAI-1 regarding an enhanced benefit from adjuvant chemotherapy has also been demonstrated in a retrospective multicenter analysis [49].

Recently, the study of 10-year long-term Chemotherapy for node-negative (N0) breast cancer patients follow-up confirmed the prognostic and predictive impact of uPA/PAI-1 in node-negative breast cancer [50]. Patients with low uPA/PAI-1 levels (uPA $\leq$ 3 ng/mg of protein and PAI-1 $\leq$ 14 ng/mg of protein) displayed 10-year recurrence-free survival of 87.1% compared to 77% in high uPA/PAI-1 level patients ($p = 0.011$). 10-year overall survival (OS) was significantly better in low uPA/PAI-1 level patients (89.8% vs. 79.1%; $p = 0.01$). Moreover, in a randomized comparison in high uPA/PAI-1 level patients, adjuvant chemotherapy significantly reduced risk of recurrence ($p = 0.019$).

In conclusion, low levels of uPA and PAI-1 are associated with a sufficiently low risk of recurrence and chemotherapy will only contribute minimal additional benefit. On the contrary, high levels of uPA and PAI-1 signify high risk of recurrence and adjuvant chemotherapy provides substantial benefit for patients. uPA and PAI-1 are defined with the highest level-of-evidence (LOE-1) [46]. Therefore, they are considered to be the only biomarkers appropriate for the routine assessment of prognosis in patients with newly diagnosed node-negative breast cancer according to the American Society of Clinical Oncology (ASCO) guidelines [34] as well as European Group on Tumor Markers (EGTM) [35].

1.2.3.3.2 Detection methods

The above clinical trials employed ELISA to measure uPA and PAI-1 levels (normalized to the total protein content of the extract) extracted from breast tumor tissue. The cut-off value defined for uPA and PAI-1 is 3 ng/mg and 14 ng/mg, respectively. Currently ELISA is the only method which is recommended by ASCO to titrate uPA and PAI-1 [34]. The commercially available ELISA test (Femtelle ®) was developed by Sekisui Diagnostics. This kit has good quality insurance and is widely used in clinic. However, ELISA requires 100-300 mg of fresh or frozen breast cancer tissue. This is problematic for two reasons. Firstly, as formalin-fixed, paraffin-embedded (FFPE) tissue is the main source of patient material worldwide, therefore, requirement of fresh or frozen tissue preclude its usage [51]. Secondly, ELISA requires large quantity of tissues. Thomassen et al. [52] used ELISA to compare the levels of uPA and PAI-1 in 10-30 mg core biopsy specimens and 90-300 mg tumor tissue taken from the same specimens. Results showed that using the smaller tissue specimen correctly classified risk in 95% of the patients surveyed; however, correlation between individual uPA and PAI-1 levels in the small biopsy specimens versus the larger tissue samples was only 0.789 and 0.907, respectively. Therefore, the feasibility of measuring the level of uPA and PAI-1 in core needle biopsy breast cancer specimens needs to be confirmed. The need for large quantity of tissue requires a surgical biopsy or vacuum-assisted core biopsy with an 8-gauge needle [53] and precludes the use of 14-gauge needle-core biopsies that are more common in clinical practice [54]. Indeed, requirement of large volume of fresh tissue becomes the main limitation of ELISA assays.

Considering the challenges faced by ELISA, several other assay formats were also used but no one method has proven to be a reliable substitute for the ELISA assay. For example, immunohistochemistry (IHC) can detect uPA and PAI-1 in frozen or formalin-fixed, paraffin-embedded (FFPE) tissues. However, there was not complete agreement in protein levels, immunohistochemical scoring, and patient outcome between IHC and ELISA. Correlation rates for uPA and PAI-1 between expression levels determined by ELISA and IHC are 0.78 and 077, respectively [55]. Furthermore, IHC analysis yields semi-quantitative information and it is impossible to estimate protein expression level above the level causing maximum staining due to saturation. All these factors indicate that the two techniques are not directly interchangeable.

Analyzing mRNA levels of uPA and PAI-1 also seems appealing, because it requires very small amounts of tumor tissue and delivers quantitative estimation of the mRNA expression. Moreover, RNA-based analysis was feasible from paraffin-embedded tissue samples. However, quantification of mRNA has also proven to be an unreliable substitute for ELISA in assessing the level of uPA and PAI-1 [56-59]. Lamy et al. compared ELISA protein levels and mRNA levels of uPA and PAI-1 in tumor tissues. Results showed that the concordance of uPA and PAI-1 is only 84% and 70%, respectively [56]. In another study [57], correlations between uPA/PAI-1 mRNA and protein were found to be distinctly weaker or not significant; no correlation between PAI-1 mRNA and protein level was also reported in [59, 58]. The discrepancy between protein and mRNA levels was possibly caused by post-transcriptional regulation, which also demonstrated that measuring mRNA levels cannot always reflect the real expression of proteins.

Protein microarrays have several advantages compared with traditional ELISA as they yield high sensitivity and consume tiny volume sample [60]. Antibody microarray was used to detect the level of uPA and PAI-1 in extracts from breast cancer tissues. Higher sensitivity was achieved compared with ELISA. However, antibody microarray could not normalize the total protein content [61]. Preliminary studies have shown reverse-phase protein arrays (RPPAs) for uPA and PAI-1 promising [62, 63]. For RPPA arrays, protein lysates are spotted onto glass slides and total protein can be measured by Sypro-Ruby protein stain. Moreover, protein extracted from formalin-fixed, paraffin-embedded primary breast cancer tissues could be used in RPPAs. As protein microarrays require tiny sample volume, they show high appealing potential for detecting uPA and PAI-1 in breast tumor tissue.

1.3 Autoantibodies (AAbs) - Diagnostic and prognostic values

As mentioned before, no-serum biomarkers were sufficiently effective for an early diagnosis and screening of breast cancer because of their low sensitivity and specificity. In recent years, numerous studies have demonstrated that serum autoantibodies could have high value as diagnostic and prognostic biomarkers for breast cancer. In the 1960s, Robert W. Baldwin found that the immune system is involved in tumor development, during which autoantibodies (AAbs) were produced against intracellular proteins that are mutated, modified, or aberrantly expressed in tumor cells. These proteins are called tumor-associated

antigens (TAAs) [64]. Several pathways can explain this regulation, including opsonization, enhancement of dendritic cell-mediated antigen presentation to T cells, recruitment of natural killer cells to perform antibody-dependent cell-mediated toxicity, generation of tumor antigen-specific $CD8^+$ T cells and complement-dependent cytotoxicity. However, these mechanisms are not sufficient to explain how exactly these natural autoantibodies originate [65].

Although little is known about the origin of this immune response, an increasing number of articles have demonstrated that autoantibodies could be used for early diagnosis and prognosis of cancer [66]. AAbs show highly appealing properties compared with current serum biomarkers. Firstly, tumor-specific immune responses seem likely to occur before clinically apparent carcinoma. For example, Lubin et al. detected P53-specific antibodies almost 1.5 years before clinically relevant lung cancer was diagnosed [67]. Thus, the identification of AAbs could potentially be used for screening and early diagnosis of cancer. Secondly, during the anti-tumoral response, the immune system performs a very efficient biological amplification, leading to high concentration of AAbs and allowing indirect detection of very small amounts of tumor antigen. Thirdly, antibodies are highly stable in serum samples and are not subject to proteolysis like other polypeptides, making sample handling much easier. They show a long lifetime ($T_{1/2}$ between 7 and 30 days, depending on the subclass of immunoglobulin) in blood and may persist as long as the corresponding autoantigen elicited specific humoral response. Finally, antibodies are biochemically well known molecules, and many reagents and techniques are available for their detection, simplifying assay development [68-73].

Thus, in recent years, numerous studies have screened various AAbs against TAAs and evaluated their diagnostic and prognostic value in breast cancer. Among them, antibodies against heat shock proteins (HSPs) received great interest. Therefore, an overview of published studies on AAbs against HSPs and other TAAs in breast cancer will be summarized in the following.

1.3.1 Heat shock proteins (HSPs) and AAbs against HSPs

HSPs were first discovered as a cohort of proteins that are powerfully induced by heat shock and other chemical and physical stresses in a wide range of species [74]. HSP are a

group of highly conserved proteins and are classified into six families according to their molecular weight (MW): HSP110, HSP90, HSP70, HSPD1, DNAJ and small HSPs (range between 13-42 kDa) including HSPB1 and HSP10. Glucose-regulated proteins (GRPs) are a related class of proteins which are localized in endoplasmic reticulum. For example, HSPA5 belongs to HSP70 family member and shares 60% amino acid identity with HSP70; HSP90B1 belongs to HSP90 family and shares 50% amino acid identity with HSP90 [75]. HSPs function predominantly as molecular chaperones. They also restore cellular homeostasis by ensuring proper formation of new proteins, preserving existing complexes, restoring function of denatured proteins, and solubilizing protein aggregates [76].

Heat shock proteins (HSPs) are overexpressed in a wide range of human cancers. Elevated HSPs expression in malignant cells plays a key role in protecting cells against the spontaneous apoptosis associated with malignancy. Several HSPs are associated with the prognosis of specific cancer. For example, the expression of HSPB1 is associated with poor prognosis in gastric, liver, and prostate carcinoma, and osteosarcomas; overexpression of HSP70 is correlated with poor prognosis in breast, endometrial, uterine cervical, and bladder carcinomas [77]. Increased HSP expression may also predict the response to some anticancer treatments. HSPB1 and HSP70 were shown to be involved in resistance to chemotherapy in breast cancer; HSPB1 predicted a poor response to chemotherapy in leukemia patients, whereas HSP70 expression predicted a better response to chemotherapy in osteosarcomas. Furthermore, implication of HSP in tumor progression and response to therapy has led to its successful targeting in therapy [77]. Thus, the detection of HSPs as biomarkers of cancer could aid early diagnosis, determining prognosis, prospectively predicting response or resistance to specific therapies, surveillance after primary surgery, and monitoring therapy in patients with advanced disease [77, 76].

Elevated HSPs expression in tumor can also stimulate the immune system to produce anti-HSP AAbs. Conroy SE et al. [78] found that AAbs against HSP90 were detectable in a significant proportion (37%) of patients with breast cancer but not in normal individuals or patients with benign breast tumors. AAbs against HSP are also related with prognosis. Same authors [79] demonstrated that mortality rate from breast carcinoma was greater in women tested positive for AAbs against HSP90 than those tested negative. Thus, AAbs against HSPs could have diagnostic and also prognostic values in cancer. In the following, we firstly give an overview of published studies on detection of single AAb against HSP for discriminating breast cancer patients from healthy controls; then multi-AAbs panels are presented.

1.3.1.1 Evaluation of single anti-HSP AAbs

We identified 6 reports describing the use of individual AAbs against HSP for discriminating breast cancer patients from healthy controls. The result is presented in Table 3. Enzyme linked immunosorbent assay (ELISA) was the most commonly used techniques. Among all 6 studies, 5 researchers utilized ELISA and only one study used western blot (WB) for the detection of anti-HSP antibody. From these data, it was observed that the frequency of a single anti-HSP antibody in breast cancer patients ranged from 8%-48%, whereas the frequency in healthy controls ranged from 0 -5% (anti-HSP70 antibodies were not included).

Table 3 *Frequency of single autoantibodies in breast cancer patients, benign subjects and healthy controls*

		sample size (N)			AAb frequency %				
HSP	method	cases	HC	benign	cases	HC	benign	P value	reference
HSPB1	ELISA	579	53	-	37.8%	1.9%	-	p<0.001	[80]
HSP70	ELISA	369	53	-	40.9%	35.9%	-	-	[80]
HSP90	ELISA	125	-	-	36.8%	-	-	-	[78]
HSPD1	WB	40	42	-	47.5%	4.7%	-	p < 0.01	[81]
HSPD1	ELISA	107	93	-	31.8%	4.3%	-	p<0.0001	[82]
HSP90	ELISA	13	22	10	8%	0	0	-	[83]

HC: healthy controls; WB: western blot; ELISA: enzyme linked immunosorbent assay.

Using ELISA, in 1995, Conroy *et al.* conducted the first study to identify anti-HSP90 autoantibody in patients diagnosed with breast cancer. They found that antibodies targeting purified HSP90 were detectable in 46/125 (36.8%) breast carcinoma patients but not in healthy individuals, or patients with benign breast tumors. Furthermore, the presence of these antibodies was found to be correlated with the development of metastasis even in patients without axillary nodal involvement [78]. Then, in another study, they analyzed the correlation between anti-HSP90 AAb and mortality rate. They found that mortality rate from breast carcinoma was greater in women tested positive for AAbs against HSP90 than those tested negative [79]. This research group also identified anti-HSPB1 and anti-HSP70 antibodies in breast cancer, still using ELISA. One of the largest sample cohorts was evaluated with 579 samples tested for anti-HSPB1 Abs and 369 samples tested for anti-HSP70 Abs. In comparison, the number of healthy controls (53 healthy female) were limited. Results showed

that there was no significant difference in the frequency of anti-HSP70 antibodies in patients with breast cancer and healthy control subjects. In contrast, anti-HSPB1 antibodies were detectable in over one-third of breast cancer patients (37.8%) while only in one healthy individual ($P < 0.001$). Furthermore, the presence of anti-HSPB1 antibodies appeared to show a significant correlation with improved survival, particularly beyond the first 5 years [80]. Hamrita *et al.* used western-blot analysis on a cohort of 40 patients with invasive breast cancer and 42 healthy controls. A significantly higher frequency of anti-HSPD1 antibodies was observed in breast cancer patients group (19/40, 47.5%), compared to control serum group (2/42, 4.7%). Thus, they suggested that the presence of circulating anti-HSPD1 antibodies could display clinical usefulness as diagnostic markers for breast cancer [81]. This was confirmed by Desmetz *et al.* in a study including 49 ductal carcinoma in situ (DCIS) patients, 58 early stage breast cancer patients, 93 healthy controls, 20 other cancer patients and 20 autoimmune diseases [82]. Anti-HSPD1 antibodies were detected in 32.6% (16/49) patients with DCIS and 31% (18/58) patients with early stage breast cancer, compared to 4.3% (4/93) in healthy controls and 0% in other control groups. Furthermore, the presence of anti- HSPD1 antibodies had a close association with disease grade in DCIS. Indeed, Anti-HSPD1 antibodies were found in 11/23 patients (47.8%) with high-grade DCIS, compared to 5/26 patients (19.2%) with low-grade DCIS ($p=0.0188$). Anti-HSPD1 antibodies displayed a specificity of 95.7%, a sensitivity of 31.8% and AUC of 63.7% for discriminating breast cancer patients from healthy controls.

As tumor is a heterogeneous disease, the use of single AAb as diagnostic biomarker remains limited due to their low sensitivity and frequency ranging from 8% to 48%. Over the past 10 years several researches demonstrated that multi-AAbs panels could greatly improve cancer sensitivity detection while preserving reasonable high level of specificity.

1.3.1.2 Evaluation of AAbs panels including anti-HSPs antibody

Thanks to novel emerging proteomic techniques, like phage display, serologic identification of antigens by recombinant expression (SEREX), serological proteome analysis (SERPA), various biomarkers which could discriminate cancers from healthy controls were discovered. In the following, we present two studies on multi-AAbs panels including one or more anti-HSPs (as shown in Table 4). One study utilized ELISA and the other utilized protein microarray.

Table 4 *Frequency of AAbs panels in breast cancer patients and controls*

methods	panel	cases	controls	SN/SP	reference
ELISA	FKBP52, PPIA, PRDX2, HSPD1 and MUC1	142	93	60.5/77.2	[84]
PM	HSPD1, P53, Her2-Fc, NY-ESO-1 and HSP70	29	28	82.7%	[32]

HC: healthy controls; ELISA: enzyme linked immunosorbent assay; WB: western blot; PM: protein microarray; SN: sensitivity; SP: specificity; AUC: Area under curve.

As could be seen on Table 4, two panels of 5 antigens, including only HSPD1 as common antigen, were evaluated [84, 32]. Although the size (143 breast cancer serum for [84] and 29 breast cancer serum for [32]), composition of patient cohorts (60 early-stage primary breast cancer and 82 carcinoma in situ (CIS) for [84], no information for [32]) and the methods used (ELISA versus protein microarray) were very different, both studies reported sensitivity and specificity of breast cancer detection in the same range. Moreover, Desmetz *et al*. have shown that their combination of 5 antigens could discriminate CIS from healthy controls in women under the age of 50 years (receiver operating characteristic area under the curve (ROC AUC), 0.85; 95% CI, 0.61-0.92) [84]. This result is very important for young women with high risk of developing invasive and aggressive tumors.

Compared with the low frequency of single AAbs in breast cancer, multi-AAbs panels could greatly improve the sensitivity of cancer detection. For example, the maximal frequency of antibodies against a single HSPD1 is 47.5% [81], whereas a panel of 5 autoantibodies (anti-HSPD1 antibodies included) can increase the sensitivity up to 82.5% [32]. Therefore, screening a panel of biomarkers was indispensable in order to have high sensitivity.

1.3.1.3 Emerging trends of anti-HSPs AAbs detection in breast cancer

We compiled and compared data on anti-HSP AAbs frequency obtained from the various studies described above. Results are presented in Table 5. The most commonly studied anti-HSP AAb in breast cancer was anti-HSPD1, which was reported in 3 separate investigations.

Some studies on AAbs panels didn’t provide the frequency for each AAbs so the frequency is unavailable. We can only compare the frequency of autoantibodies against HSPD1 and HSP90 reported in two different studies. The frequency of anti-HSPD1 antibodies was quite reproducible (range from 31.8%-47.5%), however, almost 5 times discrepancy was obtained concerning anti-HSP90 antibodies (range from 8%-36.8%). Sample sizes maybe explain this

variation. One study involved 125 breast cancer patients [78] while another study contained only 13 patients [83]. Sample size is an important factor and large-scale investigations are obligatory for verifying the real frequency of autoantibodies in serum. Anti-HSPB1, anti-HSP70 and anti-HSP90 AAbs were also studied.

Table 5 *Comparison of anti-HSP AAbs frequencies in breast cancer*

HSPs	method	sample size (N)			AAb frequency %			reference
		cases	HC	benign	cases	HC	benign	
HSPB1	ELISA	579	53	-	37.8%	1.9%	-	[80]
HSPD1	WB	40	42	-	47.5%	4.7%	-	[81]
	ELISA	107	93	-	31.8%	4.3%	-	[82]
	ELISA	142	93	-	-	-	-	[84]
	PM	29	28	-	-	-	-	[32]
HSP70	ELISA	369	53	-	40.9%	35.9%	-	[80]
	PM	29	28	-	-	-	-	[32]
HSP90	ELISA	125	-	-	36.8%	-	-	[78]
	ELISA	13	22	10	8%	0	0	[83]

HC: healthy controls; ELISA: enzyme linked immunosorbent assay; WB: western blot; PM: protein microarray.

In addition to diagnostic marker, AAbs against HSPs are also associated with tumor prognosis and could be prognostic markers. For example, antibodies against HSP90 were associated with decreased survival [79] while anti-HSPB1 antibodies were associated with improved survival [80]. AAbs against HSPD1 were also found to be correlated with breast tumor stage. They are significantly higher in higher-grade ductal carcinoma in situ [82]. So the detection of autoantibodies against HSPs can not only discriminate the breast cancer patients from healthy controls, they could also provide prognostic values.

1.3.2 Autoantibodies against other TAAs than HSPs

In addition to AAbs against HSPs, various AAbs against other tumor antigens were also reported. Tumor antigens can, in general, roughly be divided into nine subgroups: decoy proteins, stem cell antigens, viral antigens, oncogenic proteins, over-expressed proteins, frameshift antigens, nucleic acid–specific antigens, ganglioside-like antigens, and cancer-testis antigens [85]. In the following, we firstly present TAAs and related AAbs that have

been reported to discriminate breast cancer patients from healthy donors; then TAAs to tailor-made panels of AAbs will be presented. Finally, we will summarize the frequency of each AAbs and the challenges that we are facing.

1.3.2.1 TAAs and related AAbs in breast cancer

Numerous tumor specific AAbs have been identified in the serum of breast cancer patients (Table 6), but only a few of these AAbs (anti-P53, anti-Her2/neu, anti-MUC1) have been examined in detail as potential diagnostic or prognostic markers, while other anti-TAA AAbs were only reported once. More than half of studies were based on ELISA technique for screening and evaluation of AAbs frequency. In the following, we will give an overview of these reported AAbs in breast cancer patients.

Table 6 *Frequency of autoantibodies in breast cancer patients, benign subjects and healthy controls.*

TAA	sample size (N)		AAb frequency %	Reference/year
	cases	HC		
P53	101	-	7.9%	[86] 1999
	2006	-	14.7%	[87] 2000
	158	-	19%	[88] 2003
	71	205	18.3%	[89] 2003
	144	242	21.5%	[90] 2005
	50	436	34%	[91] 2006
	25	879	16%	[92] 2009
	61	20	35%	[93] 2010
HER2	20	-	55%	[94] 1994
	107	200	11.2%	[95] 1997
	37	157	7%	[96] 2000
MUC1	24	-	8.3%	[97] 1994
	40[a]	96	37.5%	[98] 1996
	140[b]		25.7%	
	61[c]		18%	
c-myb	72	49	43%	[99] 1991
fibulin	20	20	75%	[100] 2002
RPA32	801	65	10.9%	[101] 2002
lipophilin B	74	20	27%	[102] 2003

	35 [c]		37.1%	
cyclin B1	7	27	42.8%	[103] 2005
survivin	46	10	23.9%	[104] 2005
livin			32.6%	
endostatin	36 [b]	24	66.6%	[105] 2006
	59 [c]		42.4%	
GIPC1	22	10	77%	[106] 2007
IGFBP2	80	200	5%	[107] 2008
AHSG	81	73	79.1%	[108] 2009
SPAG9	100	50	80%	[109] 2009
SOX2	282	194	18.4%	[110] 2012
	78 [a]		6.4%	
p90/CIP2A	168	88	19.1%	[111] 2014

HC: healthy controls, [a] benign breast tumor, [b] early-stage breast carcinoma, [c]advanced-stage disease

1.3.2.1.1 Most studied TAAs and related AAbs in breast cancer

Tumor protein P53 belongs to an over-expressed tumor antigen and received the highest interest among all TAAs. Accumulation of the mutant P53 in tumor cells can elicit a humoral immune response leading to the production of anti-P53 AAbs. The frequency of anti-P53 antibodies in breast cancers range from 7.9% to 35%, compared with low frequency in health controls. In 2000, Soussi summarized the literature from 1979 to 1999 on anti-P53 AAbs in serum of patients with various types of cancer. Significant difference was observed between breast cancer patients and healthy controls ($P < 0.0001$) for the presence of anti-P53 AAbs [87].

Moreover, three large-scale and multi-institutional studies were conducted to identify the frequency of anti-P53 AAbs in various type of cancers [92, 89, 91]. Altogether, 1345 cancer patients and 1520 healthy controls were involved. The average of the frequency of anti-P53 AAbs in cancers ranges from 15% to 24%; while its frequency in healthy controls is very low (0%-1.02%), especially in the results reported by Muller. M, et al [91]. They found that control group (436 healthy controls) was all negative for anti-P53 antibodies, which means that this antibody response occurred only in patients with malignant disease. Therefore, anti-P53 antibodies showed high specificity for malignancy. However, low sensitivity (range from 15% to 24%) prohibits the use of single anti-P53 antibody test to screen patients. The

combination of anti-P53 antibody test and the measurement of established conventional biomarkers can increase sensitivity. As reported in [91], the occurrence of anti-P53 antibodies is independent of the elevation of conventional tumor markers (CEA and CA15-3) with significant increase of sensitivity (6-11%) and without reduction of specificity in most cancers. However, in these three large-scale studies, only limited breast cancer patients were involved (the maximal sample is 71) [92, 89, 91]. In addition to large-scale studies summarized above, several studies identified antibodies against P53 only in breast cancers [93, 86, 88, 90]. The sample size ranges from 61 [93] to 158 [88] and the frequency of anti-P53 antibodies in breast cancer ranges from 7.9% [86] to 35% [93]. Sample size has a great influence on the frequency of autoantibodies. Generally, high sample size leads to low frequency.

The association of circulating anti-P53 antibodies with clinic pathological features was also studied and conflicting results were obtained. Dalifard *et al.* found that there is no association between anti-P53 antibodies and prognostic factors [86]. In contrast, several other studies found that autoantibodies against P53 were associated with shorter survival and advanced tumor stage [93, 88, 90, 91, 112]. Conflicting results were also observed concerning to the association of circulating anti-P53 antibodies with other features like hormone receptors. A. Kulic *et al.* found autoantibodies against P53 having significant association with tumor size and tumor histological grade, while no association with ER, PR and HER-2 [93]. On the contrary, T Nozoe *et al.* found no significant association between autoantibodies against P53 and tumor size or stage. Furthermore, appearance of anti-P53 antibody is associated with negative expression of ER, PR and HER2 [112]. As anti-P53 antibody is not the only component in predicting prognosis but instead the status of a network that interact with other biomarkers, so analyzing the interactions of different biomarkers is necessary. However, both two studies involved limited sample size (61 for [93] and 42 for [112]), larger-scale studies are needed to confirm their interactions.

All these studies showed that anti-P53 antibodies display high specificity (more than 95%) and low sensitivity (average is 20.8%). Therefore, combining anti-P53 antibodies with other biomarkers could increase the sensitivity without reducing specificity. Furthermore, circulating anti-P53 antibody is associated with bad prognosis and shorter survival.

HER2/Neu/ErbB2 is a member of the epidermal growth factor receptor (EGFR) family that is amplified and over-expressed in 20%–30% of breast carcinomas. HER2-positive is

associated with poor prognosis, due to high incidence of metastasis and resistance to endocrine and conventional chemotherapy in these patients. Treatment targeting HER2 in breast cancer has shown to be a useful strategy to significantly reverse the malignancy induced by HER2 over-expression [113]. Anti-HER2 antibodies have also been detected in breast cancer patients. One research group conducted 3 studies on anti-HER2 presence in breast cancer patients over a period of 6 years (from 1994 to 2000). Results indicated that anti-HER2 antibodies were significantly present in early-stage breast carcinoma patients compared to healthy controls [94, 95] and in higher level than in advanced-stage breast carcinoma patients [96]. These studies suggest that the humoral immune response to HER2 may have a role in limiting breast carcinoma progression.

Mucin (MUC) is a family of high molecular weight glycoproteins expressed on cell surface. MUC1 has been found to be expressed abundantly in breast cancer [114]. Circulating immune complex containing MUC1 has been detected in breast carcinoma but did not correlate with the stage of disease [97]. Anti-MUC1 antibodies were detected more often among women with benign disease than in women with breast cancer. Indeed, a negative correlation was observed between the presence of anti-MUC1 antibodies and the development of disease. These results suggested that a natural humoral immune response to MUC1 should be protective against disease progression, while lack of immune reaction could be associated with unfavorable outcome [98].

1.3.2.1.2 Other TAAs and related AAbs in breast cancer

Many studies have demonstrated the potential use of other autoantibodies for breast cancer diagnosis and prognosis. These molecules included AAbs against c-myb, fibulin, RPA32, lipophilin B, cyclin B1, survivin, livin, endostatin, GIPC-1, insulin-like growth factor binding protein 2 (IGFBP-2), AHSG, SPAG9, SOX2 and p90/CIP2A, as shown in Table 4. TAAs are involved in breast carcinoma through different mechanisms.

C-myb protein, Cyclin B1, cancerous inhibitor of protein phosphatase 2A (CIP2A) and insulin-like growth factor (IGF) are molecules that control the progression of tumor through cell cycle and apoptosis [115-118]. Antibodies against c-myb were present in the serum of 31/72 (43%) breast carcinoma patients compared to 12/49 (24.5%) healthy controls (P=0.036) [99]. The frequency of AAbs against cyclin B1 is much higher with 42.8% patients strongly positive [103]. However, only 7 patients were involved in this study and the relevance of

these data could be discussed. For antibodies against CIP2A, 168 breast cancer patients and 88 normal individuals were tested and higher autoantibody was found in breast cancer (19.1%) than in normal controls (2.3%) [111]. AAbs against IGFBP-2 were present in the serum of 4/80 (5%) breast carcinoma patients, compared with 2/200 (1%) healthy controls ($P = 0.032$) [107].

Replication protein A (RPA32) and GIPC1 are involved DNA and protein metabolism, respectively [119, 120]. Anti-RPA32 antibodies were significantly higher ($P < 0.01$) among breast cancer patients (10.9%, 87/801 patients) than among non-cancer controls (0 of 65 controls) [101]. A smaller study was conducted on anti- GIPC1 antibodies. Only 22 patients were involved and 77% (17/22) breast carcinoma patients were positive [106].

Other TAAs including fibulin, alpha 2HS glycoprotein (AHSG), SPAG9, Lipophilin B, survivin and livin are involved in other mechanisms and their autoantibodies were also studied. The sample size of studies ranges from 20 to 109 and the frequency ranges from 27% to 80% [100, 102, 104, 108, 109]. Among these five studies, the frequency of anti-SPAG9 autoantibody in breast cancer is relatively high (80%) even relatively large cancer cases (100) were involved [109], therefore, the authors concluded that anti-SPAG9 autoantibody may be useful serum biomarkers for breast cancer screening and diagnosis.

In addition to diagnostic value, the prognostic value of two antibodies (against Endostatin and SOX2) was also evaluated. Endostatin is natural inhibitor of angiogenesis and is over-expressed in metastatic cancer patients [121]. AAbs against endostatin were also elevated in breast cancer. Bachelot *et al.* showed that serum antibodies against endostatin were detected in 4/24 (16%) healthy women, 24/36 (66.6%) patients with localized breast carcinoma, and 25/59 (42.4%) patients with metastatic breast carcinoma. Differences were statistically significant between all breast carcinoma patients and healthy controls ($P < 0.0001$) and between localized and metastatic breast carcinoma patients ($P = 0.03$). Furthermore, anti-endostatin antibodies were associated with better survival in metastatic breast carcinoma patients. The median survival time of the 25 patients with detectable serum anti-edostatin AAbs was 20 months compared to 7 months for the other 34 patients ($P = 0.03$). Therefore, the author concluded that serum anti-endostatin antibodies is higher in patients with localized disease and is associated with a better prognosis in patients with metastatic disease [105]. SOX2 is an embryonic stem cell marker and plays a role in breast carcinogenesis [122]. Sun *et al.* studied the presence of circulating anti-SOX2 antibodies in serum from 282 breast

cancer patients, 78 benign breast disease patients, and 194 healthy women. Results showed that antibodies against SOX2 were present in 18.4% patients with breast cancer, in 6.4% patients with benign breast disease and in 2.6% healthy women. The circulating SOX2 antibodies were able to discriminate between breast cancer patients and healthy controls ($p< 0.001$) and between breast cancer patients and those with benign breast disease ($p< 0.001$). The prevalence of SOX2 antibodies was associated with higher tumor grade and positive nodal status [110].

Although numerous tumor specific AAbs have been detected in the serum of breast cancer patients, their frequency varies greatly (from 5% to 80%). The great variation maybe resulted by various factors including tumor heterogeneity, sample sizes, sample quality and origin, healthy individuals, method and proteins used. Furthermore, some of these AAbs were also found in other cancers. For example, elevated anti-P53 antibodies were detected in head and neck carcinoma (32%), esophageal carcinoma (30%), colorectal carcinoma (24%), and carcinoma of the uterus (23%) [70]; elevated anti-HER2 antibodies were detected in prostate cancer [73]. All these results underscore the questionable utility of a single autoantibody evaluation for breast cancer diagnosis and prognosis.

1.3.2.2 The use of anti-TAA AAbs panels in breast cancer diagnosis and prognosis

AAbs against TAAs display high level of specificity while sensitivity is low because of the heterogeneity of tumor. Recently, many researchers found that multi-AAbs panels can greatly improve sensitivity while preserving a reasonable high level of specificity. In this part, we give an overview of distinct panels of AAbs which were used to discriminate breast cancer patients from healthy controls. Analysis of the literature is presented in Table 7. Overall, 12 AAbs panels were evaluated for breast cancer detection with panel size ranging from 2 to 10.

Table 7 *Frequency of anti-TAA AAbs panels in breast cancer and healthy controls*

panel	methods	cases	controls	AUC	SN/SP	Reference/year
IMP1, p62, Koc, P53, c-MYC, cyclin B1, and survivin	ELISA	64	346	-	92%/85%	[123, 124]2003
survivin and livin	ELISA	46	10	-	52.2%/-	[104] 2005
p16, P53, and c-myc	ELISA	41	82	-	43.9%/97.6%	[125] 2006
P53, c-Myc, HER2, NY-ESO-1, BRCA1, BRCA2, and MUC1	ELISA	97[a]	94	-	64%/85%	[126] 2007
		40[b]		-	45%/85%	
MUC1, HER2, P53, and IGFBP2	ELISA	184[c]	134	-	31%/-	[73] 2008
P53, HER2, IGFBP-2, and TOPO2α				0.63	-	
ASB-9, SERUMC1, and RELT	ELISA	87	87	0.861	77%/82.8%	[127] 2008
FKBP52, PPIA, PRDX2, HSPD1 and MUC1	ELISA	60[a]	93	0.73	55.2/87.9%	[84] 2009
		82[b]		0.80	72.2%/72.6%	
RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1	ELISA	59[a]	61[b]	0.749	86.1%/75%	[128] 2012
GAL3, PAK2, PHB2, RACK1 and RUVBL1	ELISA	114	68	0.81	66%/87%	[129] 2013
p62, P53, c-myc, survivin, p16, cyclin B1, cyclin D1 and CDK2	ELISA	41	82	-	61%/89%	[130] 2013
FTH1 and hnRNPF	ELISA	150	150	0.816	91.1%/72%	[131] 2013

[a] primary breast cancer patients, [b] patients with ductal carcinoma in situ (DCIS), [c] advanced-stage disease, SN: sensitivity, SP: specificity, ROC – area under ROC curve.

Among all these AAbs panels, anti-P53 antibodies were screened in half of these studies [130, 123, 73, 124-126]. Good performance was obtained by [130, 123]. One of this study

identified antibodies against seven TAAs, IMP1, p62, Koc, P53, c-MYC, cyclin B1, and survivin in 64 breast carcinoma and 346 normal subjects. It yielded a sensitivity of 92% and specificity of 85% [123]. Another group studied 8 autoantibodies in 41 cancer patients and this panel reaches a sensitivity of 61% and specificity of 89% [130]. However, both studies could cause overfitting bias. Overfitting means that if a multi-markers panel is inappropriately large with respect to the number of cases evaluated, the prediction will be out of range because of noise. Generally, when the ratio of cases to markers is less than 10, it is considered of potential bias [71]. The ratio of cases to markers in both two studies was less than 10 (about 9 for [123] and 5 for [130]), so the high level of discrimination may be caused by overfitting bias. Low multi-markers panel can avoid overfitting bias; however, small marker panel will lead to low sensitivity, as obtained by [104]. Therefore, it is better to keep a reasonable ratio between multi-markers panel and the number of cases.

The AUC of three panels reached more than 0.81 and yielded good performance for discriminating breast cancer patients from healthy controls [127, 131, 129]. No common AAbs were observed in these three panels, which also indicated that the origin of tumor is an extremely complex process and various molecules were involved in tumor genesis. In [131], they identified autoantibodies against hnRNPF and FTH1 and results showed that this panel had a low specificity. However, when CA15-3 was added to the panel, it reached a sensitivity of 89.3% and a specificity of 93.8%. From this we can see that in order to keep the specificity at a high level, different kind of markers including autoantibodies and antigens can be combined.

Four studies gave detailed information about cancer patients and evaluated the association between AAbs panel and cancer stage [126, 84, 128, 129]. Three of them found that a panel of AAbs could be used in support to mammography for the diagnosis of early primary breast cancer, especially in younger women with high risk of breast cancer where mammography is known to have reduced sensitivity and specificity [84, 129, 126]. A panel composed of seven autoantibodies was found in 64% of primary breast cancer patients and 45% of patients with (Ductal carcinoma in situ) DCIS, at a specificity of 85% [126]. In [84], five AAbs panel could also significantly discriminated primary breast cancer (AUC=0.73) and CIS (AUC=0.80) from healthy individuals. Moreover, this combination can discriminate CIS from healthy controls in women under the age of 50 years (AUC=0.85). The results are very important for young women who are at high risk of developing invasive and aggressive tumors. However, it is to note that the number of patients under the age of 50 years was limited (n = 14), so more

investigations are needed to confirmed conclusion. Alain Mangé *et al.* identified another five AAbs panel and results showed that this panel can significantly discriminate early stage cancer from healthy individuals (AUC=0.81). Moreover, this value was high in both node-negative early-stage primary breast cancer (AUC = 0.81) and DCIS (AUC = 0.85) populations. Therefore, the authors concluded that this autoantibody panel could be useful in screening strategy of early-stage invasive breast cancer and pre-invasive breast cancer [129]. The same group also identified a panel of five AAbs in ductal carcinoma in situ (DCIS) and invasive breast cancer (IBC) patients. Results showed that this signature significantly discriminated DCIS from IBC (AUC = 0.794), with an overall sensitivity of 86.1% and an overall specificity of 75.0%. Furthermore, this panel could highly distinguish low-grade DCIS from high-grade DCIS exhibiting an AUC of 0.749. Moreover, the authors compared local recurrence and absence of recurrence in a population of DCIS patients followed for 5 years. Results showed that the autoantibody signature could divide the DCIS patients into a poor-prognosis group (local recurrence) and a good-prognosis group (recurrence free, P = 0.011). Therefore, they concluded that this autoantibody panel signature could have clinical implications for the management of DCIS, which may help to avoid the over-treatment of low risk patients and dictate more intensive treatment of aggressive DCIS [128]. If we compared these two studies conducted by the same group [128, 129], no common AAbs were observed in AAbs panels. This was maybe caused by the difference of cancer patients studied and it also reflects the fact that patients in different tumor stage have their own AAbs signature. Furthermore, it maybe caused by the different methods used for choosing AAbs. In [128], they used protein microarray for choosing AAbs panel while in [129], they used two-dimensional gel electrophoresis (2D-GE) and mass spectrometry (MS) to choose the potential AAbs panel.

Compared with low sensitivity of individual anti-TAA AAb, the combination of anti-TAA AAbs panel can greatly increase the sensitivity while preserved a reasonable high level of specificity. AAbs panels' signature could be particularly appropriate in complement to mammography for women with high breast density. Moreover, they could provide clinical implications for the management of breast cancer patients, thus avoiding the over-treatment of low risk patients and dictating more intensive treatment of aggressive tumors. Two points need to be noted. Firstly, methods used to identify AAbs panel need to be improved. Indeed, ELISA is mostly employed but this technique is not adapted for large screening multiplex detection. New technologies such as protein microarrays should be more efficient to screen AAbs panels. Secondly, over-fitting bias should be avoided. Over-fitting in biomarkers

analyses occurs when a multi-marker panel is inappropriately large with respect to the number of cases being evaluated. In this case, the prediction will be out of range because of noise. Thus in order to avoid overfitting bias, size of AAbs panel and sample should be well-designed.

1.3.2.3 Conclusion

Among all anti-TAAs studied, 9 received much interest (Table 8). Anti-P53 AAb was the most reported, following by HER2, MUC1, c-myc, Survivin, cyclin B1. As shown in Table 8, a discrepancy across studies about the frequency of single anti-TAA AAb was observed. This discrepancy could be explained by various factors. 1) Study population varies greatly among studies. 2) Definition of the cutoff value is an important factor that decides the performance of the test; lower cutoff value result in higher sensitivity and lower specificity, and *vice versa*.

Table 8 *Anti-TAA AAbs identified by multiple studies for breast cancer diagnosis*

Tumor antigen	Number of studies	Range of sample size (N)		Range of sensitivity across studies	reference
		cases	HC		
P53	14	25-2006	82-346	7.9%-35%	[130, 123, 73, 124-126, 86-93]
HER2	6	20-144	157-242	7%-55%	[73, 94-96, 90, 126]
MUC1	5	24-241	93-134	8.3%-37.5%	[97, 98, 126, 73, 84]
c-myc	4	41-137	82-346	13%-22%	[123-126, 130, 104]
Survivin	3	41-64	10-346	7.8%-23.9%	[124, 130, 123, 103]
cyclin B1	3	7-64	27-346	4.7%-42.8%	[124, 130, 123]
P16	2	41	82	12.2%	[125, 130]
P62	2	41-64	82-346	7.8%-12.2%	[124, 130, 123]
IGFBP2	2	80-184	134-200	5%-7%	[103, 73]

Although little is known about the origin of the immune response against tumor, an increasing number of articles have demonstrated that autoantibodies directed against tumor antigens have great potential for early diagnosis and prognosis of cancer. In recent years, more and more anti-TAA autoantibodies have been reported. However, their real diagnostic value needs to be further confirmed by large scale investigation. Compared with individual anti-TAA autoantibody screening, anti-TAA autoantibodies panels can greatly improve the sensitivity because of heterogeneity of tumor. For example, the frequency of single anti-P53 antibody ranges from 7.9% to 35%, while the combined analysis of antibodies to a panel of

TAAs can greatly increase the sensitivity. As reported in [123, 124], the combined analysis of antibodies to P53, IMP1, p62, Koc, c-MYC, cyclin B1 and survivin increased both diagnostic specificity and sensitivity to 92% and 85% respectively.

Among all these autoantibodies reported, we chose to screen autoantibodies against heat shock proteins (HSPs) family in breast cancer serum. Among HSPs family, antibodies against several HSPs were repoted, e.g. antibodies against HSPB1, HSPD1 and HSP90 were over-expressed in breast cancers, while no reports for antibodies against other HSPs. Therefore, we want to profile antibodies against HSPs family in breast cancer serum and test the diagnostic and prognostic performance of this antibody panel. Moreover, we added anti-p53 antibody in the antibody panel because it is the most studied antibody in breast cancer.

The need of large scale and autoantibodies panels ask urgently the transition of techniques because traditional ELISA is time consuming and laborious for parallel multiple screening. New screening methods like protein microarrays are more and more developed since last 30 years. These new techniques are capable of analyzing multiple samples in parallel and require tiny volume sample. So in the following, we will focus on protein microarray.

1.4 Protein microarrays

Microarray technology is a term that refers to the miniaturization of thousands of assays on one small plate. This concept was first introduced by Tse-Wen Chang in 1983 [132]. In the following decades, this concept was successfully transformed into the DNA microarray, a technology that determines mRNA expression levels of thousands of genes in parallel. However, DNA microarray technology possesses some limitations because mRNA profiles do not always correlate with protein expression [133]. Moreover, proteins are the major driving force in almost all cellular processes. Therefore, protein microarrays were developed as a high-throughput tool to overcome the limitation of DNA microarrays and to provide a direct platform for protein function analysis.

A general scheme of typical protein array experiment is provided in Figure 4. Various proteins are printed on a solid support. After washing and blocking un-reacted surface sites, the array is incubated with a sample containing a variety of unrelated proteins. After incubation, the interaction can be detected by two methods: label-free methods (mass

spectrometry, surface plasmon resonance, etc.) or label-dependent methods (fluorescence, chemiluminescence, etc.) [134].

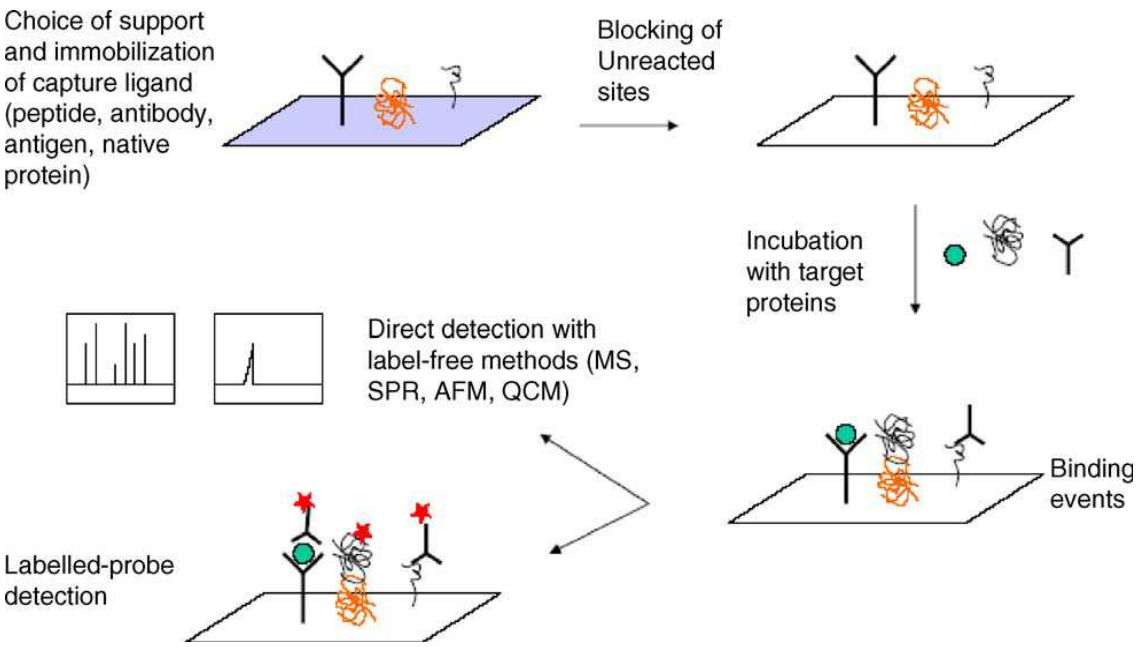


Figure 4 *General scheme of typical protein microarray experiment. A set of capture ligands (proteins, antibodies, peptides) is arrayed onto an appropriate solid support. After blocking unreacted sites of the surface, the array is probed by incubation with a sample containing the target molecules. If a molecular recognition event occurs, a signal is revealed either by direct detection or by a labelled probe. MS: mass spectrometry, SPR: surface plasmon resonance, AFM: atomic force microscopy, QCM: quartz crystal microbalance [134]*

Protein microarray provides a powerful platform for characterization of thousands of proteins in a highly parallel and high-throughput manner. It can be categorized into three major classes (Figure 5):

1) Analytical protein microarrays where antibodies or antigens are immobilized on the surface and used to detect proteins in sample by direct labeling or using secondary labeled antibody in sandwich assay format;

2) Functional protein microarrays where large number of purified proteins are immobilized on the surface for studying protein interactions (e.g. protein-protein, protein-lipid, protein-nucleic-acid and enzyme-substrate interactions);

3) Reverse-phase protein microarrays where cells, tissue or cell lysates are directly spotted on surface for the analysis of samples [135, 136].

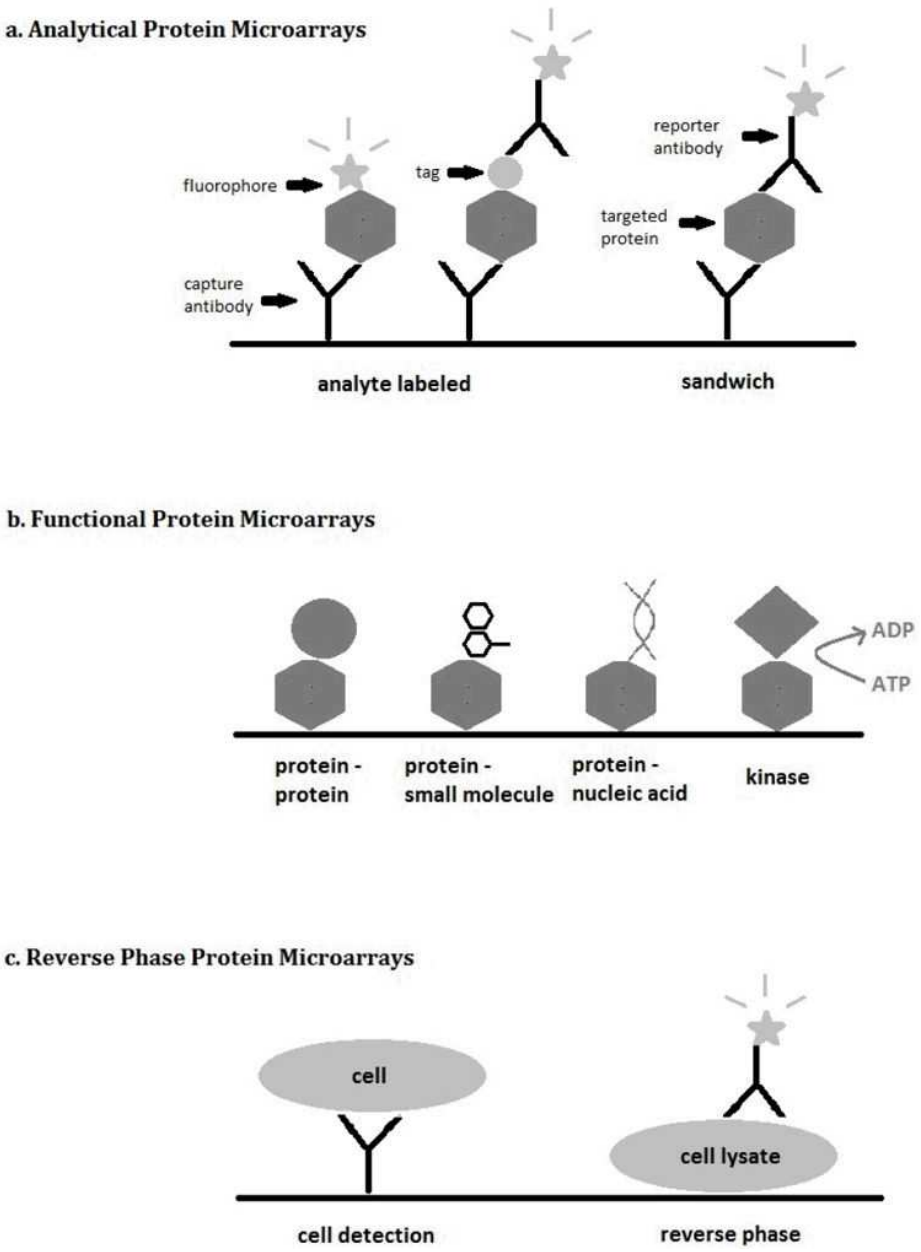


Figure 5 *Three categories of protein microarrays. (a) Analytical protein; (b) Functional protein microarrays; (c) Reverse-phase protein microarrays [135].*

Protein microarrays are presented as a very valuable tool for the study of whole proteomes, protein identification and profiling for early diagnosis of disease such as cancers, autoimmune diseases and viral infections. It is also widely used for drug identification, discovery and validation [136-140]. High throughput processing has made protein microarray become the trend due to cost reduction and high productivity of results. It is capable of speeding up new findings in protein interactions for basic research as well as clinical research purposes. Furthermore, reduction of sample volume usage is another important factor that demonstrates the superiority of this technology compared to other techniques like traditional ELISA. This factor is extremely important for clinical research in which samples are specious and limited

[60]. Despite the promising benefit, protein microarrays are still associated with numerous unsolved problems, mainly due to the complex nature of the proteins. So in the following, the challenges and limitations of proteins microarrays will be discussed.

1.4.1 Surface chemistry

Glass is typically preferred for optical sensors because of their transparency and low intrinsic fluorescence glass should be functionalized with different surface chemistries and this process is a crucial factor for the fabrication of protein microarray. An ideal surface should be compatible with diverse set of proteins while maintaining their integrity, native conformation, and biological function [141]. Unlike DNA, whose structure is uniform and exhibits a strong one-to-one interaction, the structure of proteins is much more complex and diverse, therefore, the requirements of surface for proteins' immobilization is more rigid. A good surface for proteins should satisfy the following requirements: (i) high binding capacity for target proteins and low binding capacity for un-target proteins; (ii) ability to retain activity of immobilized proteins;(iii) low variability between slides; (iv) high signal-to-noise ratios; (v) long stability for printed proteins [142].

The immobilization strategy chosen to attach proteins to the surface can greatly determine the properties of protein microarrays. There are three main strategies: physical adsorption, covalent biding and affinity based binding. Physical adsorption of proteins occurs *via* van der Waals or ionic interactions and hydrogen bonds between proteins and the chemical functions on the surface [143-145]. This is the simplest immobilization strategy; however, it is not easily controlled and may result in high variability. Covalent binding requires the presence of reactive groups on the surface allowing reaction with functional groups of proteins [146-148]. Depending on the reactive groups targeting on both surface and proteins, this strategy can led to random or oriented immobilization of proteins. However, due to the attachment to the slide at multiple sites, covalent binding is more likely to lead to a loss of activity of printed proteins. Affinity based binding lead to uniformly oriented proteins on surfaces. However, it requires a pre-treatment of printed proteins [149-151]. Each strategy induces advantages and shortcomings, as reported in Table 9. In the following, we will focus on the performance of commercial surfaces.

Table 9 *Immobilization strategies for protein immobilization*

Immobilization chemistry	Surface chemistry	Attachment site	Advantages	Disadvantages
Adsorption	Nitrocellulose, Poly-L-lysine, agarose, etc.	Electrostatic interactions, hydrogen binding, van derwaals interactions	The simplest immobilization	Random orientation, high background
Covalent binding	Maleimide, hydrazine, succinimidyl ester, epoxide, aldehyde, etc	Thiol, carbohydrate, amine	Robust immobilisation	Potential loss of activity of immobilized proteins
Affinity based binding	Protein A or G, streptavidin, glutathione, etc.	Fc region, biotin, GST tag, etc	Oriented immobilization	Pretreatment of spotted proteins

1.4.1.1 Commercial surfaces for protein microarray

There are a number of slides with various surface chemistries commercially available. In the following, we will present several representative studies to compare the performance of these commercial slides.

Eric W. Olle *et al.* compared 4 commercial slides (nitrocellulose FAST, hydrogel, SuperAldehyde and epoxy-silane ES) by printing one antibody on these solid slides. Based on spot fluorescence signal and background intensity, they concluded that the optimal slide substrate for antibody was epoxy-silane ES microarray slides [152]. Angenendt *et al.* have screened 11 different array surfaces by immobilizing five different antibodies onto each type of microarray support. Then they evaluated detection limit, inter- and intra-chip variation. Results showed that poly- L-lysine and aldehyde surfaces have good signal-to-noise ratios and low inter-field coefficients of variation (less than 20%). For polyacrylamide-coated slides, they have lower detection limits and are more suitable for the detection of very low concentrations of antigen [153]. They also investigated the properties of surface in the context of antigen microarray. Human serum albumin (HSA) was printed on 8 surfaces and mean signal to spotted concentration ratio, LOD and coefficients of variation were evaluated. Results showed that covalent binding on PEG-epoxy or dendrimer slides showed higher

signal intensities compared with non-covalently binding surfaces like amine slides or poly-L-Lysine slides [154]. These researches compared various surfaces and some of them, e.g. epoxy-silane, poly- L-lysine and aldehyde surface showed good property, however, limited proteins were evaluated. Considering the complexity and diversity of structures, these surfaces may be not applicable for other proteins.

More proteins were involved in recent studies. For example, Shannon L. Seurynck-Servoss *et al.* evaluate the performance of 23 different antibodies on 16 commercially available slides (major slide surfaces include aminosilane-coated slides, epoxysilane-coated slides, Full Moon slides, aldehyde silane slides, Poly-L-Lysine). Each antibody was spotted with 8 replications in the same experiment and 3 replicate experiments were conducted on separate days. They compared the different slide types based on spot size and morphology, slide noise, spot background, lower limit of detection and reproducibility. Results showed that the properties of the slide surface affect the activity of immobilized antibodies and the quality of data produced. Three dimensional slide surfaces tend to have higher background than two-dimensional surfaces, likely due to an inability to efficiently wash and/or block the surface. Furthermore, non-covalent chemistries for antibody immobilization work nearly as well as covalent ones [155]. This approach provides a rigorous and quantitative system for comparing the performance of commercial slide types.

In addition to antibody microarray, study on antigen microarray was also conducted. In order to identify the subtle differences of autoantibodies in serum samples, Balboni *et al.* optimized the surface conditions for their autoantigens microarray platform. Firstly, they spotted 10 autoantigens on 22 commercially available slide surfaces and evaluated overall background, uniformity, streaking, and smearing of features. Among all 22 slides, 10 slides were considered potentially suitable for printing autoantigen microarrays based on visual inspection. Secondly, these 10 surfaces were tested to confirm their suitability for autoantigen immobilization. For this round of screening, 6 antigens were spotted at different concentrations with eight replications. Considering the intra-slide and inter-slide CVs (less than 30%) and spotting smearing and streaking, FAST, poly-L-lysine and SuperEpoxy slides were chosen. However, the subsequent study found that many other antigens did not spot well on poly-L-lysine and SuperEpoxy slides, resulting to background streaking and smearing. Therefore, these two surfaces were not included for the following serum detection. Thirdly, they chose FAST and two additional surfaces (SuperEpoxy2 and PATH®protein microarray slides). Approximately 50 antigens were printed with 8 replications on each slide. Results

showed that after optimizing the major variables in autoantigen microarray platform, the variance within and among microarrays are low enough to allow detection of subtle differences within a patient over time and among patients [156]. The results are different from Angenendt *et al.*, which found that poly-L-lysine slides performed better than FAST slides. The discrepancy maybe caused by difference in spotting type and spotted antigen, among which Angenendt printed HSA with solid pins while Balboni printed 50 antigens (HSA was not included) with quill pins. Considering that they didn't analyze same antigen-antibody interactions, it is difficult to directly compare the results obtained by these two laboratories. Furthermore, it also indicated that the performance of surface slides was quite variable depending on antigen type.

Up to date, it is not possible to compare studies from different laboratories due to differences in experimental protocols and proteins used. However, one point is evident: no unique surface is suitable for all proteins' immobilization considering the complexity of protein structure. Each protein performs differently on each type of surface; therefore, it is needed to select optimal microarray coating based on experimental requirements.

1.4.2 Commercial protein microarray

Nowadays, a number of companies have developed commercial protein microarrays to detect and analyze proteins in human samples like serum, urine, tissue, etc. The commercialization of reagents and kits has undoubtedly contributed much to modern research, particularly in biological and clinical disciplines. It has enabled faster and higher-throughput experimental protocols, promoted higher uniformity and consistency between independent labs and helped to develop technologies and methodologies that would otherwise be inaccessible to individual labs. So in the following, several kinds of commercial protein microarrays that have been developed by big companies will be presented. Then their limitations and challenges will be discussed. Table 10 summarized representative commercial protein microarrays fabricated by several companies.

Table 10 *Panorama of commercial protein microarrays*

Category	Company	Products	Printed proteins	Replication of printed proteins	Price/ slide	Sample tested/ slide	Surface	Reference
Cytokine test	Whatman	FAST Quant TH1/TH2 arrays	Antibodies against 9 cytokines	3	525 €	16	Nitrocellulose	[157, 158]
		FAST Quant angiogenesis arrays	Antibodies against 9 cytokines	3	525 €	16	Nitrocellulose	[158]
	R&D system	Human Cytokine Array Panel A	Antibodies against 36 cytokines	2	128 €	1	Nitrocellulose	http://www.rndsystems.com/Products/ary005/Citations
		Human XL Cytokine Array	Antibodies against 102 cytokines	2	186 €	1	Nitrocellulose	No
	RayBiotech	Human Quantibod® Cytokine Arrays Q1	Antibodies against 20 cytokines	4	698 €	16	N.A	[159-162]
		Human Quantibod® Cytokine Arrays Q440	Antibodies against 440 cytokines	4	12900 €	16	N.A	[163]
Protein profiling	Invitrogen	ProtoArray® Human Protein Microarray	9,000 unique human proteins	2	1180 €	1	Nitrocellulose	http://www.lifetechnologies.com/fr/fr/home/life-science/protein-biology/protein-assays-analysis/protein-microarrays/technical-resources/literature-citations.html

	Sigma-Aldrich	Panorama® Antibody Microarray - Cell Signaling Kit	224 antibodies	2	discontinued	1	Nitrocellulose	http://www.sigmaaldrich.com/catalog/product/sigma/csaa1?lang=fr&region=FR
Cancer biomarker screening	RayBiotech	Human Gastric Cancer Biomarker Array Q1	Antibodies against 5 human gastric cancer biomarker	4	221 €	16	N.A	No
	Arrayit	OvaDx® Ovarian Cancer Diagnostic Test	N.A	N.A	N.A	N.A	N.A	No
Allergy microarrays	Thermo Fisher Scientific	ImmunoCAP ISAC	103 allergens	3	N.A	4	Polymer	[164-167]
	Arrayit	Allergy microarrays	123 allergens to IgE and 101 allergens to IgG	N.A	264$	1	N.A	No

N.A.: not available

1.4.2.1 Cytokines microarray

Among all these commercial protein microarrays, cytokine microarray is one of the most common kits and has been developed by several companies like Whatman, R&D Systems and RayBiotech, Inc. Cytokines play an important role in the understanding and treatment of diseases in many medical specialities and protein microarray technology is accelerating the rate at which researchers can obtain cytokine expression information.

Whatman has developed several commercial protein microarrays to test cytokine:FAST Quant human TH1/TH2 arrays, angiogenesis arrays, Human Cytokine II arrays. Each FAST Quant array contains 8 to 10 monoclonal antibodies with affinities for common human cytokines. The antibodies are arrayed in triplicate on each array. Each slide has 16 micro-wells and two wells were used to obtain standard curve which was generated by creating

dilution series from recombinant antigen mass standards. FAST Quant claims it exhibits sensitivity and reproducibility better than traditional ELISA. However, there are currently relatively few publications using FAST Quant technology. One study compared two multiplex sandwich ELISA procedures (FAST Quant and SearchLight) and a bead based assay (UpState Luminex). Results showed that all three kits differed from each other for different analytes and there was no clear pattern for any analyte studied. They concluded that results obtained from different systems cannot be combined and suggested that the dynamic range of the assay, sensitivity of the assay, cost of equipment, cost of consumables, ease of use and ease of data analysis need to be considered when choosing a system for use [157]. A more recent study was conducted by Gendie E. Lash using ELISA, FAST Quant human angiogenesis and TH1/TH2 arrays to detect cytokines. However, the author did not compare the results obtained from FAST Quant arrays and ELISA, thus the feasibility of this commercial array is unknown [158].

R&D system also developed two Human Cytokine Array kit. One kit is called XL Cytokine Array Kit and it contains 102 cytokine antibodies. R&D system claims that this array could detect multiple cytokines, chemokines, growth factors and other soluble proteins in cell culture supernatants, however, no publications were reported on this product. The other kit is called Panel A and it contains 36 different cytokine antibodies. Antibodies against human cytokines, chemokines, and acute phase proteins were spotted in duplicate on nitrocellulose membranes. Captured proteins are finally visualized using chemiluminescent detection reagents. This kit has been validated for analyte detection in cell culture supernatants, cell lysates, tissue lysates, serum, and plasma. Currently, more than 63 publications were reported on this product. Results showed that it is a powerful tool for cytokine screening in various disease conditions, like cancer [168-171] and inflammatory [172]. Furthermore, some studies validated the results by ELISA and found that ELISA data were consistent with and supported the data obtained from the Human Cytokine Array Panel A [173]. However, although array could determine the expression of multiple cytokines in a single sample, one membrane can only detect one sample, making large sample screening extra expensive.

RayBiotech Inc. has developed various series of cytokine Arrays including Quantibody®, L-Series, C-series, G-Series and E-Series. Among all these series, Quantibody® arrays were widely used by academic researches. So a detailed description about Quantibody® cytokine arrays will be presented as a representative in the following. Overall, 20 human Quantibody® cytokine arrays have been developed. The difference between different arrays is the size of

printed antibodies, which ranges from 20 (Human Cytokine Array Q1) to 440 (Human Cytokine Array Q440). The price ranges from 698€ to 12900€ correspondingly to the number of printed antibodies. Each slide has 16 micro-wells, among which eight wells were used to obtain standard curve and the resting 8 wells were used for sample detection. There are currently lots of publications using different type of Quantibody® Cytokine Arrays, more detailed information is shown on their website. RayBiotech Inc. has cooperated closely with various academic researches and been involved in many publications, which showed high quality of their commercialized products. However, results obtained by other independent research were not always satisfying. One study analyzed cytokine levels in tissue lysates using 4 different multiplex ELISA-based immunoassay arrays. They include Quansys BioSciences (microplate-based), Aushon Biosystems SearchLight (microplate-based), Milliplex MAP Sample (bead-based), and a RayBiotech Inc. (slide-based) kit. Overall, the Quansys Biosciences and SearchLight arrays screened several elevated cytokines, being more sensitive than traditional single ELISA kits. However, the Milliplex bead array technique and the RayBiotech slide technology did not measure any level of cytokines due to lower sensitivity [174]. Despite of this, we cannot conclude that RayBiotech slide is not suitable for cytokine screening, however, the variability often exists and validation by independent laboratories is needed.

1.4.2.2 Protein profiling microarray

Profiling of thousands of biochemical interactions can have wide applications including novel disease biomarker identification, drug target discovery and therapeutic antibody development. Several companies have developed commercialized products which are widely used by academic researches.

One of them is ProtoArray® Human Protein Microarray developed by Invitrogen. The newest version 5.0 contains over 9,000 unique human proteins individually purified and arrayed in duplicate under native conditions on nitrocellulose coated glass slide. This platform enables rapid profiling of thousands of biochemical interactions in as little as one day. According to their website, more than 110 publications utilized this product in different disease states including transplantation [175-177], various cancers [178-184], and autoimmune diseases [185-187]. However, discrepancy was observed when we compared independent similar researches. For example, both Michael E. Hudson *et al.* and Sacha Gnjatic *et al.* used ProtoArray® Human Protein Microarray to screen biomarkers in ovarian

cancer. In [182], they found that 94 antigens exhibited enhanced reactivity in cancer patient' sera relative to control sera. Then 4 antigens were selected and validated by using immunoblot analysis and tissue microarrays. Three of them were found to exhibit increased expression in cancer tissues relative to controls. In another study [183], 202 proteins were preferentially immunogenic in ovarian cancer sera compared with healthy controls. They validated 2 antigens by ELISA and results showed high degree of similarity between the two methods. However, no common biomarkers were found when we compared the top 15 antigens screened by these two studies. Difference on sample population (30 cancer cases for [182] and 51 cases for [183]), ProtoArray® Human Protein Microarray version (version 3.0 for [182] and version 4.0 for [183]) maybe could explain the discrepancy. However, it also showed that discrepancy exists between different laboratories even when they used products provided by the same companies. ProtoArray® Human Protein Microarray is a powerful tool for biomarker pre-screening, while further validation by classic methods like ELISA is needed.

Sigma-Aldrich has also developed Panorama® Antibody Arrays for protein expression profiling, among which Cell Signaling Kit was the mostly used by academic researches. The Cell Signaling array contains 224 different antibodies each spotted in duplicate on nitrocellulose coated glass slides. These antibodies represent biological pathways including apoptosis, cell cycle, neurobiology, cytoskeleton, signal transduction, and nuclear proteins [188]. It has been used to profile differential protein expression between normal and cancer patients, thus identifying novel potential cancer biomarkers, like in breast cancer [189-191], colorectal cancer [192], prostate cancer [193], lung cancer [194]. For breast cancer research, Julio E. Celis *et al.* used Panorama® Antibody Arrays to screen mammary adipose tissue and numerous proteins were identified, including signaling molecules, hormones, cytokines, and growth factors. Furthermore, these proteins were validated by immunoblotting [190]. In [191], they analyzed breast cancer cell line in order to find novel predictive biomarkers for target therapy. Relevant proteins were also validated by western blotting. All these researches showed that Panorama® antibody microarray is a powerful tool for profiling disease-state proteins and finding novel biomarkers of diseases, however, this array has been discontinued by Sigma-Aldrich due to lacking of sale.

1.4.2.3 Cancer biomarker screening microarray

Identifying cancer biomarkers can aid in diagnosing disease, estimating prognosis, and monitoring treatment. Recently several companies have developed some commercial cancer biomarker arrays. RayBiotech Inc. has commercialized array for gastric cancer detection which is called Human Gastric Cancer Biomarker Array Q1. Antibodies against 5 human gastric cancer biomarkers (CA19-9, CA72-4, CEA, Pepsinogen 1 and Pepsinogen 2) were arrayed on glass support in quadruplicate. However, their efficiency is unknown as there are currently no publications using this product.

Arrayit has developed OvaDx® for ovarian cancer diagnosis. Arrayit declaims that this test could monitor the response of the immune system during early stages of ovarian tumor development through measuring about 100 biomarkers in serum samples. It has high sensitivity (79.7%) and specificity (100%) for all types and stages of ovarian cancer. It is therefore an effective elective test for screening women at elevated risk for ovarian cancer. However, no publications were reported on this product.

1.4.2.4 Allergen testing microarray

Assessing allergen resources is of great importance for humans and currently lots of companies have developed allergen testing microarrays. ImmunoCAP ISAC is one of them developed by Thermo Fisher Scientific. It is a miniaturized immunoassay platform where allergen components are covalently immobilized in triplicates, on a polymer coated slide. Each slide contains 4 microarrays giving results for 4 different samples per slide. It allows the measurement of IgE antibodies to a fixed panel of 112 components from 51 allergen sources in a single step. ImmunoCAP ISAC has been used by several independent academic researches [164-167]. However, the sensibility of ImmunoCAP ISAC for latex allergen detection is lower compared with conventional methods [167]. Moreover, further improvements in threshold and better interpretation algorithms are needed to fully capitalize on the potential of microarray [166].

Arrayit has also developed protein microarray allergy tests. Allergens printed on standard glass substrate slides through covalent binding for testing 123 IgE and 101 IgG. Likewise, there is no publication for the products produced by Arrayit.

Several other companies have also developed similar commercial protein microarrays for research use only. However, assays sold as “for Research Use Only” are not regulated by the U.S. Food and Drug Administration or the equivalent European agencies as part of Health Technology Assessment. Therefore, the information provided by the manufacturer about the assay characteristics may not be adequate, and the analytical performance of the assay may not be fit for purpose [195]. Therefore, although commercially available kits might initially be viewed as a step forward by biomarker and proteomics researchers, the users of these kits are advised to proceed with great caution for several reasons.

Firstly, commercial protein microarray generally lack of replications, as shown in Table 10. The replication range from 2 to 4, which is not enough and reduce the feasibility of obtained results. Secondly, the maximal analysis of sample on each slide is 16; some slides can only analyze one sample, which reduces their functional utility and raises the actual cost per assay of interest. Thirdly, surface chemistry is also needed to be mentioned. As shown in Table 10, several commercial protein microarrays use nitrocellulose as surface. However, nitrocellulose induces some drawbacks such as considerable background auto-fluorescence in the visible spectrum using laser excitation [196]. Moreover, surface chemistry plays an important role in the performance of protein microarray, while for commercial protein microarrays, all proteins were printed on the same surface regardless of their high complexity and diversity. Therefore, future efforts should be made on using different surfaces for each targeted proteins and combining different surfaces on the same support.

All these limitations lead to the possibility of seeking custom array design or home-made printing proteins, which allows specific customization to research needs and solves the limitations. For example, we could choose printing proteins of interest instead of all proteins, thus reducing the cost; we could also increase replications of printed proteins for increasing the credibility of results obtained; furthermore, we could use different surfaces and increase number of sub-arrays on each slide.

1.4.3 Optimization of assay conditions

In addition to surface chemistry, a plethora of factors also influences microarray assay performances, such as the composition of the spotting buffer (additives and pH) [197-199, 152], humidity during spotting [200], concentration of immobilized proteins, incubation time

and drying of spotting solution [201], composition of blocking buffer and time of blocking [202, 203], buffer used for sample dilution, sample incubation time and so on [204]. A major challenge to multiplex sandwich assay development on microarray is the optimization of the assays and identifying critical parameters and optimal level for each of them.

1.4.4 Storage conditions of protein microarray

The storage of protein microarray is an important and necessary process for their development in routine use. Two main types of slides have to be considered for the storage: non-printed slides and printed protein microarrays. Various studies were reported on these two aspects.

For non-printed slides, the ability to store chemically functionalized slides prior to spotting is an important factor, since microarray technology allows mass production of slides and their subsequent continuous consumption in experimentation. Different surfaces behave differently under storage. Kusnezow compared the storage conditions of non-printed slides functionalized with (3-aminopropyl) trimethoxy silane (APTES) with cross-linker, and (3-glycidoxypropyl) trimethoxy silane (GPTS). These two types of surfaces were stored in two conditions: with or without argon. Then their performances were evaluated after 2 weeks, 1 month, 2 months and 4 months by printing 2 antibodies. Results showed that cross-linker-modified APTES slides slightly improved their performance following 2 months of storage without argon while no such effect could been seen when the slides were stored in argon atmosphere. In contrast, GPTS slides showed strong increase in signal intensities after two months of storage under argon. Without argon, less increase in signal intensities was observed. Furthermore, the performance of non-printed slides was greatly dependent on analyzed antibody. Among 2 printed antibodies, one is more affected by storage conditions [205]. Similarly, slides coated with poly-L-lysine produced better results if they were left untouched for about 1 month before being used for DNA array preparation [205]. In contrast, slides functionalized with NHS-ester are sensitive to water and performs worsen after storage unless stored under dry, cool conditions [142]. In a word, storage conditions of non-printed slides depend on surface chemistry and influences properties of immobilized proteins.

The conditions are more complex for the storage of printed protein microarray. Angenendt *et al.* immobilized 5 different antibodies on 2 types of surfaces: gel-coated surfaces and non-

gel-coated glass or plastic surfaces. Then they were stored under 2 conditions: dry at 4°C, and in blocking solution at 4°C. Results showed that all slides tested showed an unexpected increase of signal intensities after 2 weeks of storage. Furthermore, for all non-gel-coated surfaces, immobilized antibodies showed no significant loss of signal intensity over time, under these conditions tested. While for gel-coated slides, they showed higher signal intensities when stored dry at 4°C compared to those in blocking solution [154]. Kusnezow *et al.* also found that dry condition could keep better activity of printed antibody microarray. They compared storage conditions of printed antibody onto APTES and GPTS surfaces. Results showed that all microarrays could be used for at least 2 months of storage without any apparent deterioration of the performance parameters. As a matter of fact, an increase of signal intensities was observed similarly to the results obtained with the non-printed slides [205]. Based on this, Wu *et al.* also stored printed antibody microarrays at 4°C sealed under nitrogen for 1 month. Antibodies' activity decreased after 1 month of storage but reasonable high intensity can be maintained, especially when antibodies were printed with PVA additives [206]. In addition to antibody microarray, protein-protein interaction arrays and enzyme arrays were studied by Nath *et al.* [207]. They used 5 probe-target protein pairs and 3 enzyme-substrate pairs to optimize storage conditions. Results showed that unlike printed antibodies, enzymes were more sensitive to storage conditions. Printed β-Gal was completely inactive after 12 days of storage at 4°C and β-lac also lost significant amount of activity within this period. However storage of immobilized enzymes in 50% glycerol at -20°C retained their activity for up to 30 days.

In conclusion, storage efficiency of protein microarray depends greatly on surface chemistry as well as printed proteins. For printed protein microarray, the immobilization strategy between surface and proteins has great influence on the conditions of storage. Generally, immobilized proteins through covalent binding will not diffuse into the solution as may be possible with proteins just adsorbed onto the surface. Therefore, protein immobilization through physical adsorption favors dry storage conditions, e g. nitrogen or argon; in contrast, covalent binding favors storage in aqueous solutions containing glycerol, trehalose, polyvinyl alcohol addition (PVA). However, due to the diversity of proteins, it is needed to test each protein on each surface to select the best storage conditions based on experimental requirements.

1.5 Aims of the thesis

The goal of this research work concerns the development of customized multiplex protein microarrays to screen breast cancer biomarker panels for diagnosis, early detection of recurrence or therapy monitoring.

For this, three objectives have been defined:

- Firstly, although numerous candidate biomarkers have been reported as shown in the literature overview, only few biomarkers have been approved to be used in clinic. Furthermore, biomarkers used in routine are not effective for early diagnosis of breast cancer because of low sensitivity and specificity. Among potential biomarkers of interest, autoantibodies (AAbs) against HSPs seem to be good candidate biomarkers for breast cancer diagnosis and prognosis. Moreover, protein microarrays are powerful tools for the validation of candidate biomarkers**. So the first objective is to develop customized antigen microarrays to profiling anti-HSPs antibodies in breast cancer serum.**

- Secondly, uPA and PAI-1 are strong prognostic and predictive tissue biomarkers and have the highest level-of-evidence (LOE-1) in breast cancer. High level of uPA and PAI-1 are associated with high risk of recurrence and chemotherapy will contribute great benefit for patients. However, it is limited by the detection method-ELISA. ELISA requires at least 300 mg of fresh or frozen tissue. Protein microarray could overcome this limitation because it utilizes tiny sample volume. **So the second purpose is using antibody microarray to titrate uPA and PAI-1 in breast tumor tissue. We will also evaluate the performance of our protein microarray compared to commercial ELISA kit.**

- Last but not the least, because protein microarrays is influenced by various factors including storage conditions, concentration of spotted proteins, incubation time, composition of buffer, etc. **So we will optimize assay conditions of our customized protein microarrays (antigen microarray and antibody**

microarray). By optimizing these factors, the performance of protein microarray can be greatly improved.

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Chapter 2

Anti-heat shock proteins autoantibodies profiling in breast cancer serum using customized protein microarrays

2.1 Introduction

Heat shock proteins (HSPs) are induced by stress conditions such as heat shock, decrease in pH, hypersalinity, alcohols, heavy metals, oxidative stress, inhibitors of energy metabolism and fever or inflammation. They are molecular chaperones that act to assist other proteins' folding and maturation [1, 2]. HSPs are over-expressed in a wide range of human cancers leading to the production of autoantibodies against HSPs by the immune system [3, 4]. Indeed, elevated levels of anti-HSP autoantibodies (e.g. autoantibodies against HSPB1, HSPD1, HSP70 and HSP90) were found in breast cancer serum. Moreover, some of them (e.g. autoantibodies against HSPB1 and HSP90) were shown to be associated with tumor metastasis [5-11]. Therefore, the screening of antibodies against HSPs could provide information about tumor stage, development of metastasis, treatment efficiency for breast cancer patients. However, Enzyme-Linked Immunosorbent Assay (ELISA), the most commonly used format reported in the literature to screen interactions between antibodies and antigens, is expensive for multiplex analysis and consumes large amounts of biological products. In contrast, protein microarray could provide high throughput data by consuming only minute sample amounts [12, 13]. However efficient multiplex analysis still remains challenging due to biomarkers variability.

In this study, our purpose is to develop customized antigen microarray to detect anti-HSPs autoantibodies in breast cancer serum. Seven proteins belonging to HSP family (HSPB1, HSPD1, HSP70, HSP90, HSP110, HSPA5 and HSP90B1) and P53 were selected as antigens. This choice was driven in collaboration with the Institute of Cancer Research of Montpellier (IRCM). One of the key parameters that influence the performance of protein microarray is surface chemistry [14, 15]. In previous work, we have developed and characterized 6 surface chemistries for protein immobilization including carboxylic (COOH), N-hydroxy succinimide (NHS), chitosan, amine (APDMES), maleic anhydride (MAMVE) and carboxymethyl dextran (CMD) functionalized surfaces. We have shown that the extent of the interactions of immobilized antibodies with their antigens (and consequently the performance of protein microarray) was surface and protein-dependent [16]. Other parameters affect the performances of protein microarray such as pH of spotting buffer and concentration of spotted proteins [17]. Thus, firstly various immobilization conditions for each antigen were screened on the 6 different surface chemistries in order to determine optimal conditions allowing retaining biological activity. Then in these conditions, the presence of anti-HSP

autoantibodies was evaluated in 50 serum from breast cancer patients and 26 serum from healthy donors.

2.2 Materials and methods

2.2.1 Materials

Borosilicate flat glass slides were purchased from Schott. All chemicals were of reagent grade or highest available commercial-grade quality and used as received unless otherwise stated [16-17]. Chitosan was kindly provided by Dr. T. Delair (Laboratoire des Matériaux Polymères et Biomatériaux, Université de Lyon). Dextran (Mw=40000 g/mol) was obtained from Pharmacosmos and maleic anhydride-alt-methyl vinyl ether (MAMVE, Mw=216000 g/mol) from Sigma-Aldrich. HSPB1, HSP70, HSP90, mouse-anti human anti-HSPB1 antibody-biotin, mouse-anti human anti-HSP70 antibody-biotin and mouse-anti human anti-HSP90 antibody were purchased from Enzo life science (Switzerland); HSPD1, HSPA5, HSP90B1, HSP110, mouse-anti human anti-HSPD1 antibody-biotin and mouse-anti human anti-HSP90B1 antibody were obtained from Abcam (UK); P53 and mouse-anti human anti-P53 antibody-biotin were obtained from Sigma and Thermo Scientific (USA), respectively; mouse-anti human anti-HSPA5 antibody and mouse-anti human anti-HSP110 antibody were obtained from R&D Systems (USA); F555-labeled streptavidin was purchased from Invitrogen; Cy3-labeled goat anti-human antibody immunoglobulins G (IgG) and Cy3-labeled goat anti-mouse antibody IgG were purchased from Jackson Immuno Research (USA). All proteins were stored as aliquot at -20°C or -80°C following manufacturer specifications. Bovine serum albumin (BSA) lyophilized powder was obtained from Sigma.

0.01 M PBS or PBS 1X (pH 7.4) was prepared by dissolving the content of one pouch of dried powder in 1 L of ultrapure water. 0.02 M sodium carbonate buffers at pH 10.7 were prepared from 0.1 M NaHCO3 and 0.1 M Na2CO3 solutions in ultrapure water. 0.01 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH=6.2) was prepared by dissolving the content of one pouch into 1 L ultrapure water and adjust pH up to 6.2. Washing buffer contained PBS 1X and 0.1% Tween 20 (PBS-T) at pH 7.4. Blocking solution was prepared by dissolving 10 g of BSA in 100 ml of PBS-T 0.1%.

2.2.2 Serum samples

All human samples were prospectively collected between 2005 and 2007 at the CRLC Val d'Aurelle Cancer Institute (Montpellier, France) at the time of cancer diagnosis after obtaining written informed consent. Blood samples were centrifuged at 1250g for 5min, and the serum was stored at -80°C. For the multiplex immunoassay, 76 serum samples were examined: 26 healthy controls with negative mammograms, negative physical breast exams for at least 4 years, and no history of prior malignancy, and 50 patients who underwent surgery and had a histopathologic diagnosis of breast cancer. The tumor stage of breast cancer patients were summarized in Table 1.

Table 1 *Tumor stage of breast cancer patients*

Tumor stage	Number of patients
Stage I	24
Stage II	14
Stage III	10
Unknown	2
Total	50

2.2.3 Surface functionalization of glass slides

Microwells were generated on the surface of flat glass slides by photolithography and wet etching on the basis of previous work in our group [29]. There are 40 microwells on each slide. These microwells are homogenous with 3mm side length, 60 ± 1 µm depth, as well as 4.5 mm spacing between each well. The details of surface functionalization of glass slides are reported in [16-17]. Briefly, flat and microstructured glass slides were functionalized with 6 different chemistries: (Figure 1): Carboxylic surface (COOH) was obtained after hydrolysis of the tert-butyl esters from tert-butyl-11-(dimethylamino) silylundecanoate silanized surface (TDSUM surface); NHS surface was obtained from N-hydroxy succinimide activation of COOH surface; Chitosan surface was obtained by functionalization of the NHS surface with 1mg/ml chitosan solution; APDMES surface was obtained by silanization with (3-aminopropyl) dimethylethoxysilane; MAMVE and CMD surfaces were obtained by functionalization of APDMES surface with maleic anhydride-methyl vinyl ether copolymer

solution (1mg/ml in DMSO) and NHS-activated carboxymethyl dextran solution (1mg/ml in MES buffer), respectively.

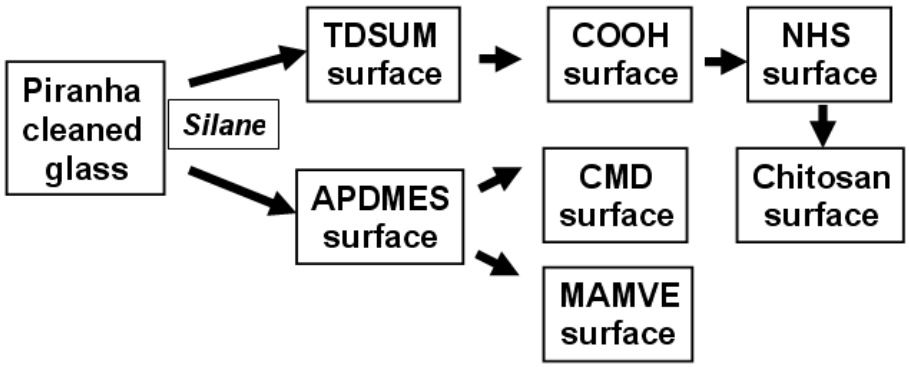


Figure 1 *Summary of surface functionalization of glass slides for protein microarray.*

2.2.4 Design of protein immobilization on flat glass slides

HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1, HSP110 and P53 were spotted (sciFLEX-ARRAYER S3, Scienion, Germany) onto chemically functionalized flat glass slides according to Figure 2. Each field contains one protein spotted at different concentrations (0.005mg/ml, 0.01mg/ml, 0.05mg/ml and 0.1mg/ml). For each concentration, 8 replicates were spotted. Streptavidin-F555 (0.01mg/ml) and buffer solution were spotted as reference for surface chemistry quality and negative controls, respectively. According to our previous results, carbonate buffer (pH=9.6) was used as spotting buffer on chitosan surface; acetate buffer (pH=4.5) was used on the other surfaces (COOH, NHS, APDMES, CMD and MAMVE surfaces). After spotting, proteins were allowed to react with surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 2 × 5 min with PBS, for 5 min with PBS-T (0.1%), and blocked with 10% BSA/PBS-T (0.1%) solution for 2h at room temperature (R.T.) to limit unspecific adsorption. Then slides were washed for 3 × 5 min with PBS-T (0.1%) and dried by centrifugation 3min at 1300rpm.

Slides were then incubated with 0.1 µM purified antibodies diluted in 4% BSA/PBS-T 0.1%. These antibodies include biotin-labeled antibodies against HSPB1, HSPD1, HSP70 and P53 and non-labeled antibodies against HSP90, HSPA5, HSP90B1 and HSP110. Each field was incubated with one antibody solution and left to react for 1h at R.T. in saturated water vapors; then slides were washed for 3 × 5 min with PBS-T 0.1% and dried.

Fields tested with purified biotin-labeled antibodies (biotin-labeled antibodies against HSPB1, HSPD1, HSP70 and P53) were then incubated with streptavidin-F555 (0.01 mg/mL diluted in 1% BSA/PBS); fields tested with purified unlabeled antibodies (antibodies against HSP90, HSPA5, HSP90B1 and HSP110) were incubated with Cy3-labeled goat anti-mouse IgG (0.01 µM in 1% BSA/PBS-T 0.1%). All incubations were left to react for 1h at R.T. in saturated water vapors and then slides were washed for 3 × 5 min with PBS-T 0.1%, 10 seconds in DI water and then dried.

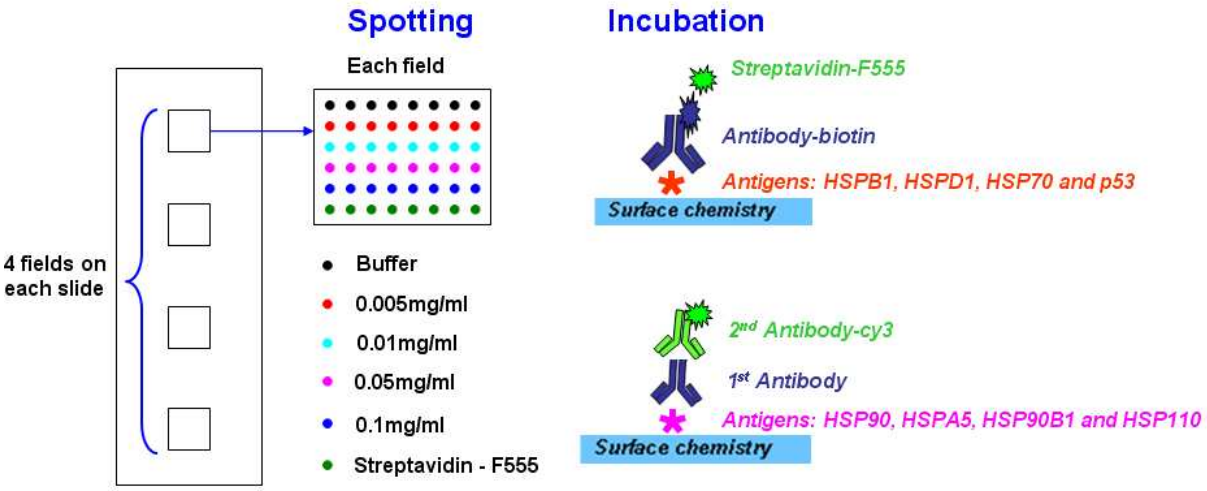


Figure 2 *Design of protein microarray. 4 proteins were spotted per slide. Each field includes buffer (negative control), streptavidin-F555 (quality control), one protein at 4 different concentrations; each solution has 8 replications. HSPB1, HSPD1, HSP70 and P53 were spotted on the same slide; HSP90, HSPA5, HSP90B1 and HSP110 were spotted on another slide. For the incubation, HSPB1, HSPD1, HSP70 and P53 were firstly incubated with biotin-labeled antibodies then with streptavidin-F555; HSP90, HSPA5, HSP90B1 and HSP110 were firstly incubated with non-labeled antibodies and then with Cy3-labeled secondary antibody.*

2.2.5 Multiplex immunoassays on microstructured protein microarray

HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1, HSP110 and P53 were spotted at their optimal concentration (sciFLEX-ARRAYER S3, Scienion, Germany) into microwells of COOH and chitosan functionalized glass slides, as indicated in Figure 3. On COOH surface, all proteins were spotted in acetate buffer (pH 4.5); on chitosan surface, carbonate buffer (pH 9.6) was used as spotting buffer; each protein was spotted in 5 replications. Buffer solution and streptavidin-F555 were spotted as negative and quality controls, respectively. After

spotting, proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 2 × 5 min with PBS, for 5 min with PBS-T (0.1%), and blocked with 10% BSA/PBS-T 0.1% solution 2h at R.T. to limit unspecific adsorption. Then slides were washed for 3 × 5 min with PBS-T 0.1% and dried by centrifugation 3min at 1300rpm.

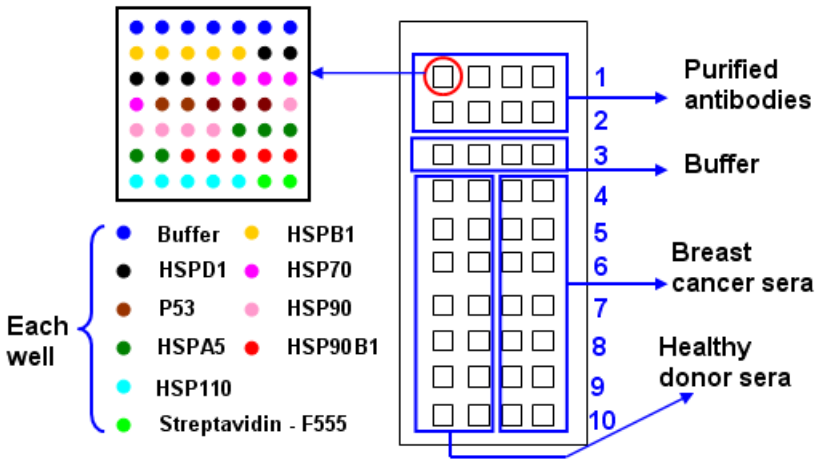


Figure 3 *Design of microstructured protein microarray. Each microwell contains streptavidin-F555, buffer solution, HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1, HSP110 and P53 spotted in 5 replicates. Line 1 and 2 were incubated with purified antibody solution for positive control; line 3 was incubated with buffer solution for negative control; lines 4 to 10 were incubated with serum from breast cancer patients and healthy donors.*

On each microstructured slide, 8 micro-wells were incubated with 0.1 µM purified antibodies diluted in 4% BSA/PBS-T 0.1% (one antibody/microwell). These antibodies include biotin-labeled antibodies against HSPB1, HSPD1, HSP70 and P53 and non-labeled antibodies against HSP90, HSPA5, HSP90B1 and HSP110. 4 microwells were incubated with buffer solution to evaluate nonspecific adsorption of detection antibodies. 14 microwells were incubated with breast cancer serum and 14 microwells with healthy donors serum (diluted with 4% BSA/PBS-T 0.1% at 1/200), as shown in Figure 3. Two cancer patients' serum and two healthy donor's serum were used as reference serum to normalize inter-slides data. Each microwell was incubated with one serum sample and then left to react for 1h at R.T. in saturated water vapors; then slides were washed for 3 × 5 min with PBS-T and dried.

Micro-wells tested with purified biotin-labeled antibodies (biotin-labeled antibodies against HSPB1, HSPD1, HSP70 and P53) were incubated with streptavidin-F555 (0.01 mg/mL diluted in 1% BSA/PBS); microwells tested with purified unlabeled antibodies (antibodies

against HSP90, HSPA5, HSP90B1 and HSP110) were incubated with Cy3-labeled goat anti-mouse IgG (0.01 µM in 1% BSA/PBS-T 0.1%); microwells tested with buffer were incubated with buffer; microwells tested with serum were incubated with Cy3-labeled goat anti-human IgG (0.01 µM in 1% BSA/PBS-T 0.1%). All incubations were left to react for 1h at R.T. in saturated water vapors, and then slides were washed for 3 × 5 min with PBS-T 0.1%, 10 seconds in DI water and dried.

2.2.6 Fluorescence scanning

After drying, slides were scanned with the Microarray scanner GenePix 4100A at wavelengths of 532 nm with the same photomultiplier tube (PMT) gain (PMT=600). Data mining was accomplished with GenePix 4100A software package (Axon Instruments). The fluorescence signal obtained for each antigen-antibody system was determined as the average of the median fluorescence signal of several replicates. The signal-to-noise ratio (SNR) was calculated as the ratio between the fluorescence signal of each antigen-antibody system and the fluorescence signal of buffer spots.

2.2.7 Data analysis

The sero-reactivities of breast cancer serum and healthy donor serum to immobilized antigens were compared using the Mann–Whitney test. Differences were considered statistically significant when $P < 0.05$. Individual and combined autoantibody performances were based on receiver operating characteristic (ROC) curves. The generalized ROC criterion finds the best linear combination (virtual marker) of tumor markers such as the area under the ROC curve (AUC) is maximized. Statistical analyses were performed using mROC [18].

2.3 Results

2.3.1 Optimization of tumor antigen microarray conditions

For the optimization of process, various concentrations of spotted tumor antigens HSPB1, HSPD1, HSP70, P53, HSP90, HSPA5, HSP90B1 and HSP110 were tested on the 6 different

chemically functionalized glass slides. The biological activity of immobilized tumor antigens was evaluated by measuring the extent of their interaction with their corresponding antibodies.

Figure 4 shows fluorescent images corresponding to HSP110/anti-HSP110 antibody interaction on the 6 surface chemistries. For each image, the top line corresponds to buffer spots and the following 4 lines correspond to 4 concentrations of spotted HSP110 (0.005 mg/mL, 0.01 mg/mL, 0.05 mg/mL, and 0.1 mg/mL). At low concentrations (0.005mg/mL, 0.01mg/mL), biological recognition between immobilized HSP110 and anti-HSP110 antibody was merely (not) detected, indicating that the surface density of HSP110 was too low for the efficient detection of the anti-HSP110 antibody. Above 0.01 mg/mL, the fluorescence signal increased with increasing concentrations of spotted HSP110 on all surface chemistries. Moreover, we can notice that fluorescent signal of buffer spots was not detectable (< 400 a.u.) indicating low non-specific adsorption.

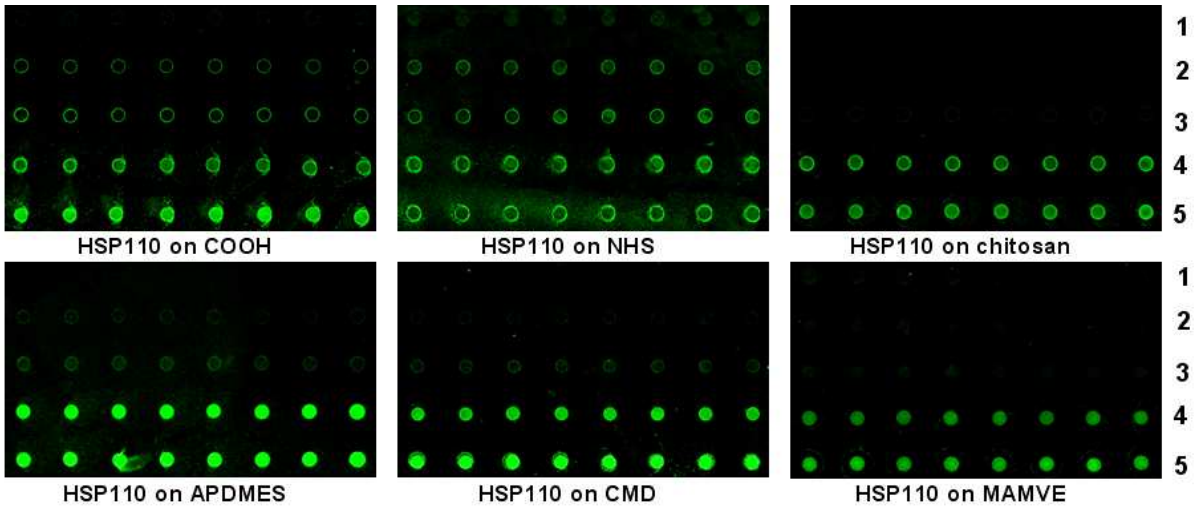


Figure 4 *Fluorescent scanning images of anti-HSP110 antibody detection on the 6 surface chemistries; line 1: buffer; line 2: 0.005 mg/ml, line3: 0.01 mg/ml, line 4: 0.05 mg/ml, line 5: 0.1 mg/ml (HSP110 spotting concentration).*

Figure 5 represents the fluorescent signal (SNR) obtained for the detection of anti-HSPD1antibody (Figure 5a) and anti-HSP70 antibody (Figure 5b) on the 6 surface chemistries, respectively. As expected, SNR increased with increasing of immobilized tumor antigen concentrations. Moreover, the dynamic range of SNR depended on microarray surface chemistry. The detection of anti-HSPD1 antibody and anti-HSP70 antibody on NHS, APDMES and MAMVE surface was not efficient whatever the spotting concentration of the tumor antigens. In contrast, the detection of anti-HSP antibodies on COOH and chitosan

surface displayed enhanced signal even when the concentration of spotted HSP was as low as 0.05mg/ml.

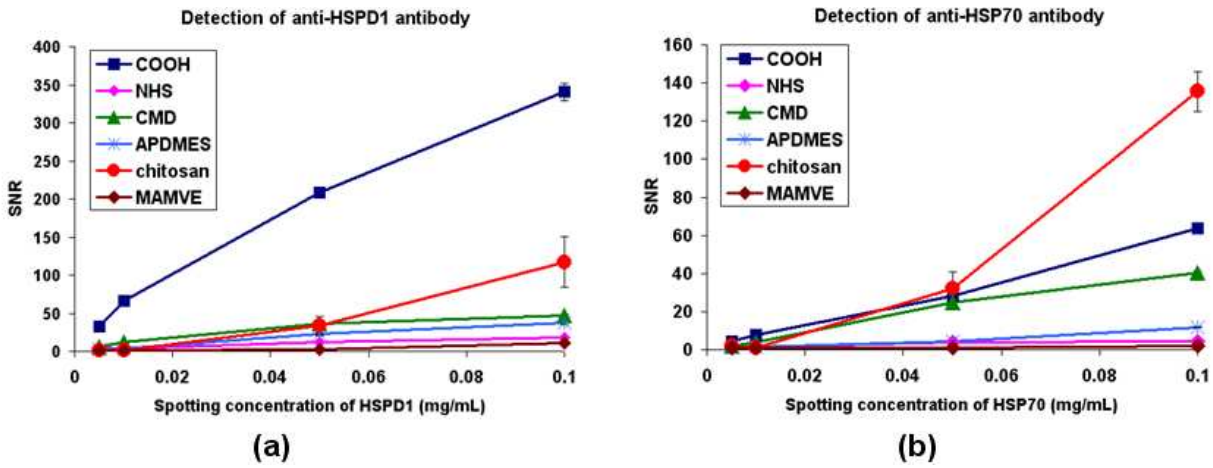


Figure 5 *Fluorescent signal analysis (SNR) of anti-HSPD1 antibody (a) and anti-HSP70 antibody (b) detection on 6 surface chemistries.*

Figure 6 gathered the results obtained for all studied antigens immobilized on the 6 different chemically functionalized glass slides, at 0.1mg/ml. For almost all antigens, NHS, APDMES and MAMVE surfaces displayed very low SNR even when the spotted antigen concentration is the highest, which suggest that these 3 surfaces didn't lead to efficient immobilization of HSPs and P53 allowing the sensitive detection of anti-HSPs and anti-P53 antibodies. In contrast, antigens immobilized on COOH and chitosan surfaces displayed enhanced detection of their corresponding antibodies. Thus surface chemistry greatly influences the performance of antigen microarray.

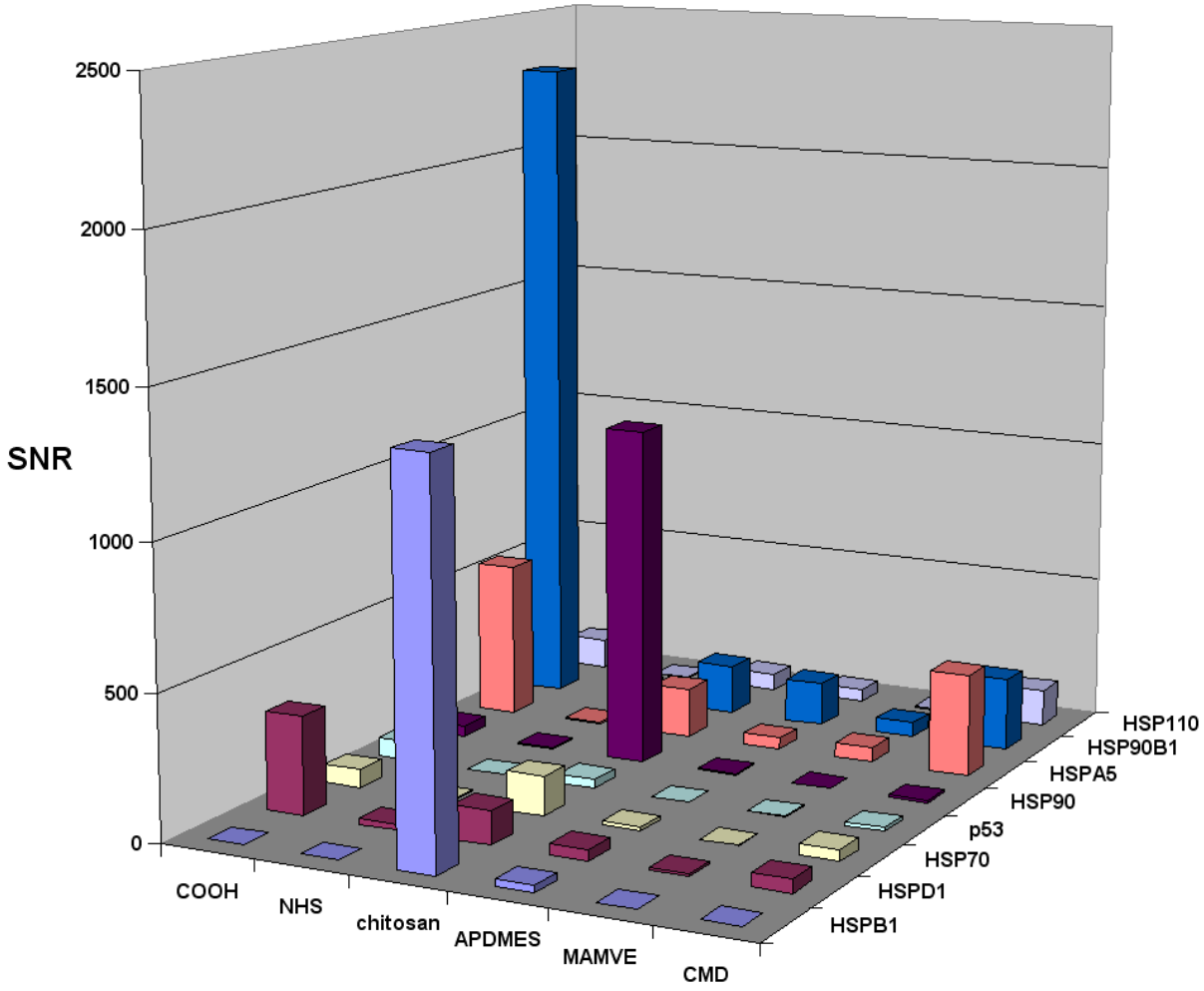


Figure 6 *Signal analysis of the recognition between 8 immobilized antigens and their antibodies on the 6 different chemically functionalized glass slides. Antigens were immobilized at 0.1 mg/ml.*

Among the 6 surface chemistries, two were composed of amino groups as functional groups (APDMES and chitosan), two were activated with NHS groups (NHS and CMD), one was composed of amine-reactive group (MAMVE), and one presented carboxylic groups (COOH). For COOH, chitosan and APDMES surfaces, the binding between surface and protein was achieved through physical adsorption; while on NHS, NHS-activated CMD and MAMVE surfaces, protein immobilization was achieved by covalent linking. Physical adsorption is the easiest way for protein immobilization and can exhibit excellent binding capacities. However, owing to random interaction, non-covalent binding does not allow to control the amount and orientation of immobilized proteins. Thus efficiency, accuracy and reproducibility of the immobilization process may be variable. Furthermore, the background level is usually higher due to a higher non-specific protein adsorption [19, 20]. Compared to physical adsorption, covalent binding represents a more robust approach. It requires the presence of reactive groups on the support which can react with probe molecules. Although

covalent binding enhances the stability of immobilized proteins, it can lead to partial loss of biological activity of proteins [21, 22]. Both binding strategies have advantages and shortcomings. Furthermore, the immobilization efficiency is closely dependent on probe proteins. There is no unique surface chemistry which is suitable for all proteins immobilization due to their highly diverse and complex structures. Our results showed that COOH and chitosan surfaces perform better for the detection of anti-HSP antibodies compared to other surfaces. This indicated that HSPs and P53 better retained their recognition activity towards anti-HSPs and anti-P53 antibodies when they were immobilized through physical adsorption. However, COOH and chitosan surfaces involved different kind of interactions as COOH is a silane monolayer with carboxylic groups, and chitosan is a polymer with amino groups. These opposite characteristics underlined the complexity of protein structure and interactions. Therefore, COOH and chitosan surfaces were selected for further evaluation of breast cancer serum (Table 2).

Next, we estimated the best immobilization concentration needed for each HSP and P53 on COOH and chitosan surface for efficient anti-HSP and anti-P53 antibodies detection. In order to reduce reagent consumption, and thus cost of the assay, we decided not to use the highest spotting concentration (0.1 mg/mL) except if fluorescence signal (SNR) is too low. From the variation of SNR with spotting concentration (Figure 5), the immobilization concentration for each HSP chosen is reported on Table 2. These concentrations allowed sensitive detection of anti-HSP and anti-P53 antibodies with high SNR values.

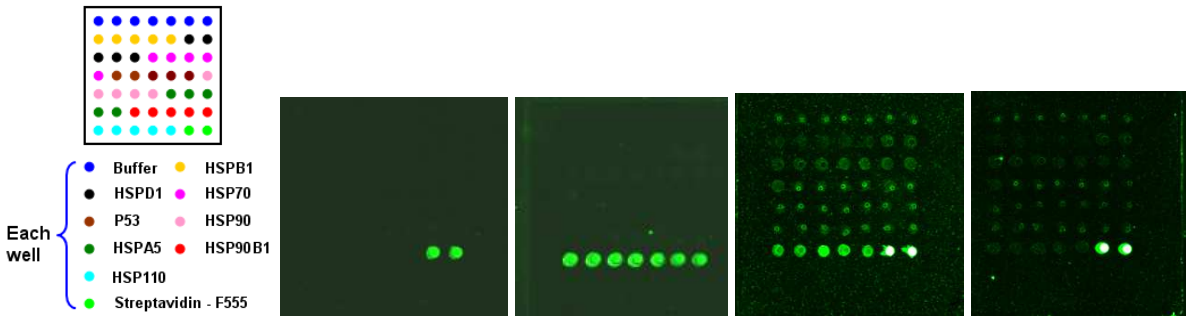
Table 2 *Optimal conditions for efficient immobilization of HSPs and P53 for screening anti-HSP and anti-P53 antibodies in breast cancer serum.*

Immobilized tumor antigen	Optimal surfaces	Optimal immobilization concentration
HSPB1	chitosan	0.05mg/mL
HSPD1	COOH	0.05mg/mL
HSP70	chitosan = COOH	0.05mg/mL
P53	COOH > chitosan	0.1mg/mL
HSP90	chitosan > COOH	0.05mg/mL
HSPA5	COOH > chitosan	0.05mg/mL
HSP90B1	COOH > chitosan	0.05mg/mL
HSP110	COOH > chitosan	0.05mg/mL

2.3.2 Detection of autoantibodies against HSPs and P53 in breast cancer serum by multiplex immunoassays on antigen microarray

2.3.2.1 Detection of autoantibodies against HSPs and P53 in breast cancer serum and healthy control serum

50 breast cancer serum and 26 healthy donor serum were tested for the presence of antibodies against HSPs and P53 by multiplex immunoassay on micro-structured glass slides functionalized either with COOH or chitosan. In Figure 7, were presented characteristic fluorescent images obtained on COOH surface after incubation with various solutions.



(a) spotting map (b) Buffer (c) anti-HSP110 antibody (d) breast cancer (e) healthy donor

Figure 7 *Fluorescence images of characteristic microwells of antigen microarray on COOH surface. (a) spotting map of each microwell, (b) microwell incubated with PBS 1X buffer solution (blank), (c) microwell incubated with purified anti-HSP110 antibody, (d) microwell incubated with breast cancer serum, (e) microwell incubated with healthy donor serum.*

Incubation with buffer solution allowed checking the quality of the surface chemistry, protein immobilization and non-specific adsorption (Figure 7b). Only the 2 spots of streptavidin-F555 were observed. Incubation with purified anti-HSP antibody allowed checking the biological activity of immobilized HSP and the cross-reactivity with the other antigen probes (Figure 7c). We can see that anti-HSP110 antibodies were well recognized by immobilized HSP110 on COOH surface and there was no cross-reactivity.

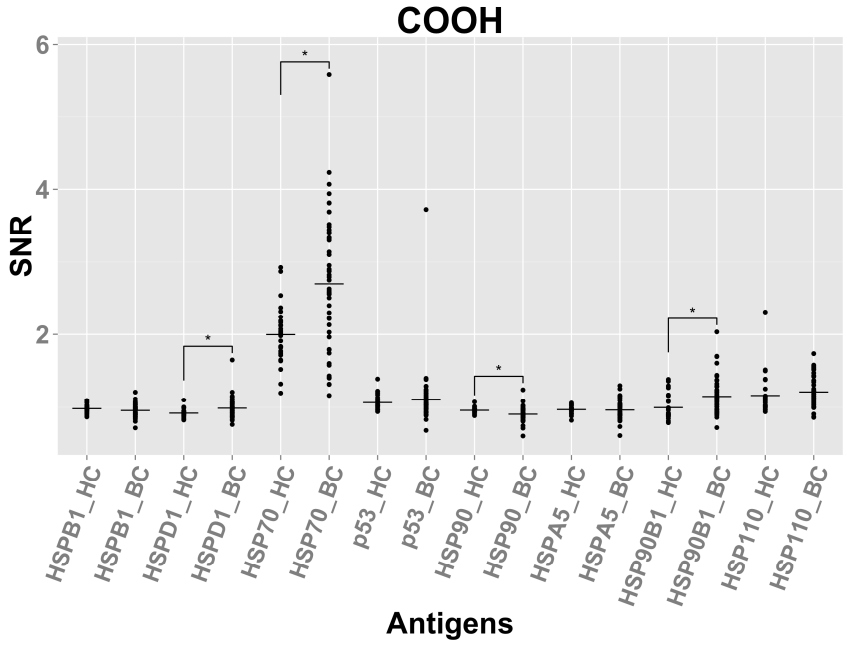
Incubation with breast cancer serum gaves the content in anti-HSP antibodies present in the tested sample (Figure 7d). In this example, breast cancer serum tested was only positive for the presence of anti-HSP110 antibody. Furthermore, the fluorescence signal is weaker compared to microwell incubated with purified antibody (Figure 7c). This was due to two reasons: on one hand, serum contained many proteins which could disturb the specific binding between antibody and its targeted antigens; on the other hand, the concentration of antibody

in serum was probably lower than purified antibody. In (Figure 7c), the concentration of purified anti-HSP110 antibody was 0.1 µM, according to the results of optimization, lower concentration of Cy3-labeled goat anti-mouse IgG (0.01 µM) was enough to detect purified anti-HSP110 antibody (chapter 4). As the concentration of anti-HSP110 antibody in serum was lower than purified antibody, therefore, 0.01 µM Cy3-labeled goat anti-human IgG was enough to test anti-HSP110 antibody in serum.

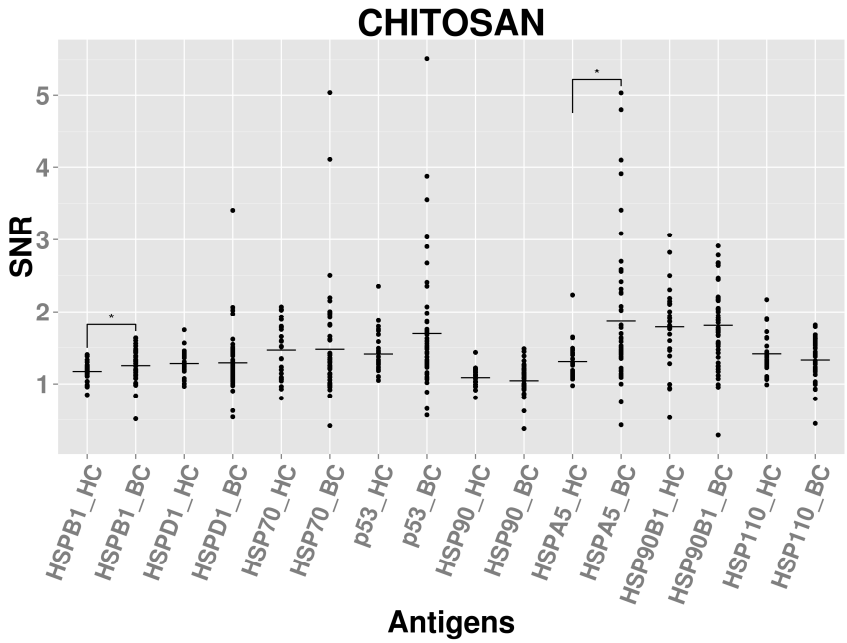
Incubation with healthy donor serum (Figure 7e) gaves the level of anti-HSPs antibodies below which the discrimination between healthy serum and cancer serum couldn't be done.

Thus, fluorescence intensities (SNR) of each tested serum (50 breast cancers (BC) and 26 healthy controls (HC)) were analyzed for the positivity to each spotted antigen (HSPs and P53) on each surface chemistry (COOH and chitosan surfaces). Results are presented in Figure 8 where each point corresponds to one serum sample. For each antigen probe, BC and HC groups were compared using statistic test (Mann-Whitney test) in order to determine if the 2 groups are statistically different for the presence of target antibody, with a probability higher than 95% confidence (p value < 0.05). On COOH surface (Figure 8a), the presence of autoantibodies against HSPD1, HSP70, HSP90 and HSP90B1 was significantly different between breast cancer patients and healthy controls ($p<0.05$). On chitosan surface (Figure 8b), the presence of autoantibodies against HSPB1 and HSPA5 was significantly different between the two groups of serum. No significant difference was obtained for the presence of autoantibodies against P53 and HSP110 between these two groups on both COOH and chitosan surfaces.

Optimal surface chemistries for the detection of anti-HSPD1, anti-HSPB1 and anti-HSP90B1 autoantibodies in serum were in accordance with conclusions drawn in Table 2. Indeed, COOH surface displayed the highest signal for detecting purified antibodies against HSPD1 and HSP90B1. Similarly, the detection of the anti-HSPD1 and anti-HSP90B1 autoantibodies in serum was significantly different between breast cancer patients and healthy controls on COOH surface. The same tendency was also observed for the detection of anti-HSPB1 antibody on chitosan surface. On the contrary, the detection of autoantibodies against HSP90 and HSPA5 was not consistent with the results obtained with purified antibodies. This inconsistence could be due to variable affinity between spotted antigen and purified antibody compared to antibodies present in serum. Moreover the complex nature of serum composition as opposed to purified model solution could induce changes in antigen/antibody interaction.



(a)



(b)

Figure 8 *Scatter plots of SNR values of autoantibodies against 7 HSPs (HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1 and HSP110) and P53 in breast cancer patients (n=50) and healthy controls (n=26); (a) and (b) represent the results on COOH and chitosan surfaces, respectively. HC: healthy controls; BC: breast cancer; *: p < 0.05 (p value was calculated by Mann–Whitney test).*

Taking into account these results, we considered each best surface chemistry/antigen probe couple which could discriminate between breast cancer serum and healthy control serum for

the presence of autoantibody, to construct the receiver operating characteristic (ROC) curve (Figure 9). The method of constructing ROC curve is shown in annex. For the detection of anti-HSP90 and anti-HSPA5 autoantibodies, we chose best surface chemistry as defined in Table 2. As individual markers, these autoantibodies showed relatively low sensitivity (<70%) and specificity (<70%) for discriminating breast cancer patients from healthy controls on both COOH and chitosan surfaces. Indeed, calculation of the area under ROC curve (AUC) for each tumor antigen/anti-tumor antigen antibody system (Table 3) indicated that it ranged from 0.581 to 0.732. Biomarkers displaying AUC≤0.75 are not clinically useful whereas biomarkers displaying AUC≥0.97 has very high clinical value [23]. However, the combination of autoantibodies panel (black line in Figure 9) displayed significantly improved performance for discriminating breast cancer patients from healthy controls. It achieved a sensitivity of 86% and a specificity of 100%. Expectedly, at higher sensitivity values, the overall specificity of the panel dropped to 96% (at 90% sensitivity) and to 70% (100% sensitivity). Moreover, AUC of 0.978 was obtained with the combination of autoantibodies panel which is nearly to best diagnosis test.

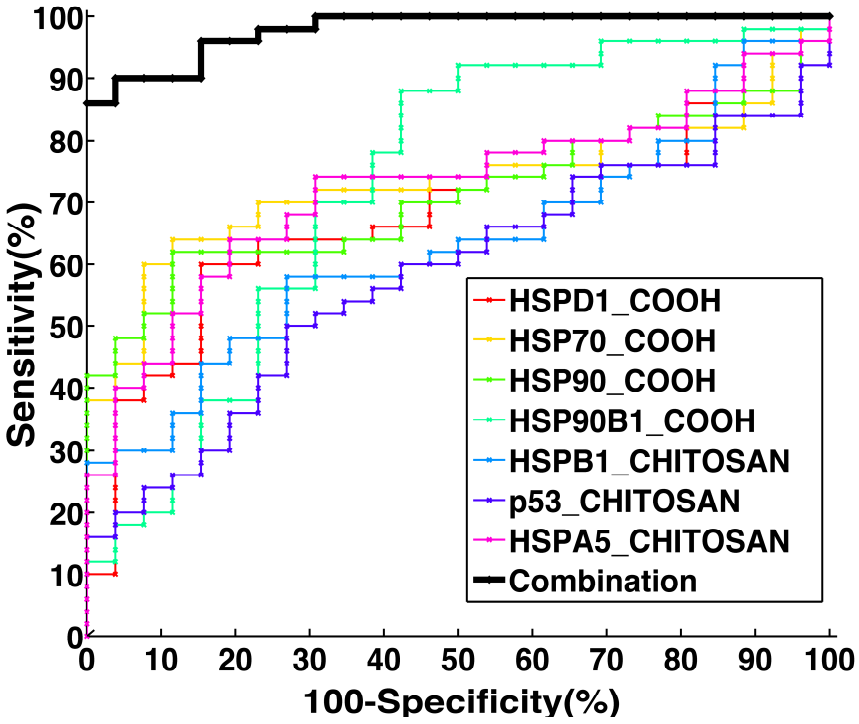


Figure 9 *Receiver operating characteristic (ROC) curve analysis of individual auto-antibody and combination of 7 auto-antibodies to discriminate breast cancer patients from healthy controls. The detection of auto-antibodies against HSPD1, HSP70, HSP90 and HSP90B1 was obtained on COOH surface; the detection of antibody against HSPB1, P53 and HSPA5 was obtained on chitosan surface*

Table 3 The area under receiver operating characteristic curve (AUC) of individual antibody and combination of 8 antibodies to discriminate breast cancer patients from healthy controls

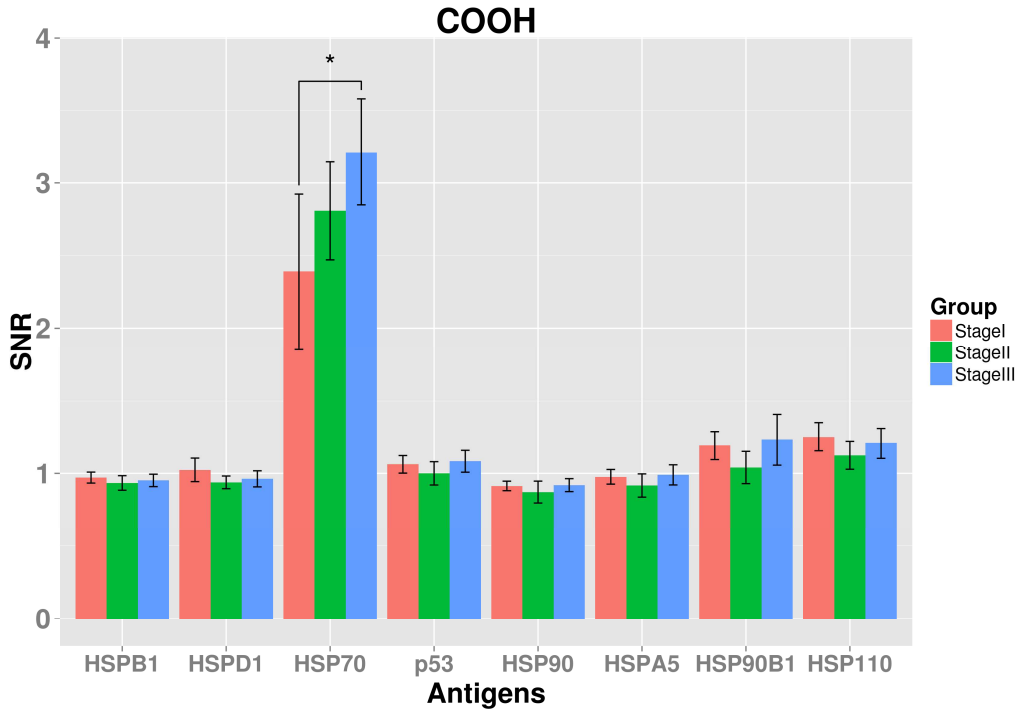
Anti-HSP antibody	AUC (95% CI)
Anti-HSPB1 antibody*	0.631 (0.528-0.743)
Anti-HSPD1 antibody**	0.683 (0.581-0.773)
Anti-HSP70 antibody**	0.732 (0.663-0.817)
Anti-P53 antibody*	0.581 (0.459-0.695)
Anti-HSP90 antibody**	0.710 (0.621-0.837)
Anti-HSPA5 antibody*	0.723 (0.567-0.836)
Anti-HSP90B1 antibody**	0.728 (0.608-0.837)
Combination of 7 antibodies	0.978 (0.938-1.000)

**The detection of antibodies was obtained on chitosan surface, **the detection of antibodies was obtained on COOH surface.*

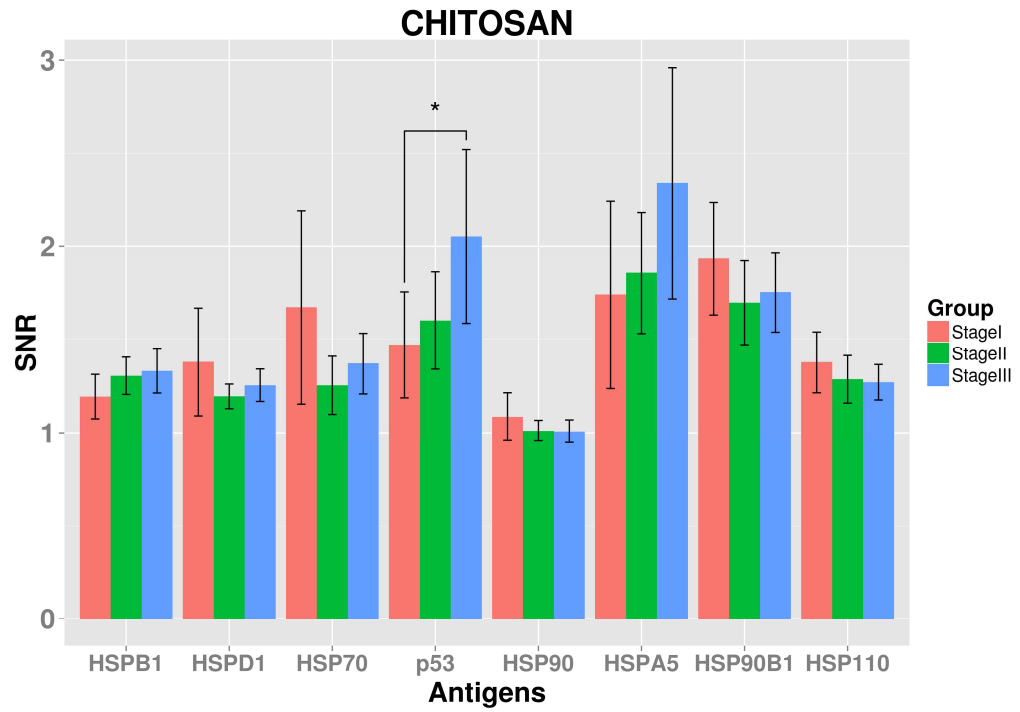
2.3.2.2 Association of anti-HSPs autoantibodies profile with tumor stage

We also assessed the correlation between the presences of autoantibodies against HSPs and P53 with tumor stage (Figure 10).

On COOH surface (Figure 10a), only the presence of autoantibodies against HSP70 was significantly different between tumor stage I and stage III ($p<0.05$) indicating that these autoantibodies could be use as prognosis biomarker. As well, the presence of autoantibodies against P53 was significantly different on chitosan surface (Figure 10b) between Stage I and stage III, and thus could be defined as prognosis biomarker. For the other auto antibodies tested, their distribution was not significantly different between all stages of breast cancer. Thus, the presence of anti-HSP antibodies seemed to be independent of tumor stage, which suggested that the detection of autoantibodies against HSPs could be useful for the diagnosis of early stage of breast cancer. However, further study with large scale population is needed to confirm this speculation.



(a)



(b)

Figure 10 *Correlation between the presence of autoantibodies against HSPs and P53 with tumor stage obtained on COOH(a) and chitosan (b) surfaces; *: $p < 0.05$ (p value was calculated by Mann–Whitney test).*

2.4 Discussion and Conclusions

In this study, we have demonstrated that customized protein microarrays could be powerful tools for the rapid screening of cancer biomarkers. The performance of protein microarray is influenced by many parameters such as surface chemistry, spotting buffer and protein concentration. 6 different surface chemistries were evaluated for the immobilization of 7 proteins belonging to the heat shock protein family and one oncoprotein, P53 in various conditions. To retain biological activity of the immobilized antigen protein, the best conditions (surface chemistry and the concentration of immobilized antigens) were determined to implement miniaturized immunoassays. Two surface chemistries (COOH and chitosan) were selected and used to detect anti-HSP and anti-P53 autoantibodies in 50 breast cancer serum and 26 healthy donors' serum. The frequency of autoantibodies against HSPs reported in literatures was compared to our results (Table 4).

Table 4 *Frequency of single anti-HSP autoantibody detected in breast cancer patients and healthy controls.*

HSPs	Methods	Sample size		AAb frequency %		p-value	Reference
		Cases	HC	Cases	HC		
HSPB1	ELISA	579	53	37.8%	1.9%	p<0.001	[6]
	PM	50	26	8%	0	0.049*	Our study
HSPD1	WB	40	42	47.5%	4.7%	p<0.01	[8]
	ELISA	107	93	31.8%	4.3%	p<0.0001	[9]
	PM	50	26	14%	3.8%	0.01**	Our study
HSP70	ELISA	369	53	40.9%	35.9%	N/A	[6]
	PM	50	26	34%	0	0.002**	Our study
HSP90	ELISA	125	N/A	36.8%	N/A	N/A	[7]
	ELISA	13	22	7.7%	N/A	N/A	[10]
	PM	50	26	4%	0	0.002**	Our study

*HC: healthy controls; ELISA: enzyme linked immunosorbent assay; PM: protein microarray; WB: western blot; N/A: not available; * Results obtained on chitosan surface, ** Results obtained on COOH surface.*

Only 4 anti-HSPs autoantibodies studied in our work were evaluated by others. Results obtained for anti-HSP70 were very close between our antigen microarray and ELISA, even if sample size were very different. The frequency of anti-HSP90 found in our study was also in

accordance with ELISA determination, only for similar sample size (less than 50 breast cancer cases and about 25 healthy controls). However, if sample size if different (2 times more in ELISA study), then the frequency of anti-HSP90 was very different suggesting that the size of the sample is a very important parameter in the reliability of the study. For the 2 other autoantibodies, anti-HSPB1 and anti-HSPD1, frequencies obtained in our work were very different from those obtained with ELISA or western-blot analysis by other groups.

As discussed above, these differences could be explained by the size and origin of the cohort: among researches, cohorts vary from 13 to 579 patients [6, 10]. The more extent is the cohort, the more reliable should be the results. The definition of the cut-off value could also lead to variable results. Indeed, cut-off value is defined as a value greater than the mean of the healthy individuals plus two standard deviations (S.D.) [9], or mean plus three standard deviations [7]. Generally, lower cut-off value results in higher sensitivity and lower specificity, and *vice versa*. Another parameter which could affect frequency of autoantibodies is the tumor stage: some studies [6, 8, 9] provide detailed characteristics about breast cancer patients, like histologic stage, lymph node status, etc; while others didn't provide any information [10, 24]. However, as the frequency of some anti-HSPs autoantibodies could vary with tumor stage, such as anti-HSP70, the choice of patients' serum and qualification are very important for data analysis and conclusion. At least, we could notice that the study methods could influence results obtained. As ELISA and western-blot analysis are gold standard methods for evaluation of immune response, they are mostly chosen by researches. However, with high throughput analysis, they became awful to use and expensive. Thus antigen microarrays could be an advantageous alternative, but process need to be standardized in order to be used in clinical trials.

Furthermore, due to the heterogeneity of tumor and individual immune response, the detection of one single autoantibody didn't allow to significantly discriminate breast cancer serum from healthy serum, whereas combining 7 autoantibodies (autoantibodies against HSPB1, HSPD1, HSP70, P53, HSP90, HSPA5 and HSP90B1) increases the sensitivity of the detection to 86% and specificity to 100%. Thus our antigen microarray achieved good sensitivity as well as the highest specificity, which is comparable with other studies obtained through ELISA [25-28]. Moreover, compared to ELISA, our customized antigen microarray is capable of providing high throughput data by consuming smaller sample amounts.

In the future, larger cohort of healthy donors and breast cancer patients are needed to validate performances of our antigen microarray. Furthermore, we will also try to detect other

antibodies against common reported antigens like HER2, MUC1. Thus, progress in technology associated to standardization effort should lead to an emerging and powerful tool for cancer diagnosis and prognosis in clinical assays.

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Chapter 3

Antibody microarray for the quantification of uPA and PAI-1 in breast tumor tissue

3.1 Introduction

As reported in Chapter 1, Urokinase type plasminogen activator (uPA) and its main inhibitor plasminogen activator inhibitor 1 (PAI-1) are involved in many human cancers, including those of breast, prostate, lung, brain, ovary. They participate in a wide variety of physiologic and pathologic processes, e.g. tumor growth, invasion and metastasis, through their effect on angiogenesis and cell migration [1, 2]. Various retrospective and prospective studies have shown that uPA and PAI-1 are good prognostic and predictive biomarkers in breast cancer. Low levels of uPA (≤3 ng/mg of protein) and PAI-1 (≤14 ng/mg of protein) are associated with low risk of recurrence and no benefit of chemotherapy for breast cancer patients. On the contrary, high levels of uPA and PAI-1 is correlated with high risk of recurrence and adjuvant chemotherapy provides substantial benefit for breast cancer patients [3-6]. Recently, these two biomarkers have been demonstrated having the highest level-of-evidence (LOE-1) for providing the prognostic and predictive value for node-negative breast cancer patients [7].

Currently ELISA is the only method which is recommended by ASCO to detect the concentration of uPA and PAI-1 in protein extraction from fresh or frozen breast tumor tissue. The commercially available ELISA test (Femtelle®) was developed by Sekisui Diagnostics. This kit has a high sensitivity. The lower limit of detection (LOD) of the assay for uPA and PAI-1 are 25pg/ml and 125pg/ml of sample respectively. However, it requires a minimum of 100-300 mg of fresh or frozen breast cancer tissue [8]. The need for large quantity of tissue requires surgical biopsy or vacuum-assisted core biopsy with an 8-gauge needle [9] and precludes the use of 14-gauge needle-core biopsies that are more common in clinical practice [10]. Indeed, requirement of large volume of fresh tissue becomes the main limitation of ELISA assays. Considering the challenges faced by ELISA, several other assay formats were also used including immunohistochemistry (IHC) [11] and analyzing mRNA levels [12-15]. However, none of them has been proven to be a reliable substitute for ELISA assay.

Protein microarrays have several advantages compared to traditional ELISA including high sensitivity and tiny volume sample consumption [16, 17]. The aim of this study was the elaboration of sensitive antibody microarray to quantify uPA and PAI-1 in protein extracts from breast tumor tissues. Various parameters such as surface chemistry, pH of spotting buffer and concentration of immobilized antibodies were evaluated in order to optimize

antibody microarray performances [18-20]. Then using the best conditions, the quantification of uPA and PAI-1 in protein extracts from breast tumor tissues was achieved and compared to classical ELISA using the Femtelle assay. In Femtelle kit, captured antibodies against uPA and PAI-1 had high affinity with uPA and PAI-1 respectively. However, the captured antibodies were unknown. So we tested several antibodies and chose those with highest performances for further experiment to measure the concentration of uPA and PAI-1 in breast tumor tissue extractions. The Femtelle assay was conducted in collaboration with the Research Cancer Institute of Montpellier (IRCM).

3.2 Experiments

3.2.1 Materials

Anti-uPA antibodies (mouse monoclonal) were obtained from Santa Cruz Biotech and Thermo Scientific (Clone number: U-16); anti-PAI-1 antibodies (mouse monoclonal) were purchased from Santa Cruz Biotech and Abcam (clone number: 3A120); anti-PAI-1 scFv antibody (sheep monoclonal, clone number: 1040.1518.5H8) was obtained from Randox Life Science; Femtelle test for uPA and PAI-1 was purchased from American Diagnostica Inc; F555-labeled streptavidin was purchased from Invitrogen. All proteins were stored as aliquot at -20°C or -80°C following manufacturer specifications. Bovine serum albumin (BSA) lyophilized powder was obtained from Sigma.

0.01 M PBS or PBS 1X (pH 7.4) was prepared by dissolving the content of one pouch of dried powder in 1 L of ultrapure water. 0.02 M sodium carbonate buffer at pH 10.7 was prepared from 0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3 solutions in ultrapure water. Washing solution contained PBS 1X and 0.1% Tween 20 (PBS-T) at pH 7.4. Blocking solution was prepared by dissolving 10 g of BSA in 100 ml of PBS-T.

3.2.2 Biological samples

All human samples were prospectively collected at Research Cancer Institute of Montpellier, France at the time of surgery after obtaining written informed consent. 50µl of cytosolic extracts were prepared from frozen tissue samples and stored at -80°C. All samples were quantified for uPA and PAI-1 using the Femtelle test.

3.2.3 Surface functionalization of microstructured glass slides

Microwells were generated on the surface of flat glass slides by photolithography and wet etching on the basis of previous work in our group [21]. Then, microstructured glass slides were functionalized with 6 different chemistries as referred in part 2.2.3. These microwells allowed testing different experimental conditions; furthermore, they allowed screening several patient sera on the same slide (one patient per microwell) when the best conditions are selected.

3.2.4 Design and Optimization of antibody microarray

3.2.4.1 Concentrations of spotted and detection antibodies

Anti-uPA and anti-PAI-1 antibodies were spotted at three different concentrations (0.33 µM, 0.67 µM and 2.5 µM) on the 6 chemically functionalized microstructured glass slides according to Figure 1. Anti-uPA antibody was spotted in the first three lines of microwells, and anti-PAI-1 antibody in the following three lines. PBS 1X was used as spotting buffer. Each antibody concentration was spotted with 6 replications in each microwell. PBS 1X and streptavidin-F555 were spotted as negative and quality controls, respectively.

After spotting, antibodies were allowed to react with surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 2 × 5 min with PBS, for 5 min with PBS-T (0.1%), and blocked with 10% BSA/PBS-T solution for 2h at room temperature (R.T.) to limit unspecific adsorption, then washed for 3 × 5 min with PBS-T and dried.

Slides were then incubated with 6 different concentrations of uPA (0 ng/ml, 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 1.5 ng/ml and 2 ng/ml) and PAI-1 (0 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml and 20 ng/ml) prepared from Femtelle kit, as shown in Figure 1. Each concentration was incubated in two microwells. Slides were left to react for 1h at R.T. in saturated water vapors, then washed for 3 × 5 min with PBS-T and dried.

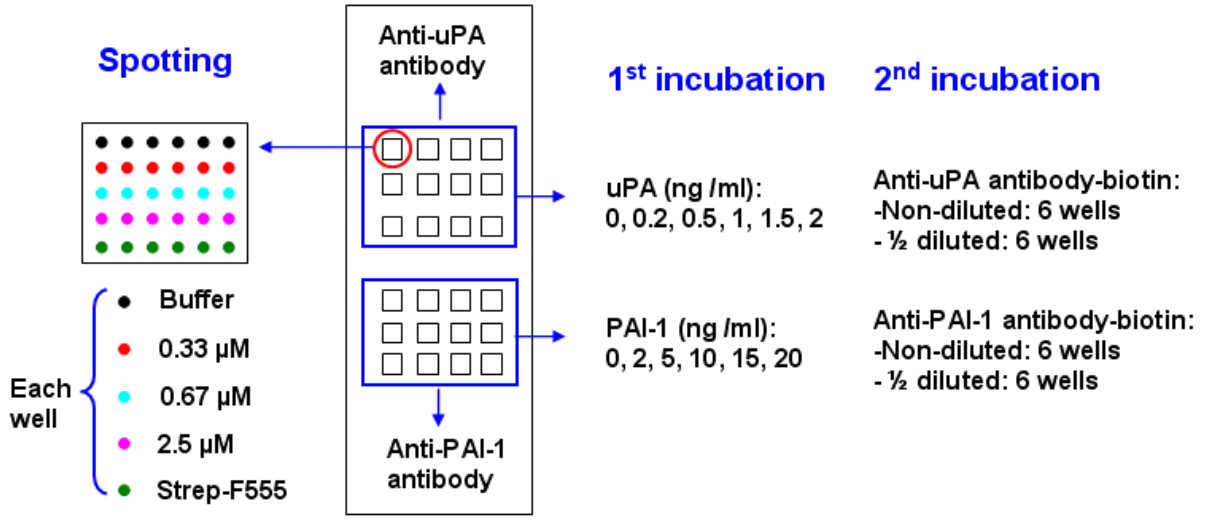


Figure 1 *Scheme of antibody microarray design for optimizing the concentration of spotted antibodies and biotin-labeled detection antibodies. Spotting: lines 1 to 3 were anti-uPA antibody; lines 4 to 6 were anti-PAI-1 antibody; each antibody was spotted in PBS buffer at 3 concentrations (0.33 µM, 0.67 µM and 2.5 µM) with 6replications for each concentration. 1st incubation: lines 1 to 3, uPA at 6 different concentrations (each concentration incubated in two microwells); lines 4 to 6, PAI-1at 6 different concentrations (each concentration incubated in two microwells). 2nd incubation was performed with not diluted and diluted (1:2) biotinylated antibodies.*

Then slides were incubated with non-diluted and diluted (1:2) biotinylated antibodies prepared from Femtelle kit as shown in Figure 1. The incubations were left to react for 1h at R.T. in saturated water vapors, and then slides were washed for 3 × 5 min with PBS-T and dried by centrifugation. Then slides were incubated with strep-F555 (0.01mg/ml diluted in 1% BSA/PBS). All incubations were left to react for 1h at R.T. in saturated water vapors, then slides were washed for 3 × 5 min with PBS-T, 10 seconds in DI water and dried.

3.2.4.2 Spotting buffer for anti-PAI-1 scFv immobilization

Three buffer solutions were tested for the immobilization of anti-PAI-1 scFv antibodies on COOH, NHS and chitosan surfaces: PBS 1X (pH=7.4), acetate buffer (pH=4.6) and carbonate buffer (pH=9.6). Anti-PAI-1 scFv antibody was spotted at 5 µM in all three buffers. The design of antibody microarray is shown in Figure 2. All incubations and washing steps were the same as previously described in part 3.2.4.1. Eight concentrations of PAI-1 were tested (incubation 1): 0, 0.5 ng/ml, 1 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml and 20 ng/ml. Biotinylated anti-PAI-1 antibody was prepared from Femtelle kit at dilution 1:2.

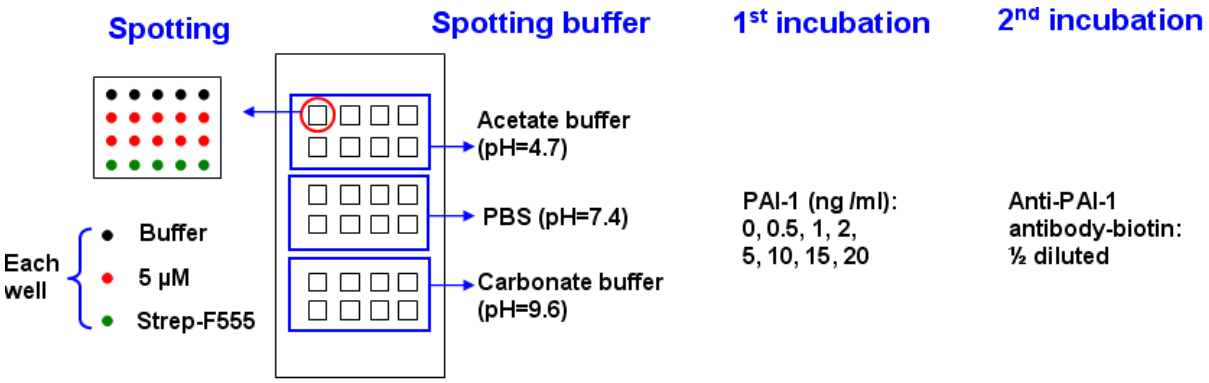


Figure 2 *Scheme of antibody microarray design for optimizing the spotting buffer of anti-PAI-1 scFv antibody. Spotting: anti-PAI-1 scFv antibody was spotted at 5 µM in all three buffers; lines 1, 2: acetate buffer (pH=4.6); lines 3, 4: PBS 1X (pH=7.4); lines 5, 6: carbonate buffer (pH=9.6). 1st incubation: 8 concentrations of PAI-1 were tested; 2nd incubation: biotinylated anti-PAI-1 antibodies were incubated at dilution 1:2.*

3.2.5 Evaluation of the biological activity of antibodies against uPA and PAI-1 with ELISA

Among five antibodies studied, no fluorescence signal was detected for the immobilization of anti-PAI-1 antibody (from both Santa Cruz Biotech and Abcam) on 6 surface chemistries. In order to figure out the reasons, we evaluated the biological activity of all antibodies by the standard method-ELISA. Also we compared them with the results obtained from Femtelle kit.

The protocol of Femtelle kit was done according to their guidelines. The protocol of ELISA is as follows:

1. Dilute the capture antibody to the appropriate concentration (20µg/ml) allowing sufficient volume for 50 µl per well.
2. Add the diluted capture antibody to the plate, cover and incubate overnight at room temperature (R.T.).
3. Remove the solution and wash the plate with 200 µl per well wash buffer (PBS 1X 0.1% Triton X-100) for 3 x 5 minutes.
4. Add 300 µl blocking buffer (1% BSA in PBS-T 0.1%) per well, cover the plate and incubate for 2 hours at R.T.
5. Remove the blocking buffer and wash the plate with 200 µl per well wash buffer.

6. Add standard solutions: adding 100 µl uPA or PAI-1 (from Femtelle kit) into wells captured with anti-uPA antibody or anti-PAI-1 antibody respectively. Cover the plate and incubate for 1 hour at RT. Both uPA and PAI-1 have 6 concentrations.
7. Remove the solution and wash the plate with 200 µl per well wash buffer for 3 x 5 minutes.
8. Add diluted (1:3) biotinylated detection antibody (from Femtelle kit) to the plate, cover and incubate for 1 hour at RT.
9. Remove the solution and wash the plate with 200 µl per well wash buffer for 3 x 5 minutes.
10. Add 100 µl diluted enzyme conjugate (from Femtelle kit: 1 µl enzyme conjugate in 1ml enzyme conjugate diluent) to the plate, cover and incubate for 1 hour at RT.
11. Remove the solution and wash the plate with 200 µl per well wash buffer for 3 x 5 minutes.
12. Add 100 µl substrate solution (from Femtelle kit) to the plate, cover and incubate for 20 minutes at RT, a blue color will develop.
13. Stop the reaction by adding 50 µl stop solution (0.5N H_2SO_4), the solution color will turn yellow.
14. Measure the absorbance at 450 nm within 30 minutes.

3.2.6 Quantification of uPA from breast tumor tissue extracts on antibody microarrays

Anti-uPA (from Thermo Scientific) was spotted using PBS 1X buffer (pH=7.4), on 3 chemically functionalized microstructured glass slides (COOH, NHS and chitosan surfaces), as shown in Figure 3. Two spotting concentrations were used: 3 µM and 6.6 µM with 14 replications each. PBS1X buffer and streptavidin-F555 were spotted as negative and quality controls, respectively. Then all incubations and washing steps were the same as previously described in part 3.2.4.1.

On each slide for the first incubation, 6 microwells were incubated with uPA prepared from Femtelle kit at six different concentrations (0 ng/ml, 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 1.5 ng/ml and 2 ng/ml) to obtain a standard curve; 2 microwells were incubated with PBS 1X (pH=7.4) buffer for negative controls; 16 microwells were incubated with non diluted breast tumor tissue extracts; 16 microwells were incubated with diluted breast tumor tissue extracts; for dilution, samples with relatively low uPA concentration (according to Femtelle kit) were diluted 2 times; while samples with relatively high concentration were diluted 5 times (Figure

3: 1st incubation). Then slides were sequentially incubated with biotinylated antibody diluted 1:2 prepared from Femtelle kit and with strep-F555 (0.01mg/ml diluted in 1% BSA/PBS).

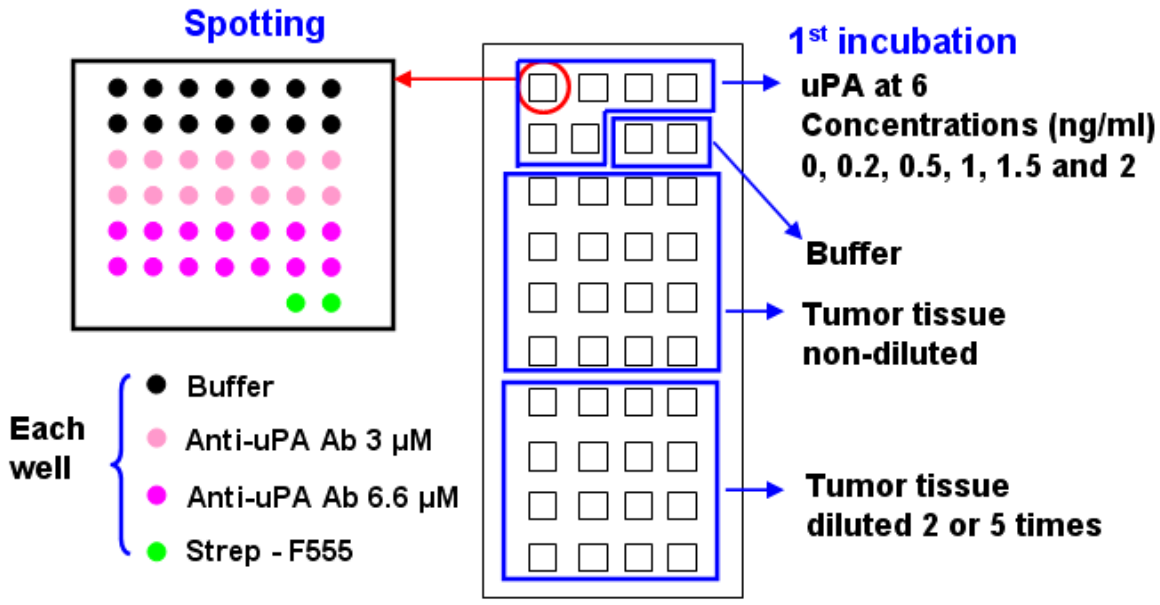


Figure 3 *Design of antibody microarray for the quantification of uPA in breast tumor tissue extracts. In each micro-well, anti-uPA antibody was spotted at two concentrations: 3 µM and 6.6 µM with 14 replications each, PBS 1X (pH=7.4) was used as spotting buffer; PBS 1X (pH=7.4) and streptavidin-F555 were spotted as negative and quality controls, respectively. First incubation was performed with uPA at six different concentrations (6 microwells)); with PBS 1X buffer (2 microwells); breast tumor tissue extracts not diluted (16 microwells); breast tumor tissue extracts diluted 1:2 (for samples with relatively low concentration) or 1:5 (for samples with relatively high concentration) (16 microwells).*

3.2.7 Fluorescence scanning and data analysis

After drying, slides were scanned with the Microarray scanner GenePix 4100A at wavelengths of 532 nm with the same photomultiplier tube (PMT) gain (PMT=600). Data mining was accomplished with GenePix 4100A software package (Axon Instruments). SNR was calculated as described in part 2.2.6. The threshold value (cut-off) for the determination of LOD (Limit of Detection) was calculated as followed: Cut off =Mean of median buffer spots + 3 SD, where SD represents standard deviation.

The standard curve was obtained by plotting the mean SNR value calculated for each uPA standard concentration.

3.3 Results and discussion

3.3.1 Optimization of antibody microarray conditions

The implementation of efficient antibody microarrays requires optimizing important parameters such as surface chemistry, concentration of spotting and detection antibody, and spotting buffer. Thus, for the detection and quantification of uPA and PAI-1, firstly the 6 surface chemistries developed in our lab were evaluated for the efficient immobilization of anti-uPA and anti-PAI-1 antibodies. The biological activity of immobilized antibodies was determined by the level of recognition with uPA or PAI-1. Secondly, various spotting concentration of the antibodies were tested on the best surface chemistries. Thirdly, concentration of the biotinylated antibodies was also optimized to obtain good detection signal. At last, 3 different spotting buffers were tested for the immobilization of anti-PAI-1 scFv antibody, the concentration of the other antibodies did not allow changing buffer. The influence of these factors will be presented as follows.

3.3.1.1 Influence of surface chemistry

Surface chemistry affected greatly the performance of antibody microarray. Among 6 surface chemistries tested, three of them (COOH, NHS and chitosan surface) showed good performances for the immobilization of anti-uPA antibody and anti-PAI-1 antibody scFv.

Anti-uPA antibody

Anti-uPA antibodies (from Santa Cruz Biotech) were spotted on the 6 different surface chemistries and its biological activity was evaluated and compared (Figure 4).

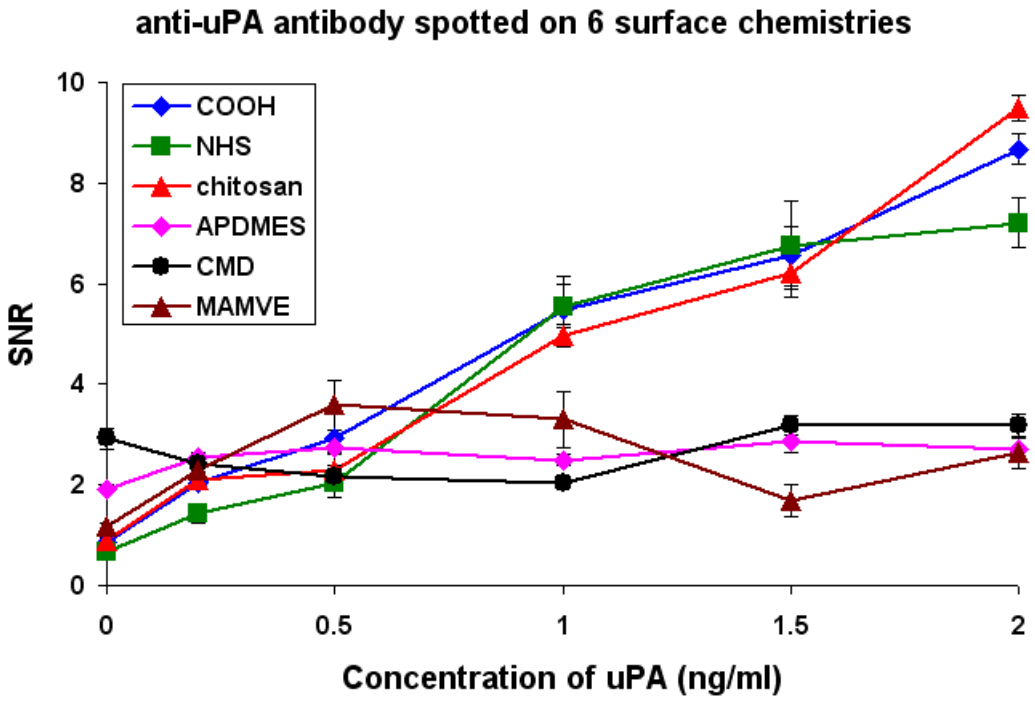
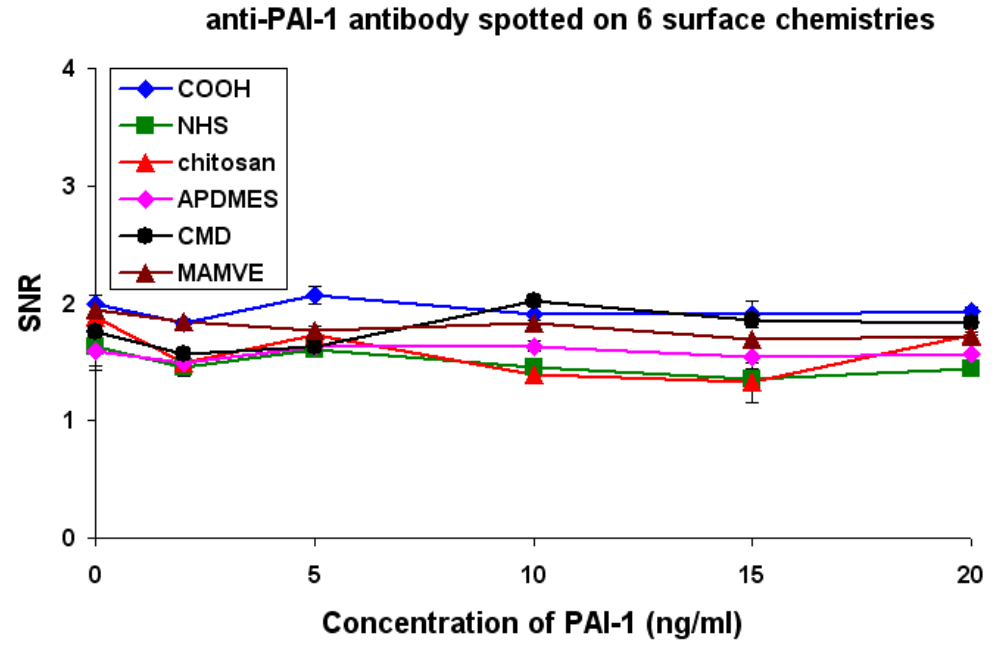


Figure 4 *Fluorescent signal analysis of uPA detection on COOH, NHS, chitosan, APDMES, CMD and MAMVE surfaces. Spotted concentration of anti-uPA antibody was 2.5μM.*

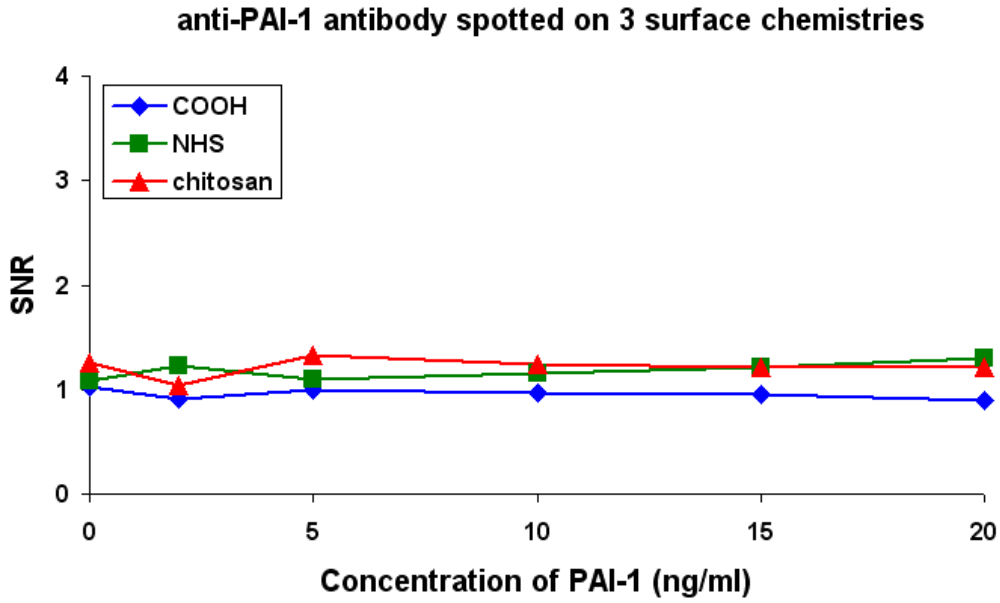
As shown in Figure 4, anti-uPA antibody spotted on APDMES, CMD and MAMVE surfaces did not allow sensitive detection of uPA. In contrast, it performed well on COOH, NHS and chitosan surfaces, fluorescence signal increasing with increasing uPA concentration. Moreover, on NHS surface, the signal reached a plateau value when uPA concentration reached 1.5ng/ml. In contrast, the dynamic range of anti-uPA antibody on COOH and chitosan surfaces was much larger compared to NHS surface. According to these results, COOH, NHS and chitosan surfaces were chosen for immobilizing anti-uPA antibody from other company (Thermo Scientific) for further experiment.

Anti-PAI-1 antibody

Two anti-PAI-1 antibodies (from Santa Cruz Biotech and Abcam) were immobilized on the various surface chemistries and tested for their biological activity to recognize PAI-1. However, none of them exhibited biological activity after surface immobilization as shown in Figure 5.



(a)



(b)

Figure 5 *Biological activity of anti-PAI-1 antibody* ***(from Santa Cruz Biotech)*** *on 6 surface chemistries (a) and anti-PAI-1 antibody* ***(from Abcam)*** *on 3 surface chemistries (COOH, NHS and chitosan surface); the spotting concentration was 2.5 μM.*

Several reasons may lead to the failures of these two anti-PAI-1 antibodies. Firstly, surface chemistries tested could modify the structure of antibodies during immobilization process, thus resulting in the loss of biological activity. Secondly, these antibodies were not biologically active before immobilization. In order to figure out the reasons, we further tested the biological activity of these 2 antibodies by ELISA. Results will be presented in later.

Anti-PAI-1 scFv antibody

As 2 entire antibodies against PAI-1 didn't exhibit biological activity, a single-chain variable fragment (scFv) against PAI-1was evaluated for the recognition towards PAI-1. scFv consists of variable regions of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker. scFv is the smallest unit of immunoglobulin molecule with antigen-binding activity [22]. Figure 6 presented performance of anti-PAI-1 scFv on the 6 surface chemistries. Only COOH surface allowed efficient immobilization of anti-PAI-1 scFv to sensitively detect PAI-1.

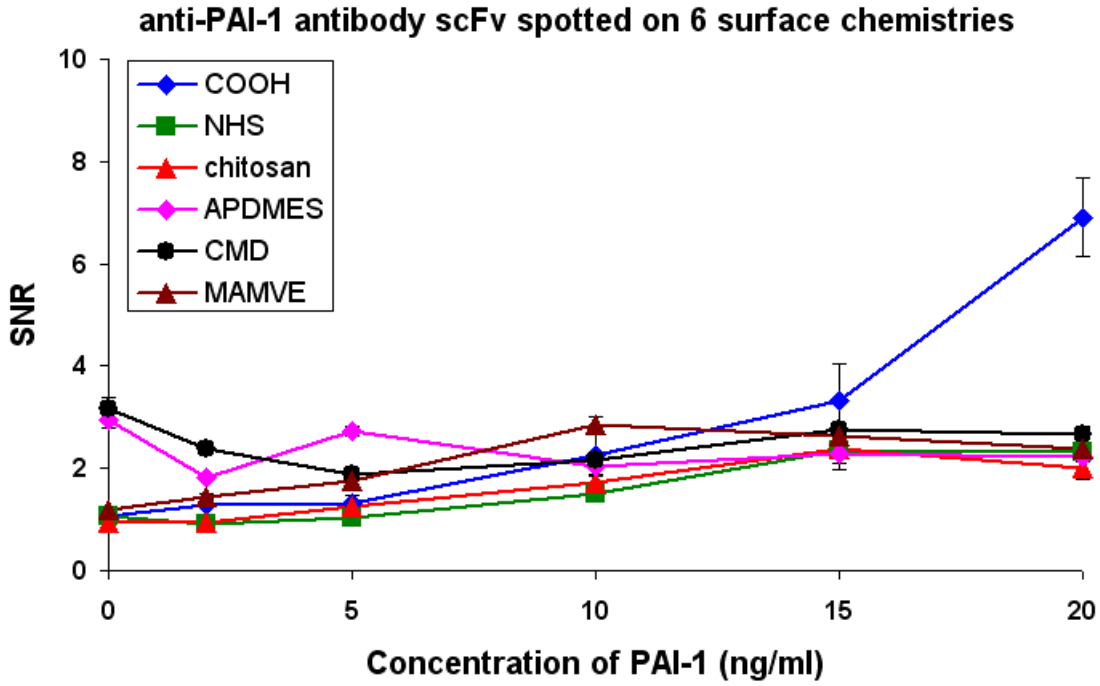
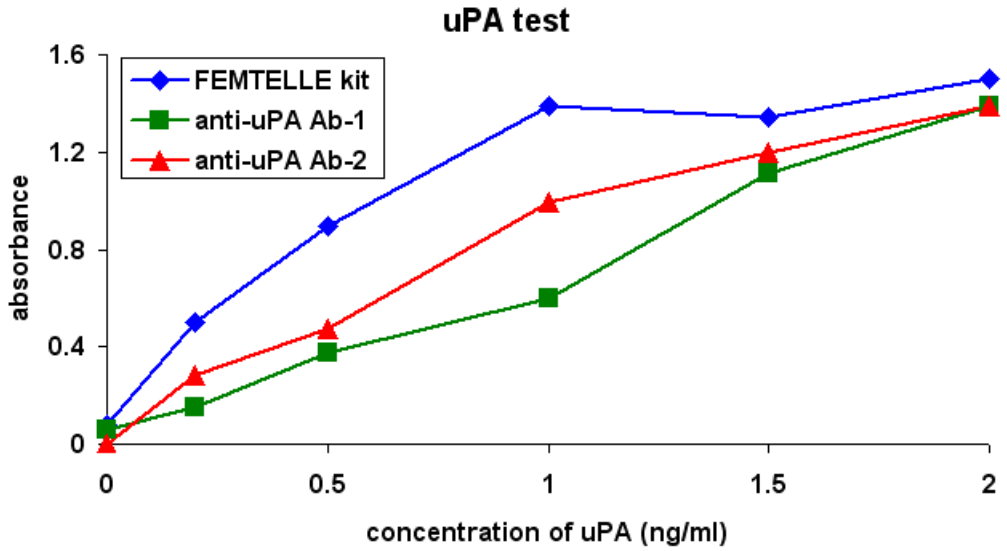


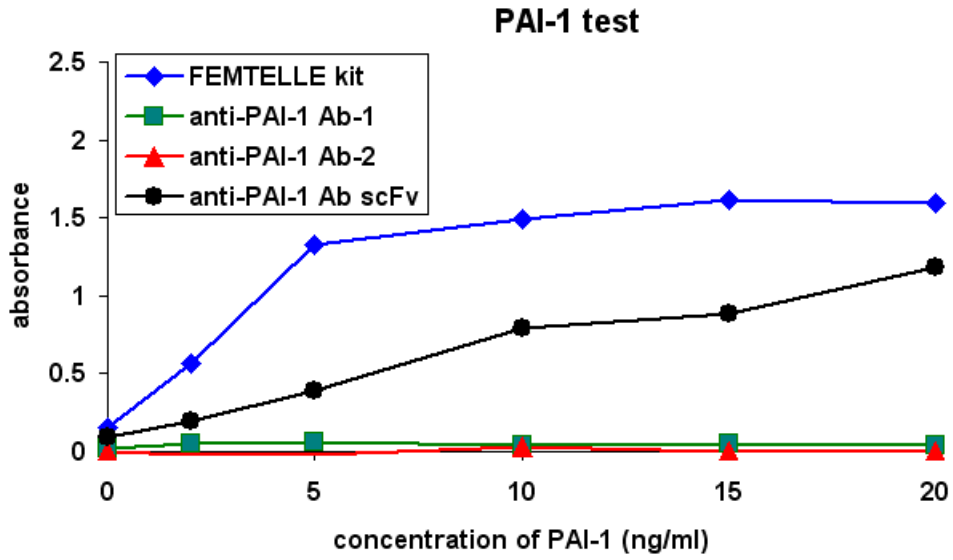
Figure 6 *Biological activity of anti-PAI-1 scFv on COOH, NHS, chitosan, APDMES, CMD and MAMVE surfaces; anti-PAI-1 scFv was spotted at 5µM.*

ELISA to test the biological activity of antibodies against uPA and PAI-1

We have tested the biological activity of five antibodies by ELISA and compared the results with those obtained from Femtelle kit. Antibodies tested include 2 anti-uPA antibodies (from Santa Cruz Biotech (Ab-1) and Thermo Scientific (Ab-2)), 2 anti-PAI-1 antibodies (from Santa Cruz Biotech (Ab-1) and Abcam (Ab-2)) and 1 anti-PAI-1 scFv (from Randox Life Science). Results of ELISA tests are presented in Figure 7.



(a)



(b)

Figure 7 *ELISA test from Femtelle kit and antibodies against uPA (a) and PAI-1 (b). In (a), anti-uPA antibody 1 and 2 are from Santa Cruz Biotech and Thermo Scientific respectively; in (b), anti-PAI-1 antibody 1 and 2 are from Santa Cruz Biotech and Abcam respectively.*

Figure 7a indicated that both anti-uPA antibodies tested displayed good biological activity for detecting uPA in ELISA, which is consistent with results obtained from antibody microarray. However, their biological recognition towards uPA appeared less sensitive compared to commercial Femtelle kit. Indeed in commercial ELISA kit such as Femtelle kit, antibody/antigen affinity is very high as the process is optimized to get best sensitivity and specificity of the assay.

Concerning anti-PAI-1 antibodies, among all three antibodies tested, only anti-PAI-1 scFv was able to recognize PAI-1, as it was observed on our antibody microarray. Moreover, as previously described for anti-uPA, the recognition activity of anti-PAI-1 in ELISA is lower than those of the Femtelle kit.

Thus, in immunoassay development, the choice of antibody is critical. High affinity between antibody and antigen is indispensable. Moreover, optimization of all the process including concentration, incubation solutions and time, is a required step to get best performance. For antibody microarray, surface chemistry is also a key parameter to improve the immunoassay. Among the 6 surface chemistries tested, COOH, NHS and chitosan surfaces showed best performance for the immobilization of anti-uPA and anti-PAI-1 scFv antibodies. Therefore, these 3 surfaces were selected for further experiments.

3.3.1.2 Influence of the concentration of captured antibodies

The concentration of capture antibody greatly influenced the lowest limit of detection (LOD) of antibody microarray. The threshold value of LOD was defined as mean of fluorescence signal (SNR) of buffer plus 3 S.D., where S.D. represents standard deviation. Thus, the lowest detected concentration displaying a SNR value higher than the threshold value, is defined as LOD. Moreover, spotting concentration influenced the dynamic range of detection. Dynamic range is a critical factor to evaluate the performance of assay. Generally, wider dynamic range corresponds to higher ability to test concentrations of uPA and PAI-1 in breast tumor tissue extraction.

Anti-uPA antibody (from Thermo Scientific) was spotted at 2 concentrations (3 µM and 6.6 µM) on the 3 selected surface chemistries, and detection of uPA was evaluated as a function of the concentration. Results obtained on chitosan surface are presented in Figure 8. As can be see, the detection of uPA is better when the spotted concentration of anti-uPA antibody is higher, reaching 0.2 ng/ml as LOD. Furthermore, spotting concentration affected the dynamic range of uPA detection. At the highest spotted antibody concentration (6.6 µM), the dynamic range was wider: 0.2 ng/ml-1.5 ng/ml. Therefore, in order to obtain high sensitivity of detection, high concentration of immobilized anti-uPA antibody is necessary.

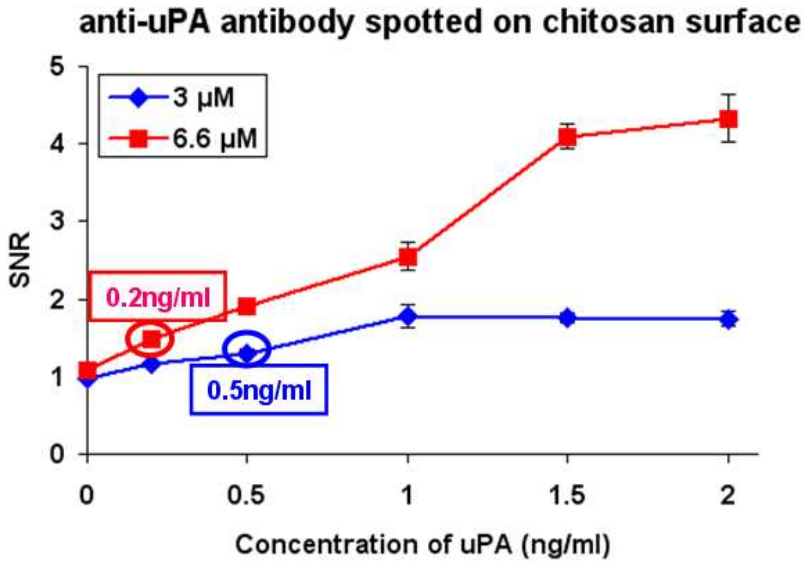


Figure 8 *Detection of uPA on chitosan surface as a function of anti-uPA antibody spotted concentration of (3µM and 6.6 µM); limit of detection (LOD) of uPA for each spotted concentration was indicated.*

Results obtained with anti-PAI-1 scFv spotted at 2 concentrations (5 µM and 10 µM) on chitosan surface are presented in Figure 9. As previously described for anti-uPA, the sensitivity of the detection of PAI-1 was dependent on the capture antibody spotted concentration. Thus, at the highest spotted concentration of anti-PAI-1 scFv, LOD of PAI-1 reached was as low as 2ng/ml, with a dynamic range varying from 2 ng/ml to 20 ng/ml.

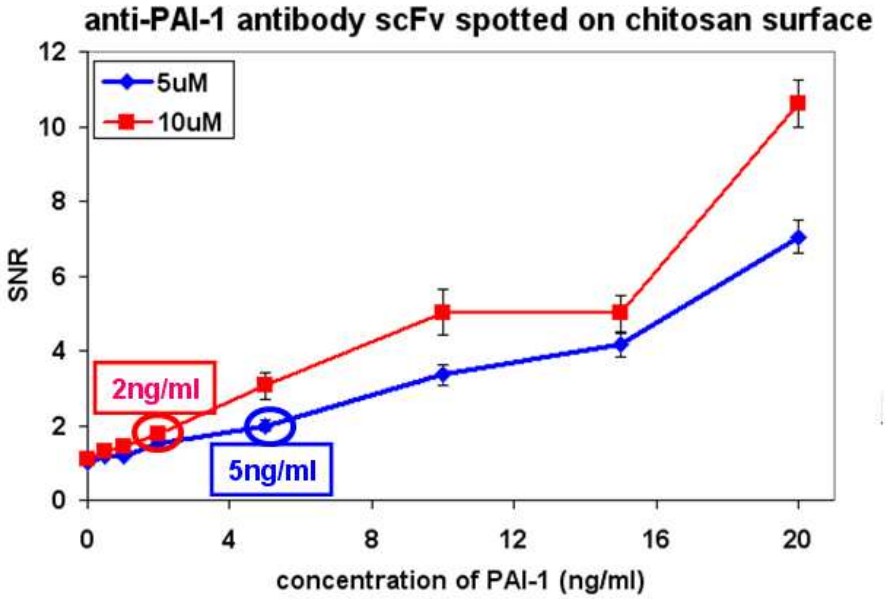
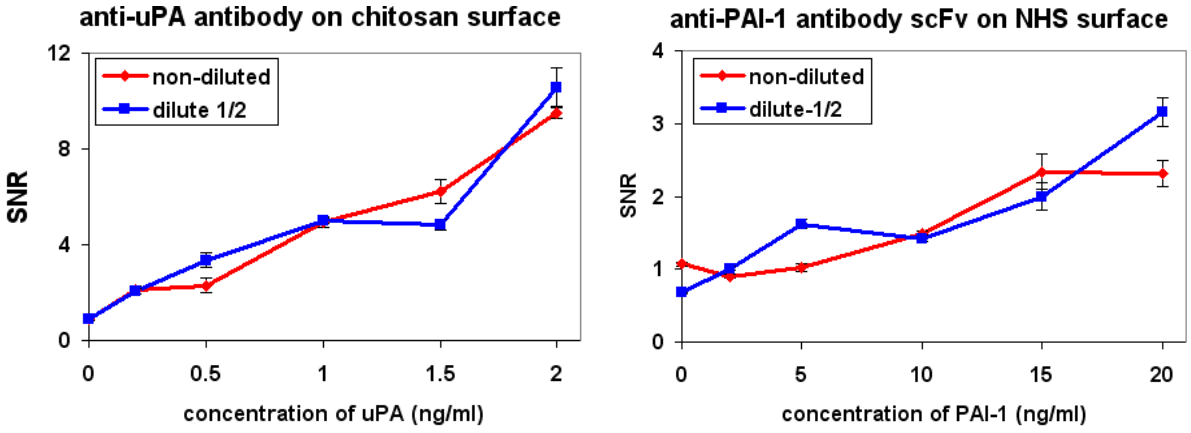


Figure 9 *Detection of PAI-1 on chitosan surface, as a function of anti-PAI-1scFv spotted (5µM and 10µM); limit of detection (LOD) of PAI-1 for each concentration was indicated.*

In conclusion, higher spotting concentration of antibodies results to better LOD and wide dynamic range of detection. Therefore, high spotted concentrations of anti-uPA were chosen to titrate uPA in breast tumor tissue extraction.

3.3.1.3 Influence of the concentration of biotin labeled detection antibody

The concentration of detection antibody also affected the performance of antibody microarray. In our study, biotinylated antibodies against uPA and PAI-1 were obtained from Femtelle kit. However, no information about their concentration was given by the supplier. Thus, two conditions were tested: not diluted and diluted 1:2 in 4% BSA/PBS-T 0.1% solutions. Figure 10 showed the results obtained for anti-uPA antibody (from Santa Cruz Biotech) spotted on chitosan surface at 2.5 µM (Figure 10a) and results obtained for anti-PAI-1 scFv spotted on NHS surface at 5 µM (Figure 10b). For both antibody/antigen systems no significant difference was observed between the two conditions. Therefore, diluted (1:2) biotinylated antibodies were chosen for further experiments from the economical point of view.



(a) (b)

Figure 10 *Comparison of diluted and non-diluted biotinylated antibody against uPA and PAI-1 on the detection of uPA with capture anti-uPA (from Santa Cruz Biotech) immobilized on chitosan surface at 2.5 µM (a) and on the detection of PAI-1 with capture anti-PAI-1 scFv immobilized on NHS surface at 5 µM (b).*

3.3.1.4 Influence of spotting buffer for anti-PAI-1 scFv immobilization

According to previous results, the best spotting buffer for antibody on COOH, NHS and chitosan surface is carbonate buffer (pH=9.6) [20]. However, anti-PAI-1 scFv is closer to

antigen protein in terms of size and structure than to native antibody [22]. Therefore, 3 buffer solutions [PBS 1X (pH=7.4), acetate buffer (pH=4.6) and carbonate buffer (pH=9.6)] were tested for the immobilization of anti-PAI-1 scFv on COOH, NHS and chitosan surfaces. Anti-PAI-1 scFv antibody was spotted at 5 µM in all 3 buffers. Then biological activity of the immobilized anti-PAI-1 scFv was evaluated by mean of level of recognition of PAI-1.

Results are presented on Figure 11. On both COOH (Figure 11a) and NHS (Figure 11b) surfaces, only immobilization of anti-PAI-1 scFv with carbonate buffer allowed retaining the biological activity of the scFv. In contrast, on chitosan surface (Figure 11c) the best biological activity was obtained with PBS 1X.

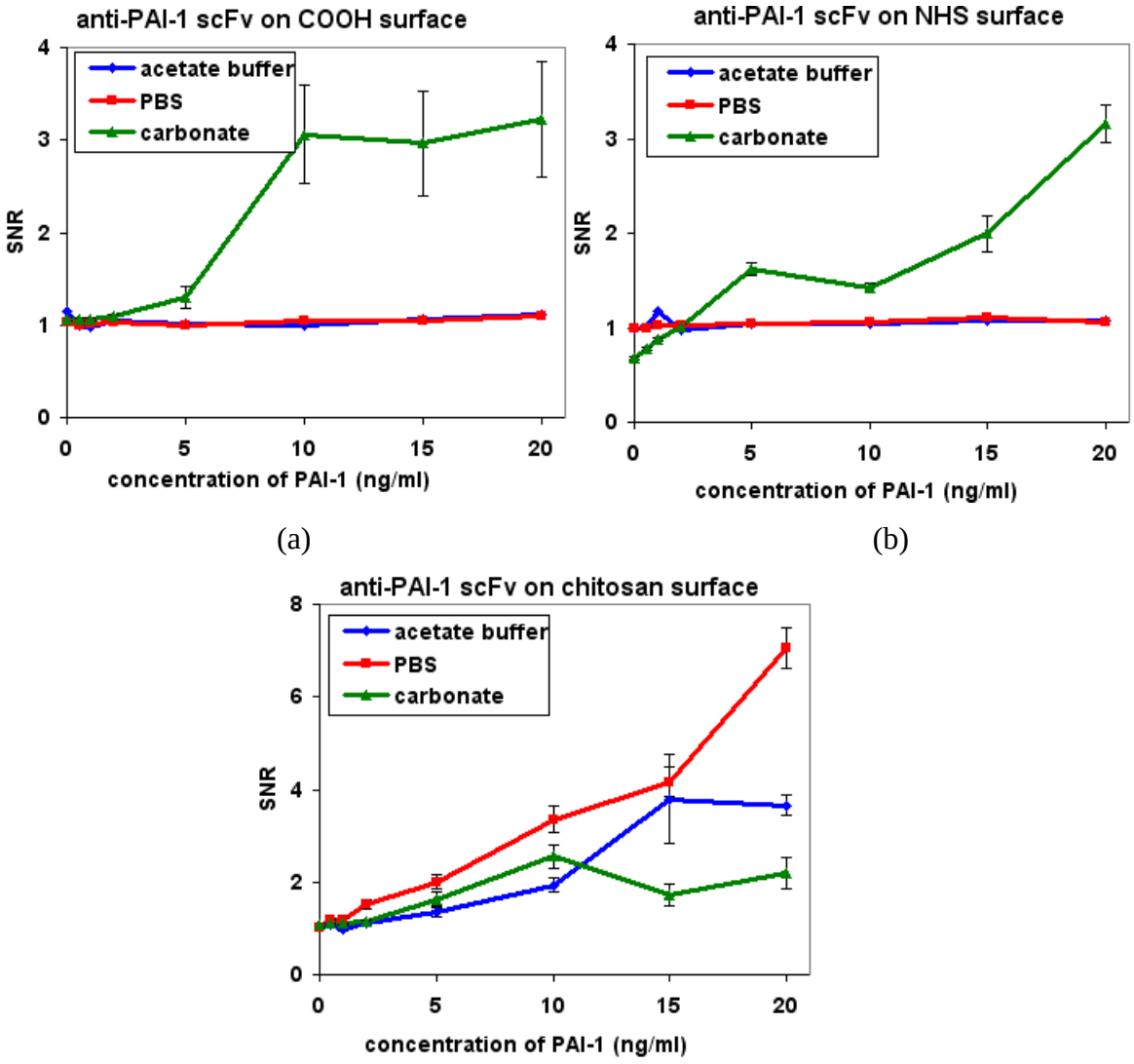


Figure 11 *Evaluation of the biological activity of immobilized anti-PAI-1 antibody scFv (5 µM) on COOH (a), NHS (b) and chitosan (c) surfaces using three different spotting buffers: acetate buffer (pH=4.6), PBS 1X (pH=7.4), carbonate buffer (pH=9.6).*

COOH and NHS surfaces are both silane monolayers, NHS surface was obtained by NHS activation of the COOH surface. Protein immobilization on COOH surface was achieved through physical adsorption, whereas on NHS surface it was through covalent binding. These 2 ways of immobilization on silane monolayer seemed to lead to complete denaturation of immobilized anti-PAI-1 scFv, resulting to complete loss of biological activity, or to very low amount of immobilized anti-PAI-1 scFv, resulting in not detectable signal, when acetate buffer or PBS 1X was used as spotting buffer. However, chitosan surface is a polymeric surface obtained from functionalization of NHS surface with chitosan polymer, a natural polysaccharide. The immobilization on chitosan surface was achieved through physical adsorption as well, but the surface area developed and hydrophilic character was higher than COOH surface [20]. Moreover, chitosan surface presents NH_2 groups instead of COOH groups on COOH surface. Thus, physical adsorption on chitosan surface should involve different mechanisms leading to better level and orientation of immobilized anti-PAI-1 scFv. This immobilization process seems to be enhanced using PBS 1X as spotting buffer.

3.3.1.5 Conclusion

Performances of antibody microarrays are greatly influenced by the biological activity of immobilized antibodies and by the sensitivity of the detection system. Among parameters involved in biological activity of immobilized antibodies, surface chemistry as well as spotting conditions (concentration and buffer solution) are ones of the most critical. Thus, to elaborate efficient antibody microarrays for the quantification of uPA and PAI-1, these parameters were optimized and results are reported in Table 1. Among the 6 surface chemistries tested, 3 of them (COOH, NHS and chitosan surfaces) displayed good performances to retain biological activity of immobilized anti-uPA antibody and anti-PAI-1 antibody scFv. Moreover, higher spotting concentration of antibodies results in better LOD and wider dynamic range of detection under optimal spotting buffer. At last, same detection level was obtained with biotinylated antibody diluted 1:2 allowing to reduce consumption of biological reactive.

Table 1 *Optimal conditions for the immobilization of anti-uPA antibody and anti-PAI-1 ScFv antibody.*

Antibody/spotted concentration	Surface	Spotting buffer	LOD (ng/ml)	Dynamic range
anti-uPA antibody **at 6.6 µM**	COOH	PBS	0.2	0.2 – 1.5ng/ml
	NHS	PBS	0.2	0.2 – 2ng/ml
	chitosan	PBS	0.2	0.2 – 1.5ng/ml
anti-PAI-1 antibody (scFv) **at 10 µM**	COOH	carbonate	2	2 – 20 ng/ml
	NHS	carbonate	2	2 – 20 ng/ml
	chitosan	PBS	2	2 – 20 ng/ml

3.3.2 Quantification of uPA in breast tumor tissue extracts

16 cytosolic extracts were obtained from frozen breast tumor tissues by 10% Triton X-100 (recommended by Femtelle kit). The concentration of uPA in each cytosolic extracts was determined by Femtelle kit and ranked from 0.4 ng/ml to 8 ng/ml (see Table 2).

Table 2 *The concentration of uPA in 16 cytosolic extracts determined by Femtelle kit*

No. of patient	Concentration of uPA (ng/ml)
1	0.4
2	0.9
3	1.4
4	1.5
5	2.1
6	2.3
7	2.4
8	3.1
9	3.3
10	3.6
11	3.9
12	4.3
13	4.6
14	6.4
15	7
16	8

The goal was to quantify the concentration of uPA in each cytosolic extracts by our customized antibody microarray and compare the value to those obtained with Femtelle kit. For this, we elaborated three types of antibody microarrays, with COOH, NHS and chitosan surface chemistries considered as best surfaces according to previous results. Thus, the design of customized antibody microarrays was pictured in Figure 12. 10 lines of 4 microwells were etched on glass slides. Inside each microwell, the anti-uPA antibodies were spotted in PBS 1X buffer at two concentrations (3 µM and 6.6 µM) and buffer and strep F555 were added as negative and positive controls of microarray quality.

In order to elaborate nano-FLISA (Fluorescent immunoassay) for the quantification of uPA, titration curve should be included on the microarray. So, on each microstructured slide, the two first lines of microwells were dedicated to construct standard titration curve. 6 microwells were incubated with 6 different concentrations of uPA including 0 ng/ml, 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 1.5 ng/ml and 2 ng/ml.

Moreover, in order to match the dynamic range of the standard titration curve, and taking account of the expected value given by Femtelle tests, cytosolic extracts were tested on antibody microarray under two conditions: non-diluted (microwell lines 3 to 6) and diluted 1:2 (No.1-11) or 1:5 (No.12-16) (microwell lines 7 to 10).

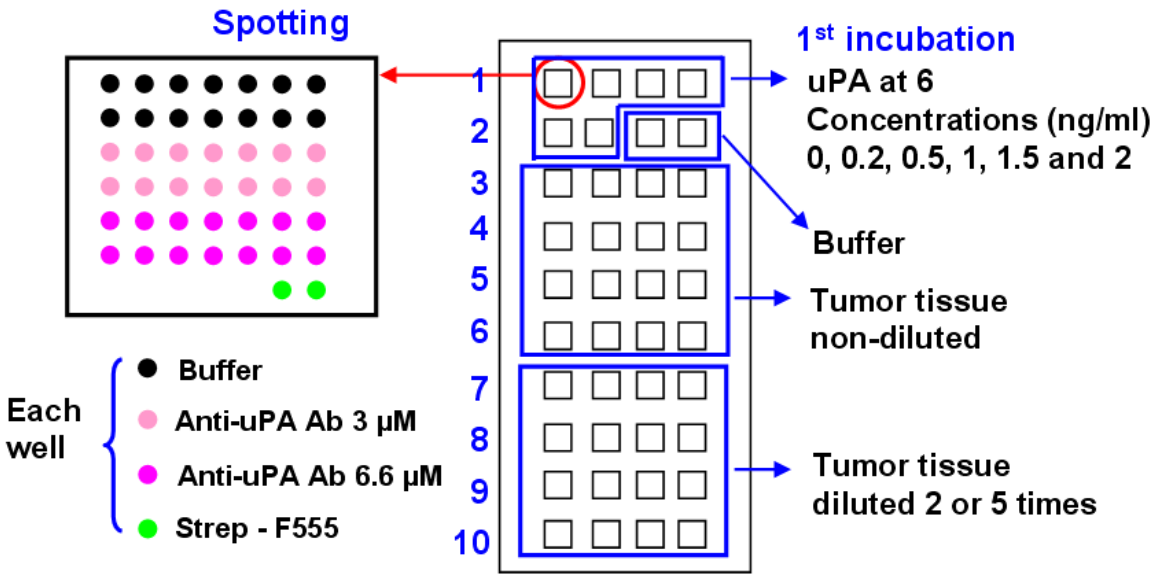


Figure 12 *Scheme of antibody microarray design for uPA concentration measurement*

3.3.2.1 Standard titration curve of uPA on antibody microarray

Figure 13 gives the fluorescence image obtained with the same PMT (photomultiplier tube coefficient of fluorescence scanner) when anti-uPA antibody was immobilized on COOH, NHS and Chitosan surface respectively. The microwells were incubated with standard uPA solutions of 6 different concentrations; the last two microwells were incubated with buffer.

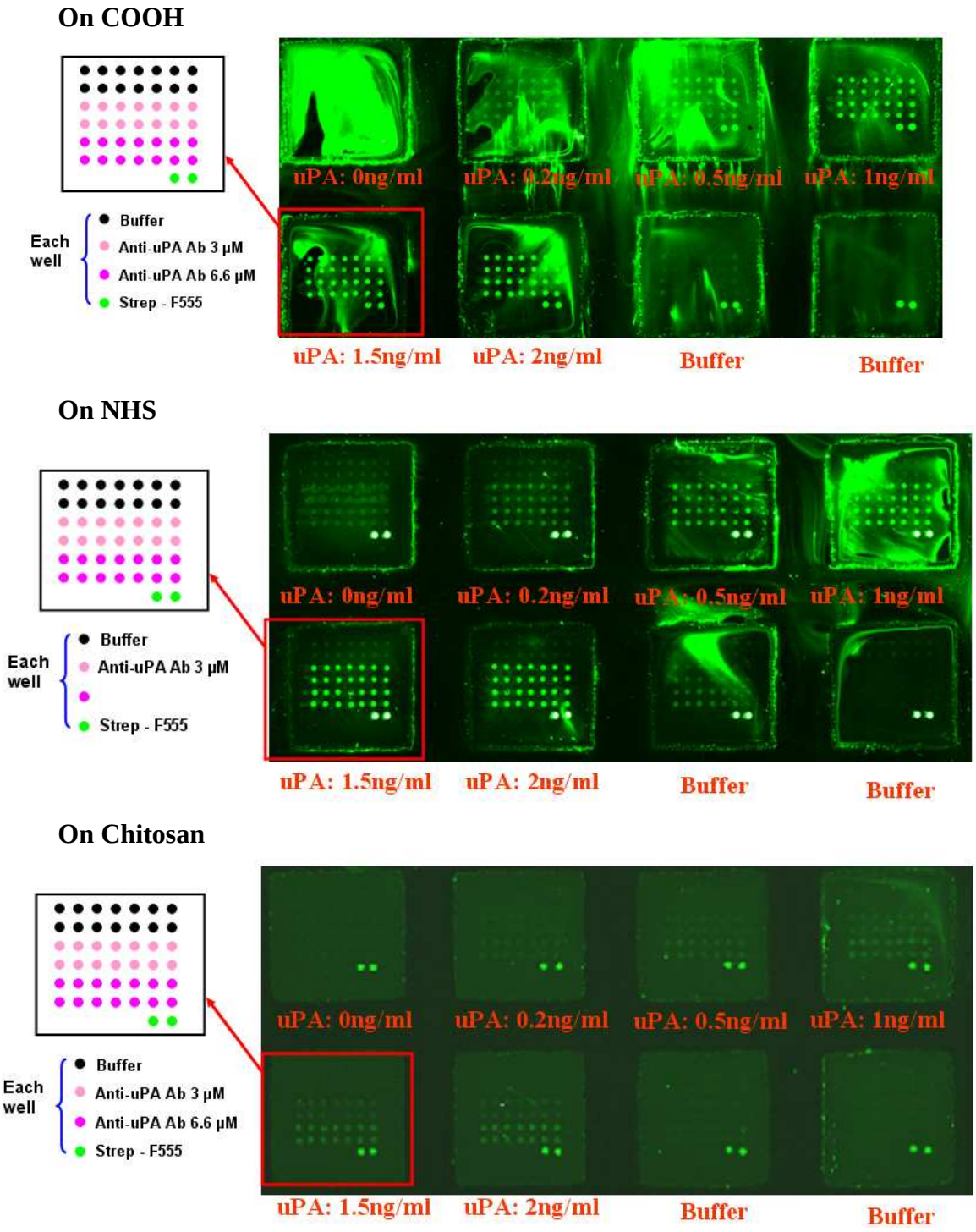


Figure 13 *Fluorescence image obtained on COOH surface; microwells were incubated with uPA including 0 ng/ml, 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 1.5 ng/ml and 2 ng/ml and buffer (PBS 1X) (two microwells).*

Whatever the surface chemistry, in each microwell, the 2 spots of streptavidin-F555 were easily detected which means that these surfaces had a good ability for protein immobilization. Microwells incubated with buffer exhibited weak signal. Unfortunately, Figure 13 exhibits also a spurious fluorescence which affected the quantitative analysis of fluorescence. This high unspecific binding was observed when the microwell was incubated with uPA, which should be caused by inefficient blocking process or more probably by bad washing steps. Remember that washing and rinsing step were performed without agitation. Fluorescent intensity signals were higher on COOH and NHS compared to chitosan surfaces. But whatever the surface chemistry, a similar behavior could be noticed. For microwells incubated with uPA, fluorescence signal obtained from immobilized anti-uPA antibody increased with the increasing concentration of incubated uPA.

Figure 14 gives the fluorescent signal (SNR) obtained for the detection of uPA on COOH, NHS and chitosan surfaces when the concentration of spotted anti-uPA antibody was at 3 µM and at 6.6 µM). SNR is defined as the ratio between the fluorescence signals of antibody spots over the fluorescent signals of buffer spots. As expected, SNR increased with the increasing of uPA concentrations. Among all three surfaces tested, the fluorescence signal obtained from COOH surface is the highest, followed by NHS and chitosan surface. Although SNR is different, the lowest limit of detection (LOD) of uPA on these three surfaces is the same, about 0.2 ng/ml. However, the dynamic range is strongly depending on microarray surface chemistry. On COOH surface, SNR reached a plateau when uPA is at 1.5 ng/ml, therefore, the dynamic range of uPA detection is between 0.2 ng/ml to 1.5 ng/ml. The same range was obtained for chitosan surface. In contrast, the dynamic range is wider on NHS surface, between 0.2 ng/ml to 2 ng/ml. Moreover, according to the trend of the curve, higher concentrations of uPA could be also detected on NHS surface but with weaker signals. Comparison between the curves obtained from two different concentrations of spotted antibodies did not exhibit crucial discreapancy. The difference concerned mainly the high concentrations of uPA which exhibited stronger SNR for 6.6 µM of spotted anti-uPA antibodies.

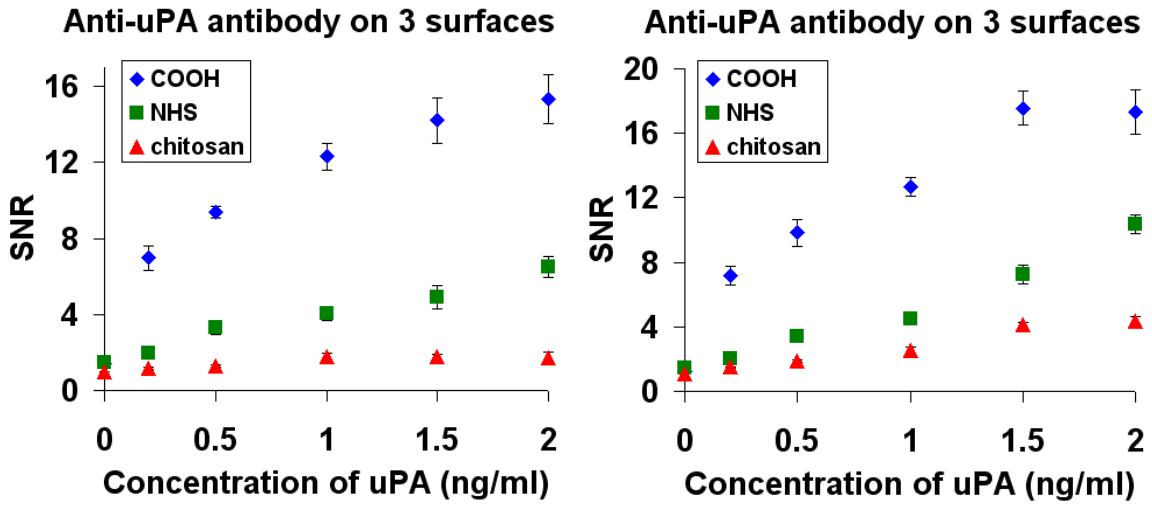
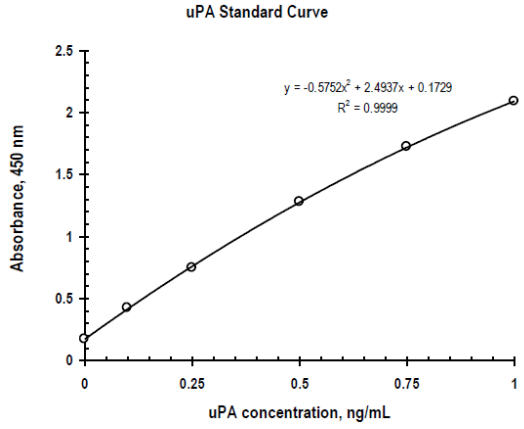
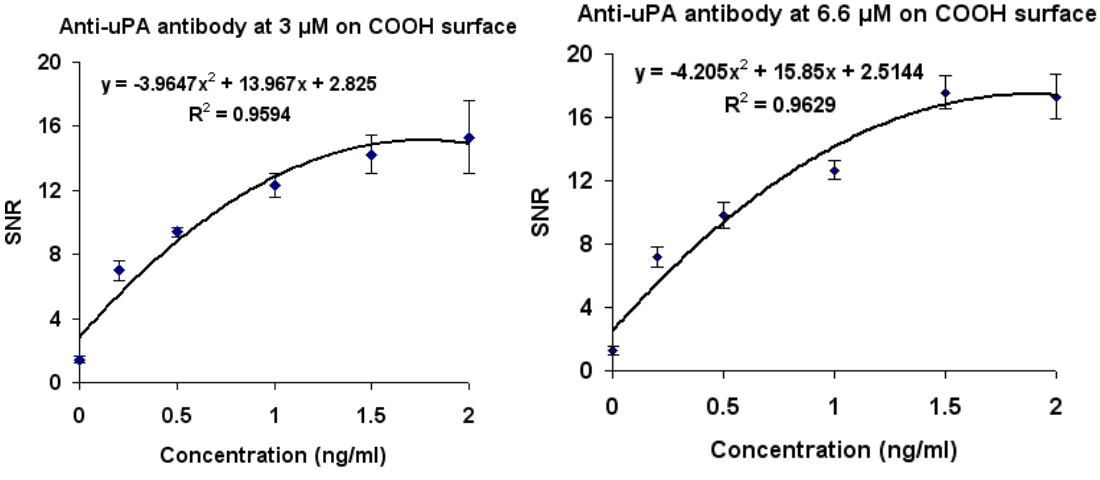


Figure 14 *Fluorescent signal analysis (SNR) of uPA detection on COOH, NHS and chitosan surface when the concentration of spotted anti-uPA antibody was at 3 µM (left) and at 6.6 µM (right).*

For providing the quantification of uPA in cytosolic extracts, standard titration curve issued from our customized microarray should be drawn and also compared with uPA standard curve obtained from Femtelle kit. Figure 15 gives all of the titration curves obtained from antibody microarray made with different surface chemistries.



(a)



(b) (c)

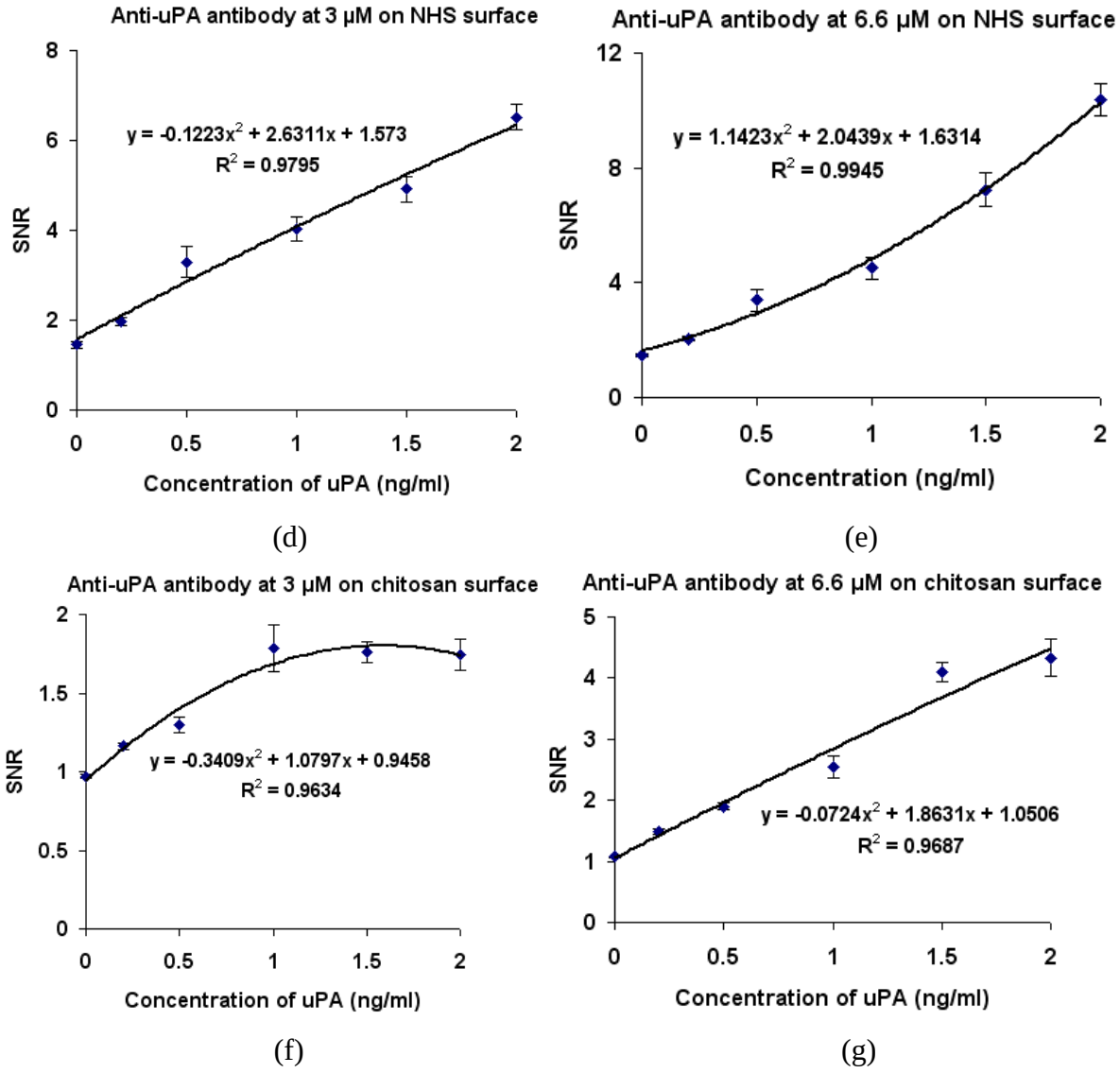


Figure 15 *(a) represented uPA standard curve obtained from Femtelle kit; (b) and (c) represented uPA standard curve obtained from our customized antibody microarray when anti-uPA antibody was immobilized on COOH surface at 3 µM and 6.6 µM respectively. (d) and (e) corresponds to NHS surface and (f) and (g) to chitosan surface.*

According to the protocol of Femtelle kit, the lowest limit of detection (LOD) for the assay for uPA is 0.025 ng/ml, which seems much lower than the results obtained by our antibody microarray (0.2 ng/ml). However, 100 µl uPA standard solutions were needed to add in order to obtain the standard titration curve in Femtelle kit. In contrast, only 1 µl uPA standard solution was used in our antibody microarray. Therefore, if we consider the total amount of uPA, LOD of our antibody microarray (0.2ng) is 10 times less than Femtelle kit (2.5ng).

For the dynamic range, we can see that it ranges between 0.01 ng/ml to 1 ng/ml for Femtelle kit (Figure 15 a). In comparison, the dynamic range of uPA detection by our antibody microarray is wider, for example, it ranges from 0.2 – 2ng/ml on NHS surface

(Figure 15 d and e). Furthermore, fluorescent signal (SNR) didn't reach a plateau at 2ng/ml, therefore, higher uPA concentration could be tested on NHS surface in order to increase the dynamic range.

Furthermore, we compared correlation coefficient (R^2) of ELISA kit and our antibody microarray. Correlation coefficient (R^2) defines the association between x and y. An R^2 value of exactly 1 indicates the all data points lie exactly on a straight line. Generally, a correlation greater than 0.8 is described as strong, whereas a correlation less than 0.5 is described as weak. The R^2 of ELISA kit is 0.9999 (Figure 15a), very close to 1. In comparison, the R^2 obtained from our customized antibody microarray was also high; it was 0.9945 when the spotting concentration of anti-uPA antibody was at 6.6 µM on NHS surface (Figure 15 e).

Table 3 summarized LOD, dynamic range of uPA detection and its corresponding SNR range on COOH, NHS and chitosan surface when the concentration of spotted anti-uPA antibody was at both 3µM and 6.6 µM. Among all three surfaces tested, NHS surface had the widest dynamic range for uPA detection at both two spotted concentrations of anti-uPA antibody. Moreover, the dynamic range is wider on chitosan surface when the concentration of spotted anti-uPA antibody is higher (at 6.6 µM) but fluorescent signals were weaker.

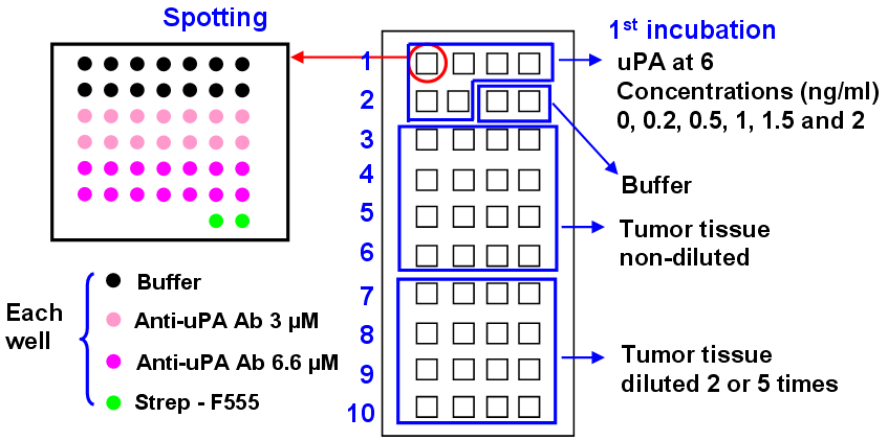
Table 3 *LOD and dynamic range of uPA detection on COOH, NHS and chitosan surface when the concentration of spotted anti-uPA antibody was at 3µM and 6.6 µM respectively.*

Anti-uPA Antibody spotted concentration	Surface	LOD	Dynamic range of uPA	Range of SNR
3 µM	COOH	0.2 ng/ml	0.2 – 1.5ng/ml	5.5-14.9
6.6 µM	COOH	0.2 ng/ml	0.2 – 1.5ng/ml	5.5-16.8
3 µM	NHS	0.2 ng/ml	0.2 – 2ng/ml	2.1-6.3
6.6 µM	NHS	0.2 ng/ml	0.2 – 2ng/ml	2.1-10.3
3 µM	chitosan	0.2 ng/ml	0.2 – 1ng/ml	1.1-1.8
6.6 µM	chitosan	0.2 ng/ml	0.2 – 1.5ng/ml	1.4-4.5

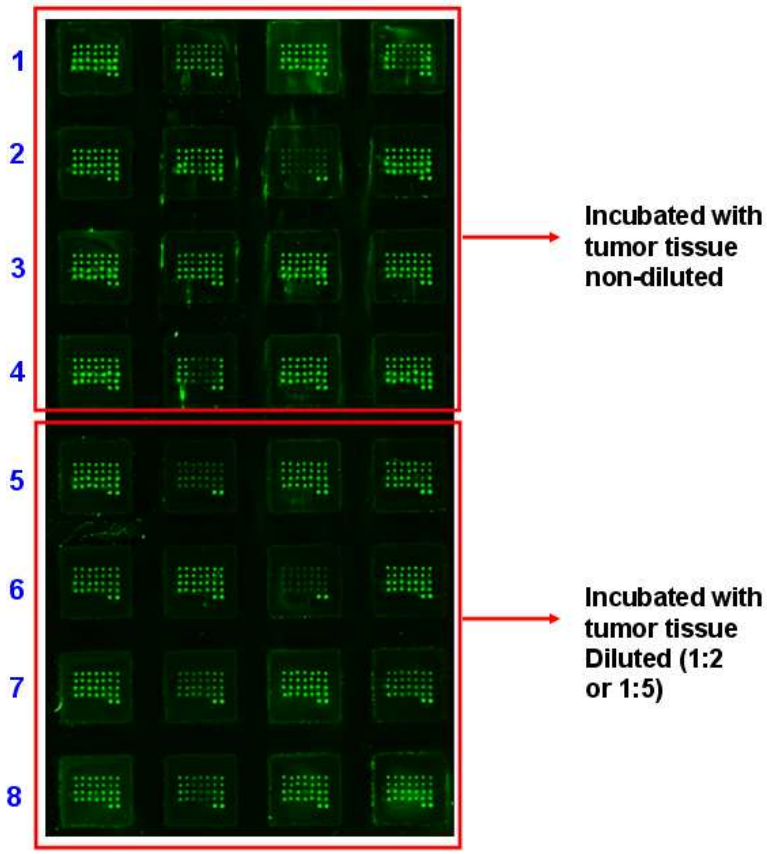
3.3.2.2 Quantification of uPA in breast tumor tissue extracts

Figure 16 (a) gives the design of antibody microarray; (b) represents the fluorescence image obtained on COOH surface for detecting the concentration of uPA in tumor cytosolic

extracts, among which line 1-4 were incubated with non-diluted cytosolic extracts, whereas line 5-8 were incubated with corresponding diluted cytosolic extracts. (c) and (d) correspond to the pictures obtained on NHS and chitosan surfaces respectively. High unspecific binding on chitosan surface were noticed (markerd with white square), which maybe caused by inefficient washing process.

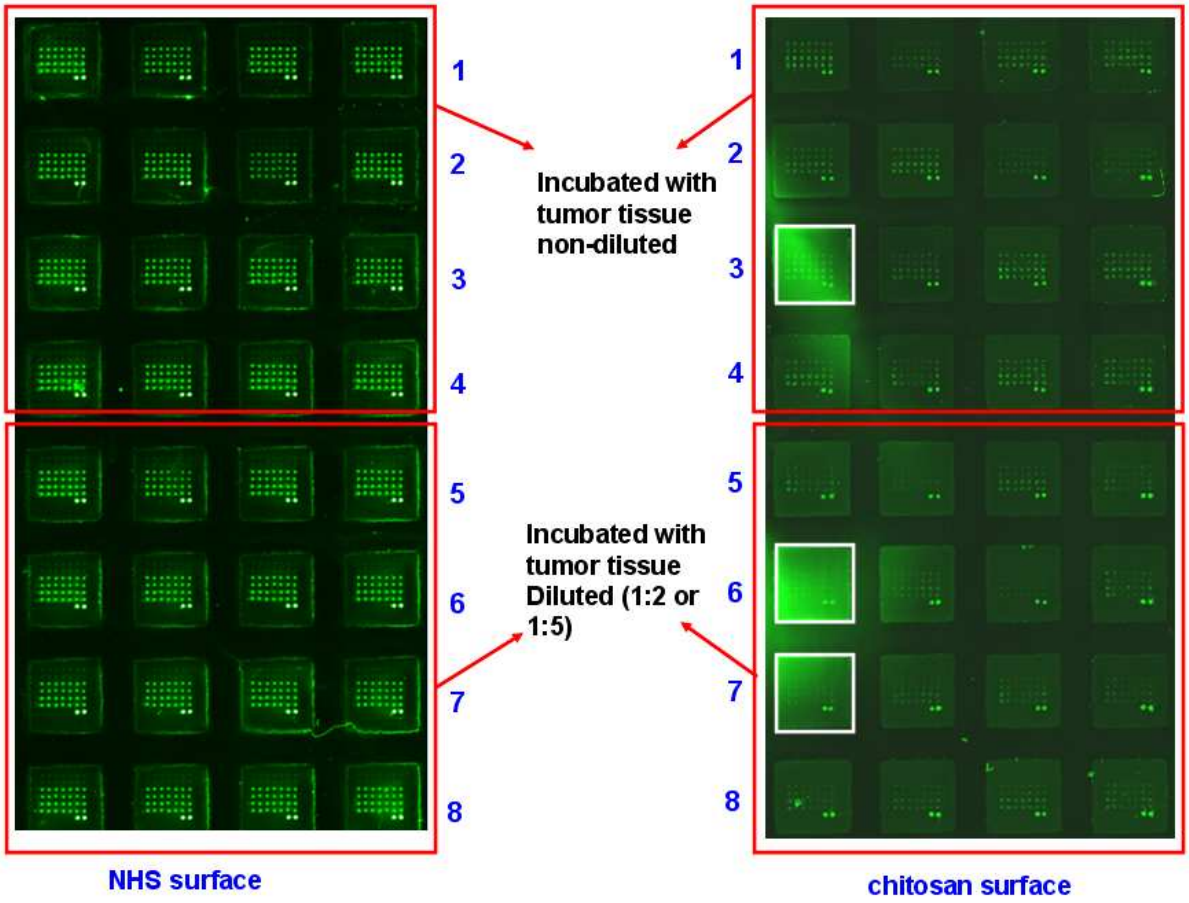


(a)



(b) **COOH surface**

Figure 16 *(a) represents the scheme of antibody microarray design; (b) represents the fluorescence image obtained on COOH surface for detecting the concentration of uPA in tumor cytosolic extracts: line 1-4 were incubated with non-diluted cytosolic extracts; line 5-8 were incubated with corresponding diluted cytosolic extracts.*



(c) (d)

Figure 16 *(c) represents the fluorescence image obtained on NHS surface (d) on chitosan for detecting the concentration of uPA in tumor cytosolic extracts: line 1-4 were incubated with non-diluted cytosolic extracts; line 5-8 were incubated with corresponding diluted cytosolic extracts.*

According to the standard curve obtained from each surface, if the SNR of one sample is in SNR range (shown in Table 3), its concentration could be calculated by the formula obtained from the standard curve (shown in Figure 15), otherwise not. Therotically, as uPA concentration of all samples are in the dynamic range after dilution, therefore, their corresponding SNR are also in the SNR range. However, several samples whose SNR is out of range, therefore, their concentrations could not be calculated by the formula. We summarized the samples whose SNR are in the range, and then calculated the concentration of each sample and compared the results with those obtained from ELISA. Figure 17 (a) (c) and (e) compared the results of ELISA with those obtained from protein microarray with COOH, NHS and chitosan surface, respectively.

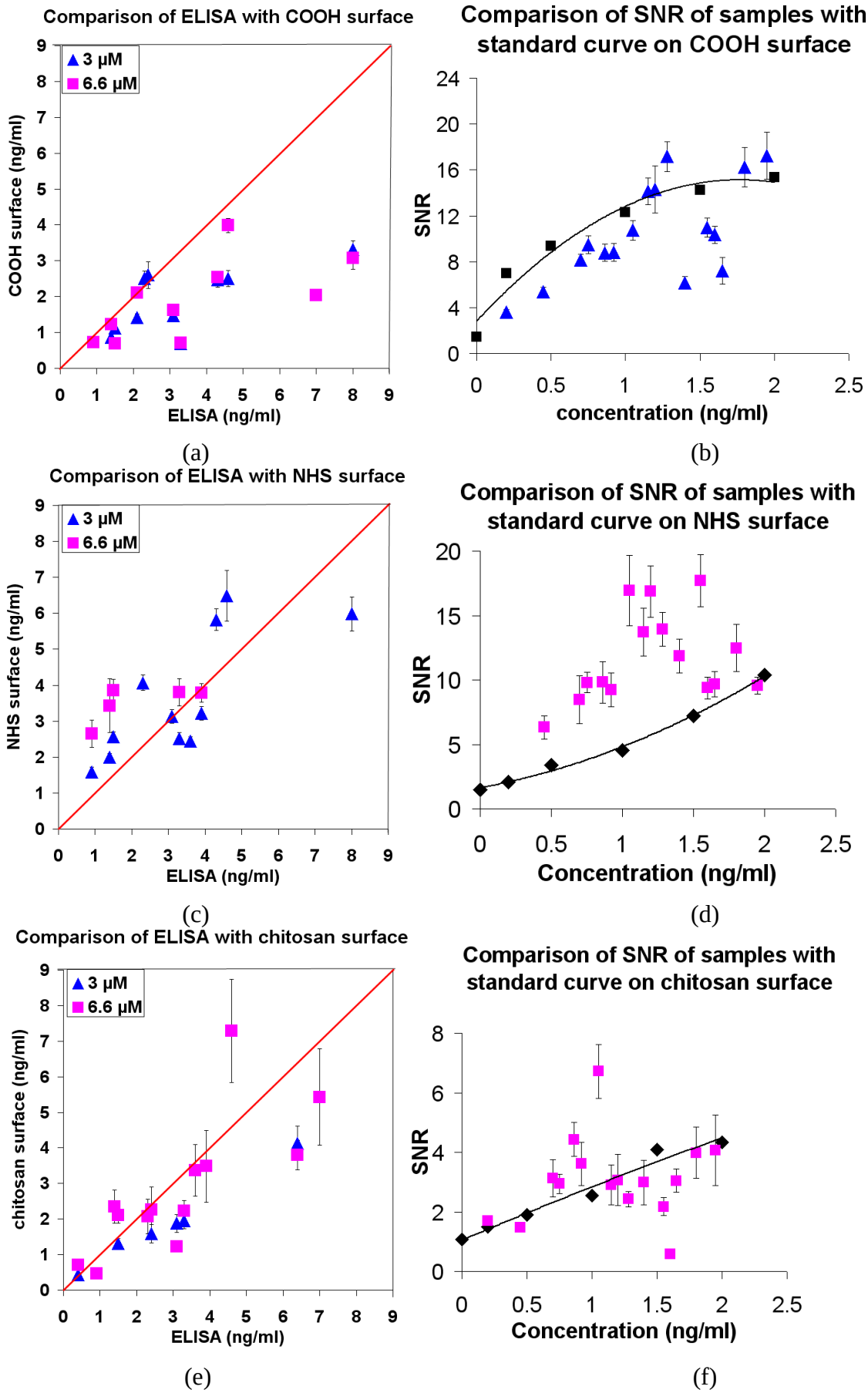


Figure 17 *(a) (c) and (e) compared the results of ELISA with those obtained from protein microarray with COOH, NHS and chitosan surface, respectively; (b) (d) and (f) compared the SNR of samples with standard curve obtained from COOH, NHS and chitosan surface, respectively.*

About half samples tested on COOH surface, displayed results consistent with those from ELISA; while the results of the other samples were lower than ELISA (Figure 17a). Thus we compared SNR values of tested samples with the standard curve obtained at 3 µM anti-uPA antibody spotting concentration (Figure 17b). It appeared that SNR values of most samples were lower than SNR values of standard curve. This result could be explained by the high unspecific binding of standard samples on COOH surface (Figure 13). In contrast, on NHS surface, about half tested samples displayed higher concentration than with ELISA (Figure 17c). In this case, the SNR values of most samples were higher than the SNR values obtained with standard samples at 6.6 µM anti-uPA antibody spotting concentration (Figure 17d). These differences could be due to the partial lost of activity of standard uPA solutions with time; therefore, new standard uPA solutions should be used for further experiment.

At last, most samples tested on chitosan surface displayed results consistent with ELISA (Figure 17e). Moreover, SNR values of these samples were consistent with standard curve (Figure 17f). Although SNR values obtained on chitosan surface were the lowest (Table 3), the quantification of uPA of most samples were the best; therefore, SNR value is not the deciding factor for quantifying uPA. In contrast, the quality of standard curve is more critical. Among all 3 surfaces tested, unspecific binding was the lowest on chitosan surface (Figure 13).

Standard curve is critical for quantifying uPA and two points need to be improved in further experiments. Firstly, high unspecific binding was observed on COOH and NHS surface (Figure 13) and it greatly influences the quality of standard curve; therefore, it important to reduce unspecific binding, e.g increasing washing time and/or washing buffer composition. Secondly, on NHS and chitosan surfaces, higher uPA concentrations could be tested in order to increase their dynamic ranges (Figure 15 d, e and g).

In the literature, only one study reported on the use of antibody microarray to quantify uPA in breast tumor tissue extracts. They used commercial surface and tested 50 tissue samples. Results showed that uPA values measured by antibody microarray were 40–50% lower than those obtained from ELISA. Furthermore, they analyzed the association between protein microarray and ELISA and found that the results obtained from protein microarray equal 0.06 + 0.56 ELISA [23]. In our study, we found that results obtained from antibody microarray were also different from ELISA and furthermore, surface dependent. For example, results obtained on NHS are higher than ELISA while those obtained on COOH surface are lower.

However, as our study is limited by sample size, we could not obtain the real association between our home-made surface with ELISA; therefore, future large scale study is needed.

3.4 Conclusions

In this study, we aimed at developing antibody microarray for the titration of uPA and PAI-1 in breast tumor tissue extracts. Using microstructured glass slides, we could rapidly screen various conditions (surface chemistries, antibody spotting concentrations and spotting buffers) to optimize performances of the antibody microarray. Among the six surface chemistries tested, three were selected (COOH, NHS and chitosan surfaces) for the titration of uPA in cytosolic extracts. The best antibody concentration was found to be 6.6 µM spotted in PBS 1X (pH=7.4). In these conditions, LOD was determined at 0.2 ng/mL and the dynamic range was 0.2-2 ng/mL. Then 16 breast tumor tissue extracts were titrated for uPA on our antibody microarray. The results indicated that the performances of our antibody microarray are surface dependent. However, as our study is limited by sample size, we could not obtain the representative association between antibody microarrays and ELISA; further large scale investigation is needed. Furthermore, high unspecific binding was observed on COOH surface; therefore, unspecific binding should be reduced in order to improve the quality of standard curve and quantification.

Concerning the quantification of PAI-1, among 3 anti-PAI-1 antibodies tested, only anti-PAI-1 scFv could retain its biological activity following immobilization on surfaces (chemically functionalized microarray and ELISA plates). Best performances were obtained with 10 µM anti-PAI-1 scFv spotted in PBS 1X (pH=7.4) leading to LOD of 2 ng/mL. In comparison, LOD for the Femtelle kit for PAI-1 is 0.125 ng/ml. As discussed before, if we consider the total amount of PAI-1, LOD of our antibody microarray is almost 6 times less than those from Femtelle kit.

These results are very promising for the implementation of a nano-FLISA test. Indeed, our antibody microarray showed a higher sensitivity and a wider dynamic range compared to Femtelle ELISA kit. Furthermore, considering that one of the main limitations of Femtelle kit is that it requires 100-300mg of fresh or frozen samples, our antibody microarray shows high potential as it consumes 25 times less sample volume. Moreover, we planned to use formalin-fixed paraffin-embedded (FFPE) tissue which is more easy to obtain and main source of patient material worldwide [24].

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Chapter 4

Optimization of protein microarray elaboration and processing

4.1 Introduction

Although protein microarrays show high potential for biomarkers screening, various factors influence microarray performances such as surface chemistry [1], humidity during spotting [2], composition of the spotting buffer (additives and pH) [3-6], concentration of immobilized proteins [7]. Considering protein microarray processing, critical factors are composition of blocking solution and duration [8, 9], concentration of incubated solution and incubation time [10], buffer solution used for sample dilution, washing time, and so on.

A major challenge for miniaturized multiplex sandwich assay development is the optimization of these parameters in order to reduce processing time keeping high performances. In this chapter, we study the influence of several critical factors (blocking and incubation duration, concentration of incubated solutions) on the performances of protein microarray. We chose one-factor-at-a-time (OFAT) method for designing experiment. OFAT method is based on the variation of one single factor in each experiment while keeping the others constant and measuring the process output [11].

Furthermore, due to the physico-chemical nature of proteins, performances may also be affected by the delay between microarray fabrication and their use. Therefore on a practical point of view, storage condition of protein microarray slides is an important issue for preserving the integrity of microarray performances. It is a major concern for both microarray manufacturers and users. Generally, arrays are stored under aqueous conditions (glycerol, blocking solution, polyvinyl alcohol, etc) or dry condition (sealed under nitrogen or not) [12-15]. However, proteins have diverse structure and physico-chemical properties. Therefore, optimal storage condition is expected to be protein and surface dependent.

Herein, various storage conditions for antigen microarrays were studied. Our microarrays are designed for cancer diagnosis and are based on immobilized heat shock proteins (HSPs) microarray for the multiplex profiling of anti-HSPs antibodies in serum. These arrays will be usable for medical applications (not only research) providing that their performances should be maintained upon storage for a reasonable time span.

4.2 Experiments

4.2.1 Materials

The proteins used were as followers: HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1, HSP110, p53, mouse-anti human anti-HSPB1 antibody-biotin, mouse-anti human anti-HSPD1 antibody-biotin, mouse-anti human anti-HSP70 antibody-biotin, mouse-anti human anti-HSP90 antibody, mouse-anti human anti-HSPA5 antibody, mouse-anti human anti-HSP90B1 antibody, mouse-anti human anti-HSP110 antibody, mouse-anti human anti-p53 antibody-biotin, F555-labeled streptavidin and Cy3-labeled goat anti-mouse antibody IgG. Detailed information of these proteins can be referred in part 2.2.1. All proteins were stored as aliquot at -20°C or -80°C following manufacturer specifications. Bovine serum albumin (BSA) lyophilized powder was obtained from Sigma.

0.01 M PBS or PBS 1X (pH 7.4) was prepared by dissolving the content of one pouch of dried powder in 1 L of ultrapure water. 0.02 M sodium carbonate buffers at pH 10.7 were prepared from 0.1 M NaHCO3 and 0.1 M Na2CO3 solutions in ultrapure water. Washing buffer contained PBS 1X and 0.1% Tween 20 (PBS-T) at pH 7.4. Blocking solution was prepared by dissolving 10 g of BSA in 100 ml of PBS-T 0.1%.

4.2.2 Surface functionalization of flat and microstructured glass slides

The details of surface functionalization of glass slides can be referred in part 2.2.3. Glass slides were functionalized with the 6 chemistries including COOH, NHS, chitosan, APDMES, MAMVE and CMD surfaces.

4.2.3 Elaboration and processing of protein microarray

Antigen proteins were spotted (between 4 to 8 replicates for each protein) on functionalized glass slides at different concentrations (0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml and 0.1 mg/ml) in acetate buffer (pH=4.6), using sciFLEX-ARRAYER S3 (Scienion, Germany). Streptavidin-

F555 (0.01mg/ml) and acetate buffer (pH=4.6) were used as quality and negative controls, respectively. After spotting, proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 2 × 5 min with PBS, for 1 × 5 min with PBS-T, and then blocked (blocking step) with 10% BSA/PBS-T solution at various time (30 min, 1 h, 2 h) and then washed for 3 × 5 min with PBS-T and dried.

For the biological recognition step, purified biotin-labeled antibodies or purified unlabeled antibodies were incubated at various times (30 min, 1 h). Different antibody concentration diluted in 1% BSA/PBS-T 0.1% were tested (0.1µM and 0.5µM). Then slides were washed for 3 × 5 min with PBS-T and dried.

For the detection step, streptavidin-F555 at various concentrations, diluted in 1% BSA/PBS (0.05µM, 0.19µM), or IgG-Cy3 at various concentrations (0.01µM, 0.05µM) was incubated for 30 min or 1 hr. Then slides were washed for 3 × 5 min with PBS-T, 10 seconds in DI water and then dried. The different experimental conditions used are reported in Table 1, (a) and (b) represent the design for the optimization of experimental time and concentration of solution respectively.

Table 1 *Experiment design of optimization. (a) and (b) represent the optimization of experimental time and concentration of solution respectively.*

Blocking process	1st incubation	2nd incubation
2hr	1hr	1hr
1hr	30min	30min
30min		

(a)

Antigens	Concentration of first Ab / Ab-biotin	Concentration of strep-F555	Concentration of second Ab: IgG –cy3
HSPB1, HSPD1, HSP70 and p53	0.1 and 0.5µM	0.05 and 0.19 µM	-
HSP90, HSPA5, HSP90B1 and HSP110		-	0.01 and 0.05µM

(b)

4.2.4 Stability of chemically functionalized glass slides

Chemically functionalized glass slides were stored under dry condition and then evaluated for chemical stability by contact angle measurement and by their ability to immobilize probe molecules (oligonucleotides (ODN) and proteins).

4.2.4.1 Contact angle measurement

Surface energy of chemically functionalized flat glass slides was followed by contact angle measurements (Digidrop Goniometer, GBX, France) as a function of time (1 month, 2 months, 3 months and 4 months). De-ionized water, ethylene-glycol and diiodomethane were used in all measurements. To minimize the experimental error, the contact angle was measured at three random locations for each sample and the average value was calculated. Surface energy was determined according to Owens-Wendt model [16].

4.2.4.2 Immobilization of probe molecules

3 different fluorescent labeled molecules (oligonucleotide-Cy3 (ODN-Cy3), streptavidin-Cy3 (Strep-Cy3), Immunoglobulin-Cy3 (IgG-Cy3) were spotted at various time (1 month, 2 months, 3 months, 4 months), as described in Figure 1. According to previous results, Strep-cy3 (0.01mg/ml, 0.005mg/ml) was diluted in acetate buffer (pH=4.6), IgG-cy3 ($6X10^5$ µM) was diluted in carbonate buffer (pH=9.6) and ODN-Cy3 (5µM) was diluted in PBS 1X (pH=7.4). Buffer spots include two spots of acetate buffer, one carbonate buffer and one PBS 1X.

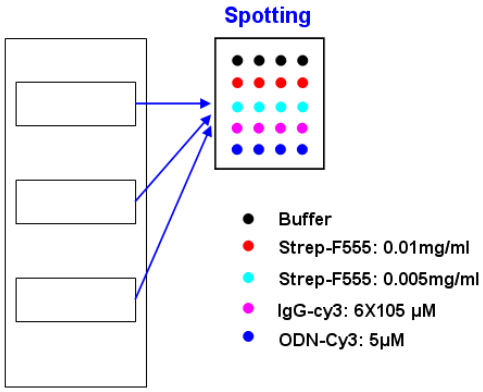


Figure 1 *Protein microarray design for evaluation of surface chemistry stability. Three replicates of the same array were spotted on each flat slide. One array contains 4 replicates of each molecule: Strep-cy3 (0.01mg/ml and 0.005mg/ml), IgG-cy3 ($6*10^5$ µM) and DNA-cy3 (5µM). Buffer spots include two spots of acetate buffer (pH=4.6), one of carbonate buffer (pH=9.6) and one of PBS 1X (pH=7.4).*

After spotting, proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 15 min with 2% BSA (PBS-T 0.1%), for 2 × 10 min with PBS-T 0.1%, and then 10 seconds in DI water and dried for scanning.

4.2.5 Storage of spotted slides

In a first test, micro-structured glass slides were functionalized with both NHS and chitosan. P53 and HSPD1 were spotted in acetate buffer (pH=4.6) at 0.1 mg/ml; streptavidin-Cy3 (0.1mg/ml) and acetate buffer were spotted as quality and negative control, respectively, as shown in Figure 2. After spotting, proteins were allowed to react with functionalized surfaces under saturated water vapors overnight at 4°C. Then slides were washed sequentially for 2 × 5 min with PBS, for 5 min with PBS-T, and then dried by centrifugation for 3 min at 1300 rpm. Slides were stored for 1 month at 4°C without blocking or after blocking with 10% BSA/PBS-T solution for 2hrs at room temperature to limit unspecific adsorption. Storage conditions tested were: dry under nitrogen atmosphere, in 5% threhalose solution and in 50% glycerol solution.

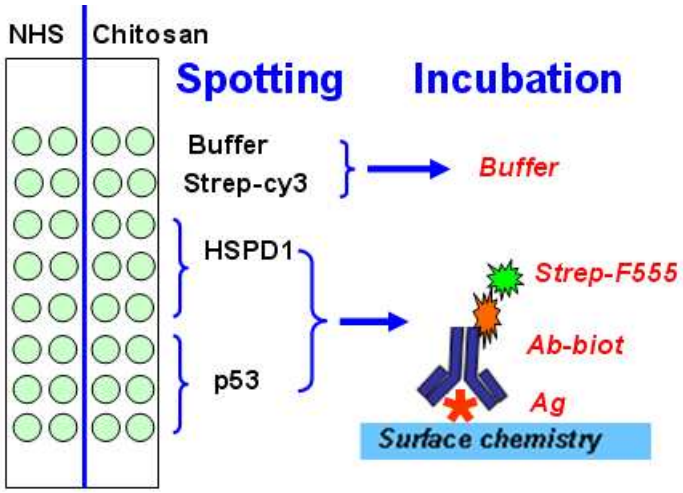


Figure 2 *Scheme of protein microarray design. Buffer solution, streptavidin-Cy3 (Strep-cy3) and 2 antigen proteins (HSPD1 and P53) were spotted at 0.1mg/ml on micro-structured glass slide functionalized with both NHS and chitosan. Then buffer and Strep-Cy3 spots were incubated with buffer solution; HSPD1 and P53 spots were firstly incubated with biotinylated antibody and then with streptavidin-F555.*

In a second test, four antigen proteins (HSPD1, HSP70, HSPA5 and HSP110) were spotted at two concentrations (0.05 mg/ml and 0.1mg/ml) on flat glass slide functionalized with COOH and CMD surface chemistries, shown in Figure 3. Each concentration has 48 replications; streptavidin-F555 (Strep-cy3) at 0.01mg/ml and acetate buffer (pH=4.6) were spotted as quality and negative control, respectively. After blocking with 10% BSA/PBS-T solution, slides were stored at various times (1 month, 2 months, 3 months and 6 months) in 50% glycerol solution at 4°C.

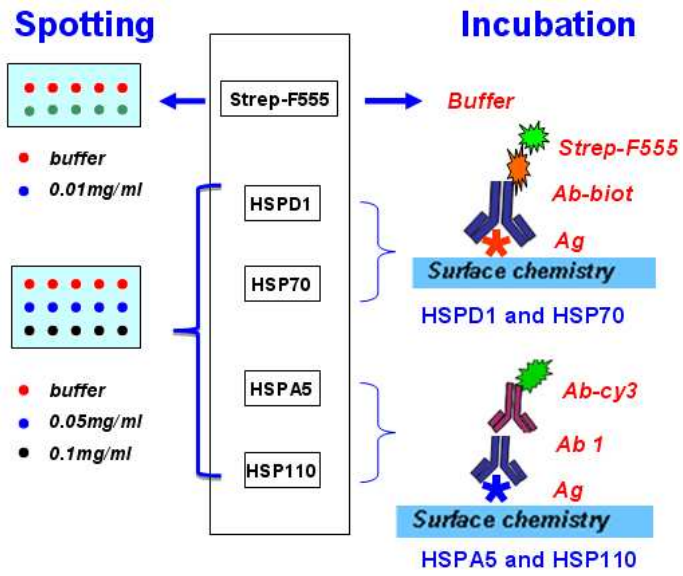
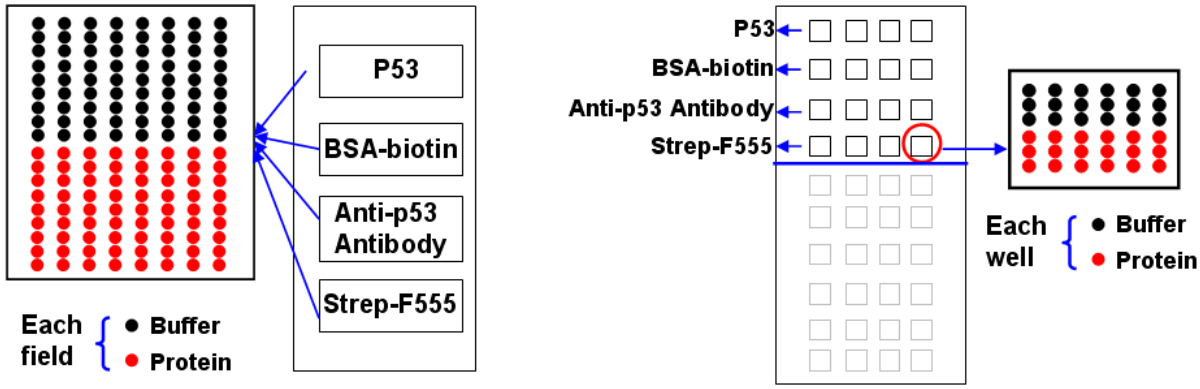


Figure 3 *Scheme of protein microarray design. 4 antigen proteins (HSPD1, HSP70, HSPA5 and HSP110) and streptavidin-cy3 (Strep-cy3) were spotted onto COOH and CMD functionalized flat glass slides. Each field contains buffer spots and one protein at two concentrations (0.05 mg/mL and 0.1 mg/mL), each concentration has 48 replications."Strep-cy3" field was incubated with buffer solution; HSPD1 and HSP70 fields were firstly incubated with biotinylated antibody and then with streptavidin-F555; HSPA5 and HSP110 fields were firstly incubated with unlabeled mouse antibody and then with goat-anti-mouse IgG-cy3.*

Reference biological activity of spotted antigens (T0) was obtained immediately after protein immobilization and blocking with 10% BSA/PBS-T (no storage). After storage, unblocked slides were blocked with 10% BSA/PBS-T solution 2hr at room temperature, then washed with PBS-T for 3 × 5 min and dried. "Strep-F555" spots were always incubated with buffer solution. HSPD1 and HSP70 spots were incubated with 0.5µM biotinylated-antibody, 1hr at room temperature (RT). After washing, they were incubated with streptavidin-cy3 (0.01mg/ml), 1hr at RT. HSPA5 and HSP110 spots were incubated with 0.5µM unlabeled antibody, 1hr at room RT and washed. Then they were incubated with goat-anti-mouse IgG-cy3 at 0.01µM, 1hr at RT. Then slides were washed for 3 × 5 min with PBS-T, 10 seconds in DI water and then dried.

4.2.6 Evaluation of protein microarray reproducibility

P53 (0.1mg/ml), biotin-labeled BSA (0.05mg/ml), anti-P53 antibody (0.15mg/ml) and streptavidin–F555 (0.01mg/ml) were spotted onto 3 chemically functionalized (COOH, NHS and chitosan surfaces) flat and micro-structured glass slides. On flat glass slides, 4 fields containing 72 replications of each spotted solution were defined according to Figure 4a. On micro-structured glass slides, each micro-well contains 18 replications of each spotted solution according to Figure 4b. As each protein was spotted in 4 micro-wells, so in total, each protein also had 72 replications. For chitosan surface, carbonate buffer (pH=9.6) was used as spotting buffer while for COOH and NHS surface, acetate buffer (pH=4.5) was used. In order to compare inter- and intra-coefficient variation for each surface chemistry, 2 flat glass slides and 2 micro-structured glass slides were evaluated for each surface chemistry.



(a) (b)

Figure 4 *Design of protein spotting on flat glass slide (a) and micro-structured glass slide (b). On both slides, 4 proteins (P53, BSA-biotin, anti-P53 antibody and streptavidin–F555) were spotted and each of them has 72 replications.*

Table 2 *Incubation process for protein microarray reproducibility*

Spotted proteins	Biological recognition step	Detection step	Detection step
P53	Anti-P53 Ab-biotin: 0.5µM	Strep-F555: 0.19 µM	Buffer
BSA-biotin	Buffer	Strep-F555: 0.19 µM	Buffer
Anti-P53 antibody	P53: 0.05 µM	Anti-p53 Ab-biotin: 0.5µM	Strep-F555: 0.19 µM
Streptavidin–F555	Buffer	Buffer	Buffer

The process of incubation for both flat glass slides and micro-structured slides is shown in Table 2. The detail of incubation and washing step is the same as in part 2.2.4.

4.2.7 Fluorescence scanning and data analysis

After drying, slides were scanned with the Microarray scanner GenePix 4100A at wavelengths of 532 nm with the same photomultiplier tube (PMT) gain (PMT=600). Data mining was accomplished with GenePix 4100A software package (Axon Instruments). SNR were calculated as referred in part 2.2.6.

4.3 Results and discussion

4.3.1 Optimization of elaboration and processing of protein microarray

Enzyme-linked immunosorbent assays (ELISA) performed in a 96-well plate is routinely used for detection of proteins. Figure 5 showed the general protocol of ELISA, during which antigen was captured on well, then the first antibody was added and then followed by a second labeled antibody. Differences on protocol exist between different commercial ELISA kit and home-made ELISA, e.g. blocking time, blocking solution, incubation time and the concentration of incubation solution.

ELISA is limited to screen large numbers of proteins in an efficient manner, particularly when sample volumes are limited. In contrast, protein microarray could simultaneous screen multiple proteins in small sample volumes. Furthermore, the theoretical detection limit for microarrays is significantly lower than that for 96-well plate assay [17]. However, microarrays have not matched their theoretical predictions. One reason is that their current experimental protocols originate from 96-well plate ELISA protocols and have not been fully optimized [18, 19]. Various factors influence the performance of microarray and in this study, our interests focus on optimizing factors including time of incubation time and the concentration of incubated solution. We chose one-factor-at-a-time (OFAT) method for designing experiment. During the experiment design, we vary one single factor each time

while keeping the others constant and then measure the process output. We determine the best condition for each factor one by one.

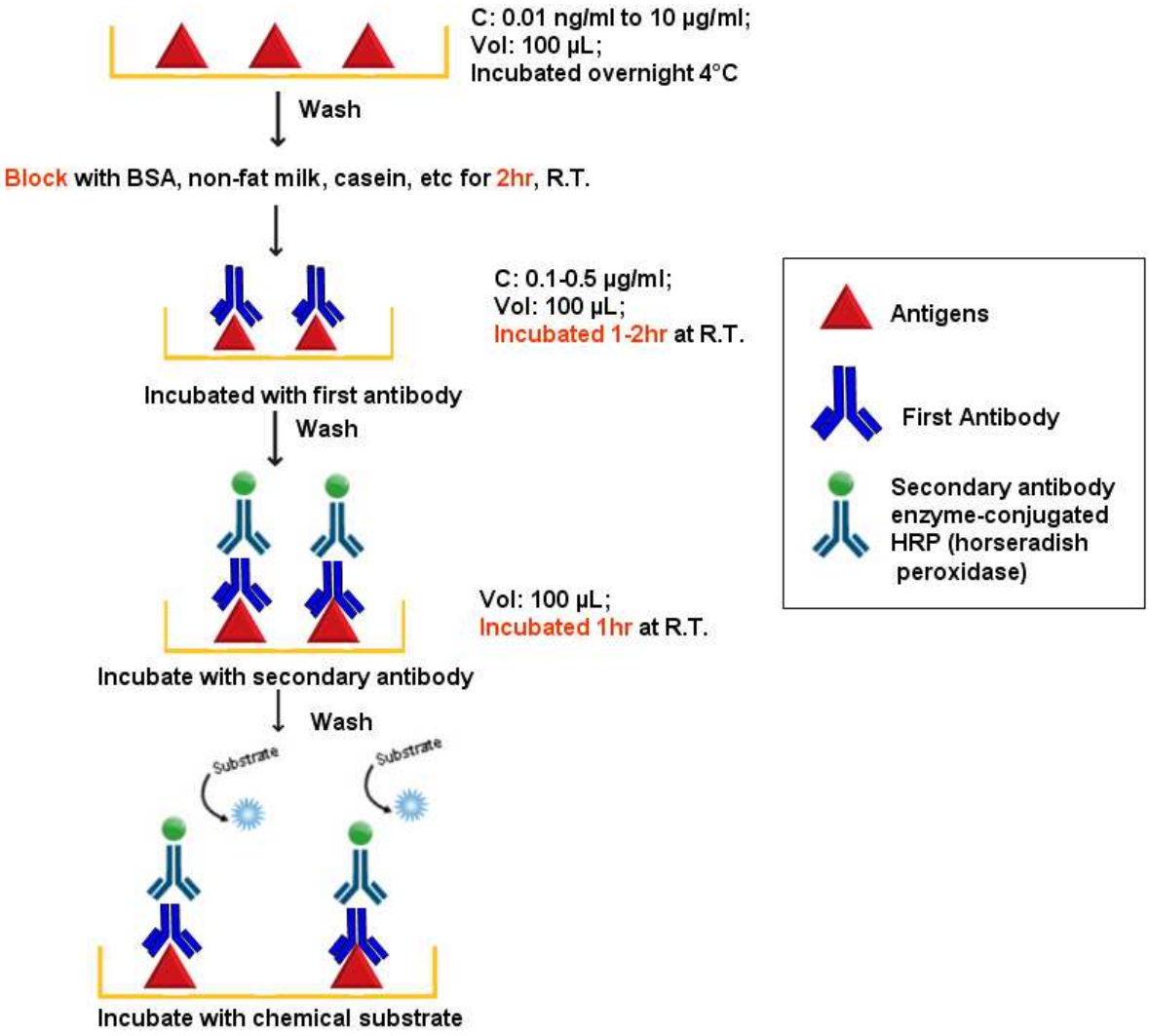


Figure 5 *Scheme of ELISA; C: concentration; Vol: volume; R.T. room temperature.*

4.3.1.1 Influence of blocking, antibody and detection incubation times

In order to evaluate the duration of experiment, we spotted two antigens (HSPD1 and P53) on NHS surface at four concentrations (0.01mg/ml, 0.025mg/ml, 0.05mg/ml and 0.1mg/ml) and then evaluated the blocking and incubation process at different time period, as described in Figure 6.

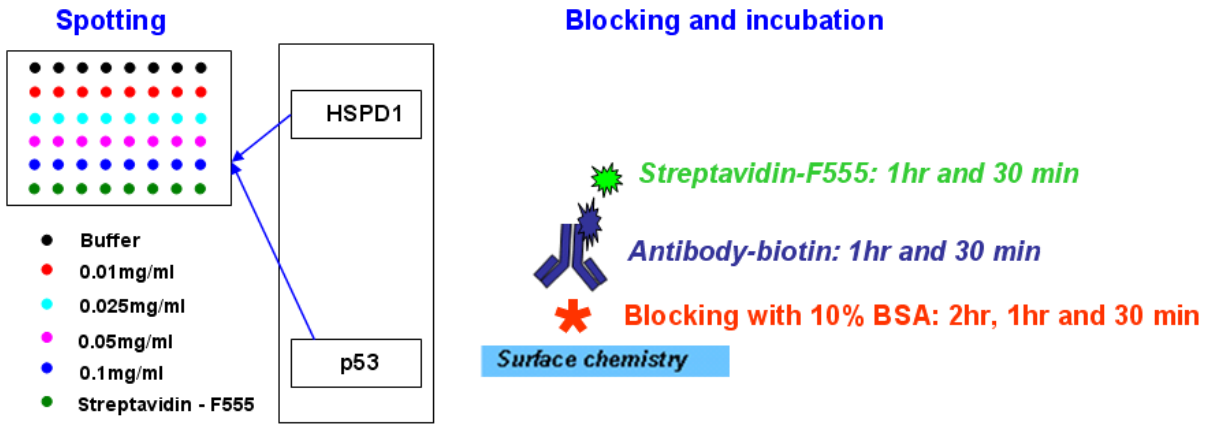
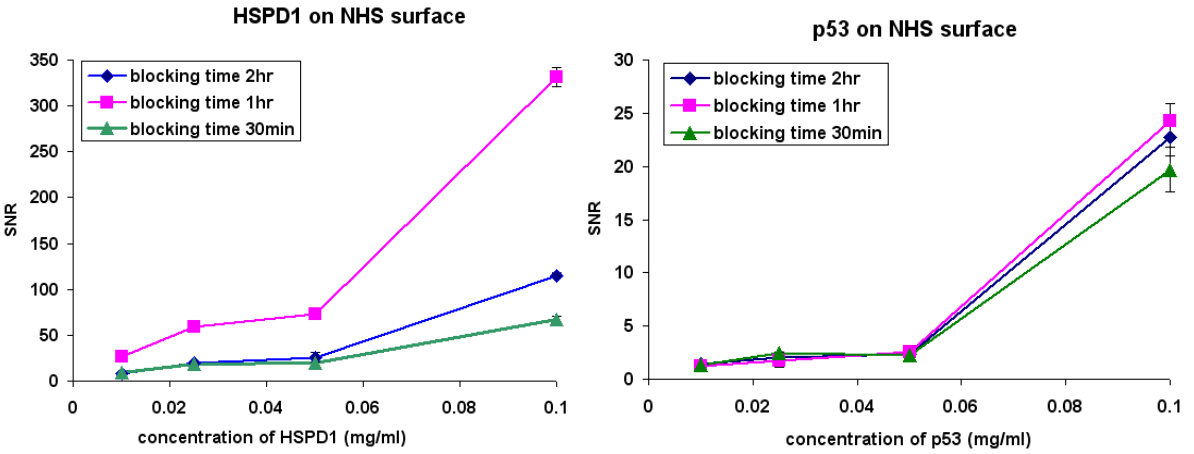


Figure 6 *Protein microarray design for evaluation of blocking and incubation time. HSPD1 and P53 were spotted at 4 different concentrations on NHS surface; streptavidin-F555 (0.01mg/ml) and acetate buffer were used as quality and negative control, respectively. Three time periods (2hr, 1hr and 30min) were tested for blocking process; two time periods (1hr and 30min) were tested for the incubation of biotinylated antibody as well as streptavidin-F555.*

Blocking process

The influence of blocking time on the efficiency of antigen/antibody recognition is presented in Figure 7. Firstly, for both antigens, biological recognition with their corresponding antibody is improved at 0.1 mg/mL of spotted concentration. Secondly, the influence of blocking incubation time on the level of antigen/antibody recognition is protein dependent. Indeed, P53/anti-P53 antibody system doesn't seem to be affected by blocking time (Figure 7a), whereas HSPD1/anti-HSPD1 antibody system is greatly influenced by blocking time (Figure 7b). 1hr of blocking leads to the best performance for HSPD1/anti-HSPD1 antibody system.



(a) (b)

Figure 7 *Influence of blocking time (2hr, 1hr and 30min) on the efficiency of antigen/antibody recognition on NHS surface, as a function of spotted antigen concentration. (a) HSPD1/anti-HSPD1 antibody recognition; (b) P53/anti-P53 antibody recognition.*

Blocking process is critical because it could reduce unspecific binding and improve the performance of microarrays. As shown in Figure 8, the fluorescence signal of buffer decreased with the increasing of blocking time for both HSPD1 and p53. However, after 1 hr of incubation with blocking solution, no significant benefit is observed. Same results were obtained from [9]. They found that long time of blocking could reduce the background; however, it could also block the reactive sites of spotted proteins and lead to the decrease of signal. Therefore, 1hr of blocking is the best for our microarray performance, because it could reduce unspecific binding without affecting the efficiency of biological recognition.

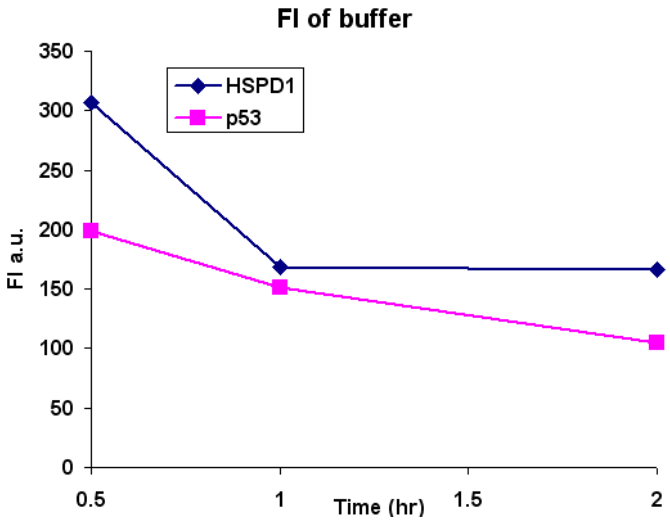
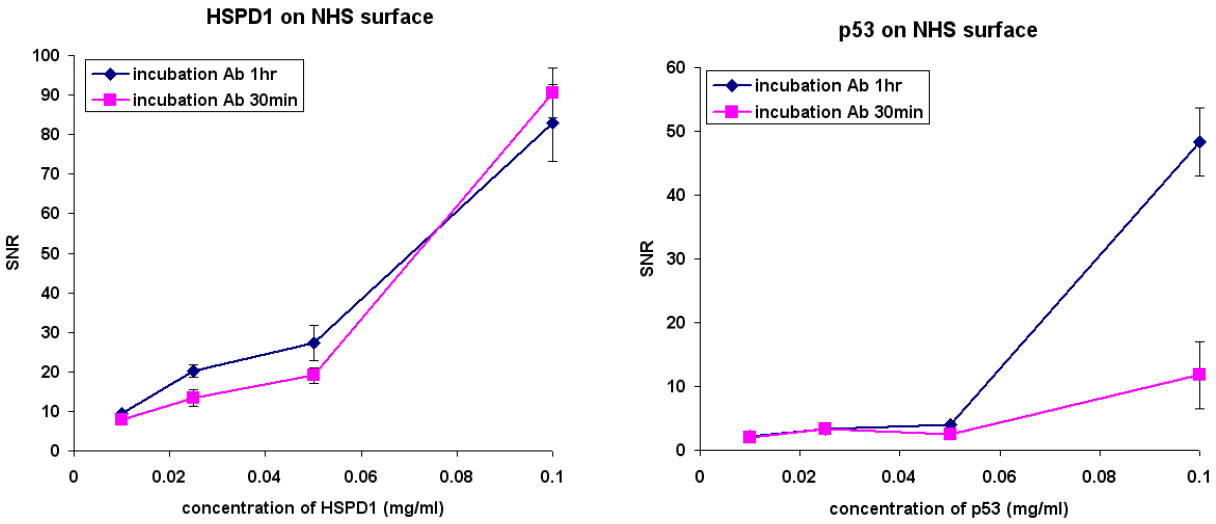


Figure 8 *Influence of blocking time (30min, 1hr and 2hr) on the fluorescence signal of buffer of HSPD1 and p53.*

Incubation process with antibody

Figure 9 showed the influence of recognition antibody incubation time (biotin-labeled antibody) on the efficiency of the biological recognition. Again, it is dependent on detection system and spotted concentration. In all conditions, biological recognition of both systems is improved at 0.1 mg/mL spotted antigen and with 1hr of incubation time with biotin-labeled antibody.



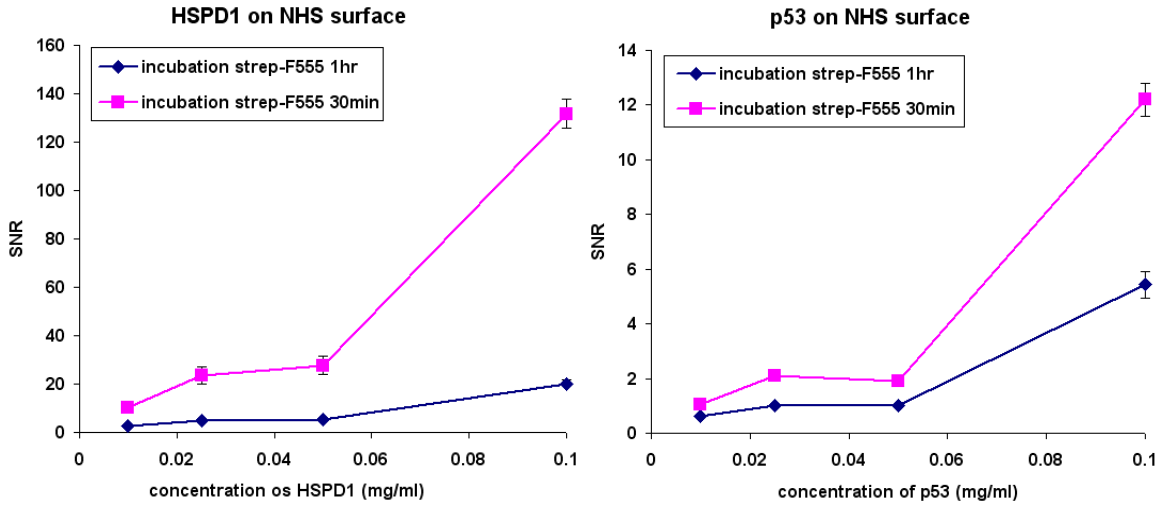
(a) (b)

Figure 9 *Influence of antibody incubation time (30min and 1hr) on the efficiency of antigen/antibody recognition on NHS surface. (a) HSPD1/anti-HSPD1 antibody recognition, (b) P53/anti-P53 antibody recognition*

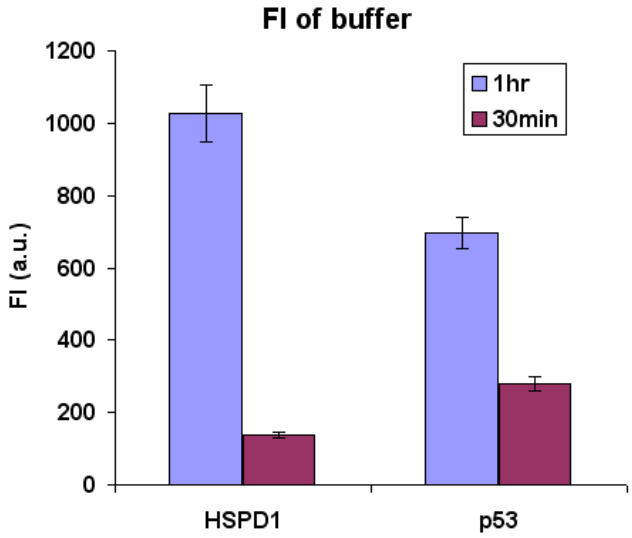
The incubation time is dependent on antigen-antibody micro-spot kinetics. It is influenced by two factors: (i) the transport of the analyte from the solution to the surface reaction area; (ii) the subsequent binding process [10]. Binding process depends on the affinity between antigens and antibodies. P53/anti-P53 antibody recognition performs better under 1hr of incubation maybe because of the low affinity between both parts compared to HSPD1/anti-HSPD1 system.

Incubation process with streptavidin-F555

For the detection step, both antigen/antibody systems showed improved detection level after 30 min incubation with Strep-F555 instead of 1hr, and with 0.1 mg/mL spotted concentration. Figure 10 showed the influence of second incubation (strep-F555) on microarray performance. From the results, we can conclude that for both HSPD1 (Figure 10a) and P53 (Figure 10b), 30 min of incubation is better than 1hr. When we compared the fluorescent signal of buffer, we found that longer time of incubation leaded to higher signal of buffer (Figure 10c). Therefore, shorter time of incubation is better and it could reduce the unspecific binding.



(a) (b)



(c)

Figure 10 *Influence of detection time (30min and 1hr) on the efficiency of antigen/antibody recognition on NHS surface. (a) HSPD1/anti-HSPD1recognition, (b) P53/anti-P53 recognition, (c) Fluorescence intensity (FI) of buffer*

During our antigen microarray processing, the process of incubation with antibody is essential. We could not reduce the incubation time because of the antigen-antibody micro-spot kinetics. Unlike the recognition between antigen and antibody, the affinity between biotin and streptavidin is strong, thus short time of incubation is efficient; furthermore, it could reduce unspecific binding of streptavidin. For the blocking process, long time of blocking could not reduce the background. Therefore, taken all these experimental duration together, we could reduce experimental time from 4h to 2h30, as shown in Table 3.

Table 3 *Experimental time in the protocols before and after optimization*

Factors	Initial protocol	Optimized protocol
Blocking time (hr)	2	1
Antibody incubation time (hr)	1	1
Streptavidin-F555 incubation time (min)	60	30

4.3.1.2 Influence of detection antibodies and streptavidin-F555 concentrations

From previous results, it was shown that the efficiency of protein microarrays is very dependent on the biological system studied and on the surface chemistry. Thus, to optimize costs related to the elaboration and processing of protein microarray, we studied the influence of detection antibodies and streptavidin-F555 concentrations on the level of detection of interactions of all antigen/antibody systems studied. The 8 antigens were spotted at 2 concentrations on NHS, COOH and APDMES surfaces and processed according to Figure 11.

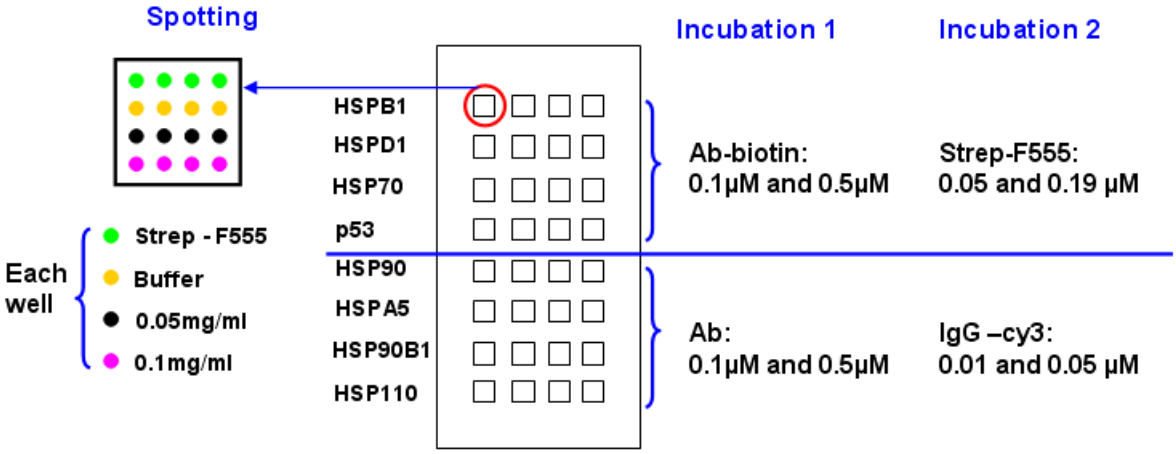
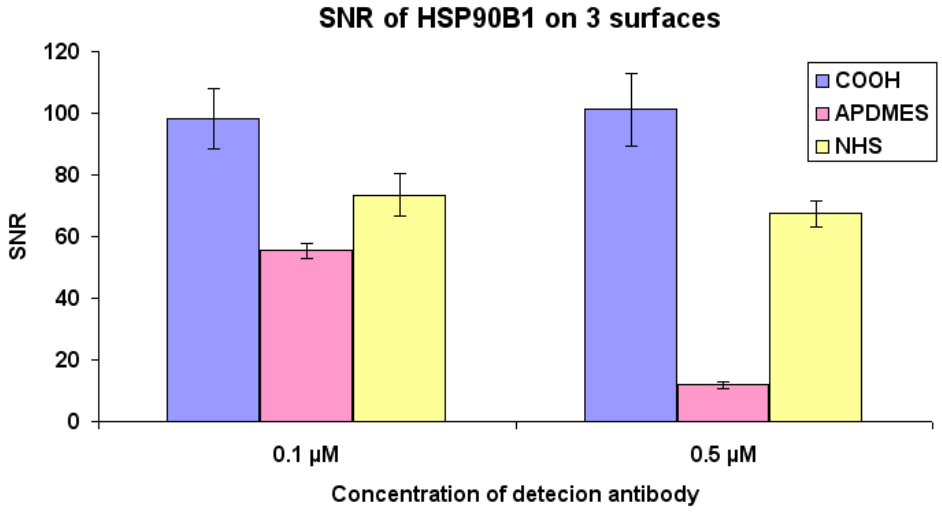


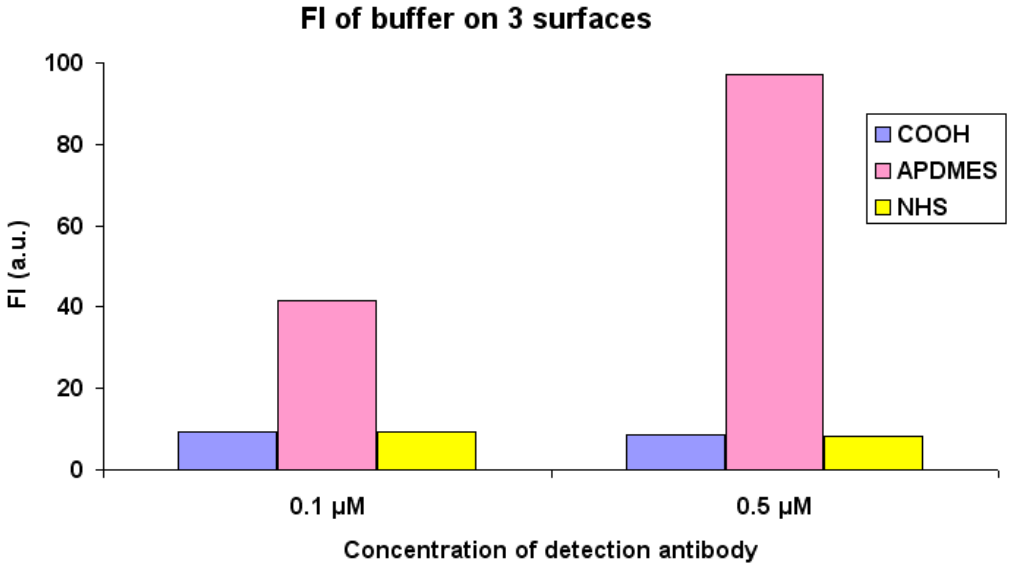
Figure 11 *Scheme of protein microarray design for optimization of detection antibody and streptavidin-F555 concentrations. 8 antigens were spotted at 2 concentrations (0.05mg/ml and 0.1mg/ml) in acetate buffer (pH=4.6) on COOH, NHS and APDMES surfaces. In each microwell, acetate buffer and strepdavidin-F555 were used as negative control and quality control respectively; each solution was spotted in 4 replicates. Biotin-labeled or unlabeled antibodies were tested (Incubation 1) at two concentrations (0.1µM and 0.5µM); detection step (Incubation 2) was performed using strep-F555 (0.05µM and 0.19µM) or IgG-cy3 (0.01µM and 0.05µM).*

As expected, the best detection signal for all antigens was obtained with 0.1 mg/mL of spotting concentration. Therefore, only results obtained with this concentration will be presented.

Figure 12 showed the influence of the concentration of incubated antibodies on the performance of antigen microarray. Figure 12a represents the fluorescence signal (SNR) obtained for HSP90B1/anti-HSP90B1 on COOH, NHS and APDMES surfaces at 2 different concentrations (0.1 µM and 0.5 µM) of first antibody. On COOH and NHS surfaces, no significant difference was observed between the both concentrations of first antibody. In contrast, the signal on APDMES surface was higher when the concentration of first antibody was lower (0.1 µM), which was caused by lower fluorescence signal of buffer, as shown in Figure 12b. Higher concentration of first antibody could increase unspecific binding; therefore, in order to improve the performance as well as to be more economic, 0.1 µM of first antibody will be used in further experiment.



(a)



(b)

Figure 12 *Influence of the concentration of detection antibodies on the performance of antigen microarray. SNR of HSP90B1/anti-HSP90B (a) and fluorescence signal of buffer (b) on COOH, NHS and APDMES surfaces, using 2 different concentrations of first antibody (0.1 µM and 0.5 µM).*

Concerning the influence of the concentration of streptavidin-F555 on the detection level, Figure 13 showed the performance of HSP70/anti-HSP70 detection on APDMES surface using 2 streptavidin-F555 concentrations (0.05 µM and 0.19 µM). Fluorescence intensity of buffer was lower when the concentration of streptavidin-F555 was lower (0.05 µM) indicating that less unspecific adsorption was obtained. Thus to the detection signal (SNR) of HSP70/anti-HSP70 antibody interaction was higher. Same results were obtained for other proteins (HSPB1, HSPD1 and P53) on APDMES surface. In contrast, no significant difference was observed when the 4 antigens (HSPB1, HSPD1, HSP70 and P53) were immobilized on COOH and NHS surfaces. This could be due to higher unspecific adsorption of streptavidin-F555 on APDMES surface compared to COOH and NHS surfaces. Therefore, in order to reduce the unspecific adsorption, low concentration of strep-F555 (0.05 µM) will be used in further experiment.

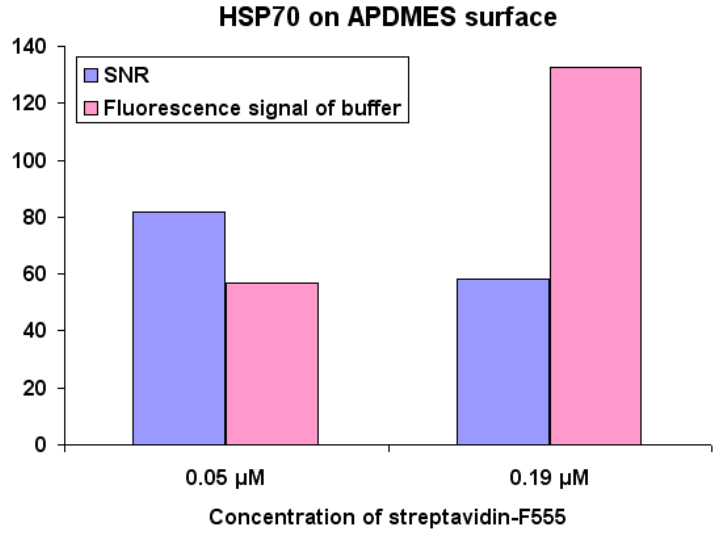


Figure 13 *Influence of the concentration of streptavidin-F555 on the performance of HSP70/anti-HSP70 detection; 2 streptavidin-F555 concentrations (0.05µM and 0.19µM) were tested.*

At last, the concentration of second antibody (IgG-Cy3) could also influence the detection level of antigen/antibody recognition. Figure 14 showed the performance of HSP90/anti-HSP90 antibody recognition on COOH surface using 2 concentrations of IgG-cy3 (0.01 µM and 0.05 µM). As previously observed for streptavidin-F555, at high IgG-Cy3 concentration (0.05 µM), unspecific binding is higher (high fluorescence signal of buffer) leading to lower specific signal (SNR). Same results were obtained for other proteins (HSPA5, HSP90B1 and HSP110) on COOH, NHS and APDMES surfaces. Therefore, in order to reduce the unspecific binding, low concentration of IgG-Cy3 (0.05 µM) will be used in further experiment.

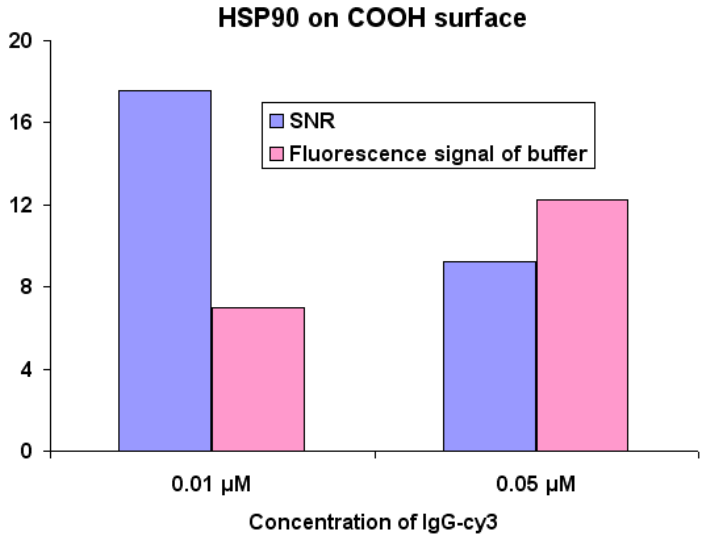


Figure 14 *Influence of the concentration of IgG-cy3 on the performance of HSP90/anti-HSP90 detection; 2 concentrations (0.01µM and 0.05µM) were tested.*

Table 4 *Experimental conditions for optimal detection of antigen/antibody interactions studied.*

Concentration of spotted antigens	Concentration of first Ab / Ab-biotin	Concentration of strep-F555	Concentration of second antibody: IgG –cy3
1.4-1.8 µM (HSPB1, HSPD1, HSP70 and p53)	0.1µM	0.05 µM	-
1-1.2 µM (HSP90, HSPA5, HSP90B1 and HSP110)		-	0.01 µM

Table 4 summarized the optimized concentrations of spotting antigens, recognition antibodies and detection molecules. We can see that the ratio between spotted antigen concentration and first antibody concentration ranges between 10:1 and 18:1. For HSPB1, HSPD1, HSP70 and P53, the ratio between first antibody and streptavidin-F555 was 2:1; for HSP90, HSPA5, HSP90B1 and HSP110, the ratio between first antibody and second antibody (IgG-cy3) was 10:1. Among the results, the ratio between first antibody and streptavidin-F555 is the highest; maybe we could further reduce the concentration of streptavidin-F555. During the protocol, washing step will remove the majority of proteins in each step, therefore, less concentrated solutions are needed in the following step; otherwise, higher concentrated solution could lead to high unspecific binding. According to our results, a ratio about 10:1 on the concentration between the former and later solution was recommended. In addition to reduce cost, it could also improve the performance of antigen microarray.

4.3.2 Evaluation of the stability of protein microarray under storage

Storing protein microarray is a major concern for both microarray manufacturers and users. The stability of protein microarray under storage can be studied at 2 levels: the stability of the surface chemistry, and the stability of spotted proteins.

4.3.2.1 Stability of the surface chemistries of protein microarray

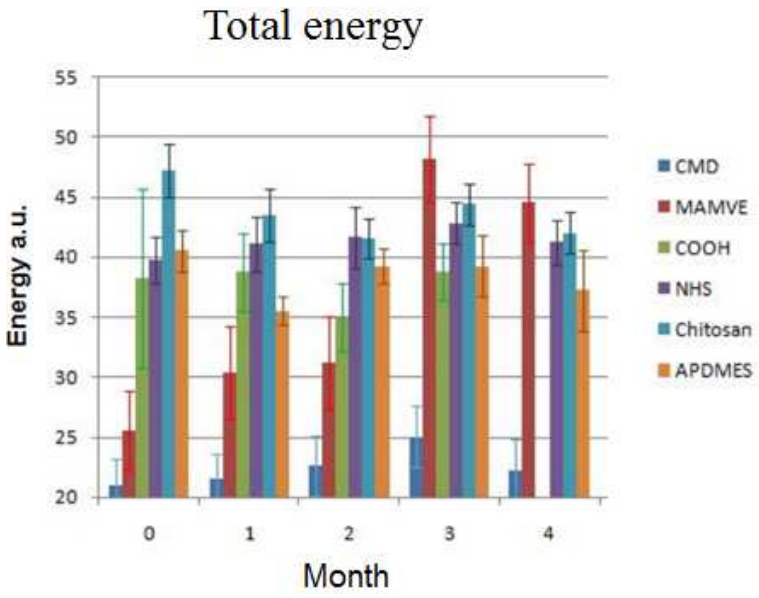
The 6 surface chemistries used for the elaboration of protein microarray were developed in previous work [20]. They were chemically characterized by X-ray photoelectron spectroscopy (XPS), infrared spectroscopy (IR), mass spectrometry (Tof-SIMS). They were also characterized for their surface energy by contact angle measurement. Indeed, contact angle provides macroscopic information of the surface energy and it is related to the chemical characteristics of the surface. Measuring contact angle is a rapid characterization technique, and any change indicates modification of chemical functions on the surface. Thus, first the stability of the 6 surface chemistries was evaluated, under storage (in dry condition) at different times (from 0 to 4 months), from a macroscopic point of view by surface energy calculation. Second, the stability of surfaces was also studied from the molecular point of view by evaluating their capacity to immobilize proteins.

Total surface energy of all six surfaces was calculated from contact angle measurements according to Owens-Wendt model [16]. 3 different liquids (water, diiodomethane, ethylene glycol) were used in order to determine the dispersive and polar contributions to total surface energy. Results presented in Figure 15 showed that total surface energy remains constant until 4 months of storage in dry condition, for 3 surface chemistries (COOH, NHS and chitosan surfaces, but not for MAMVE, APDMES and CMD surfaces (Figure 15a). The drastic increase of total surface energy of MAMVE surface was mainly due to the increase of polar energy (Figure 15b). MAMVE polymer contains anhydride units which, in the presence of water, could be hydrolyzed leading to 2 carboxylic acid groups. The storage of chemically functionalized surfaces was performed under dry condition but not under sealed nitrogen atmosphere. It is likely that water molecules contained in ambient atmosphere could contribute to the hydrolysis of anhydride units. Then, the formation of carboxylic acid groups led to increase the amount of hydrogen bonds, so the polar energy of the surface. It is to note that the increase of polar energy of MAMVE surface is detected after 3 months of storage indicating that the hydrolysis of anhydride units should be very slow. The dispersive energy

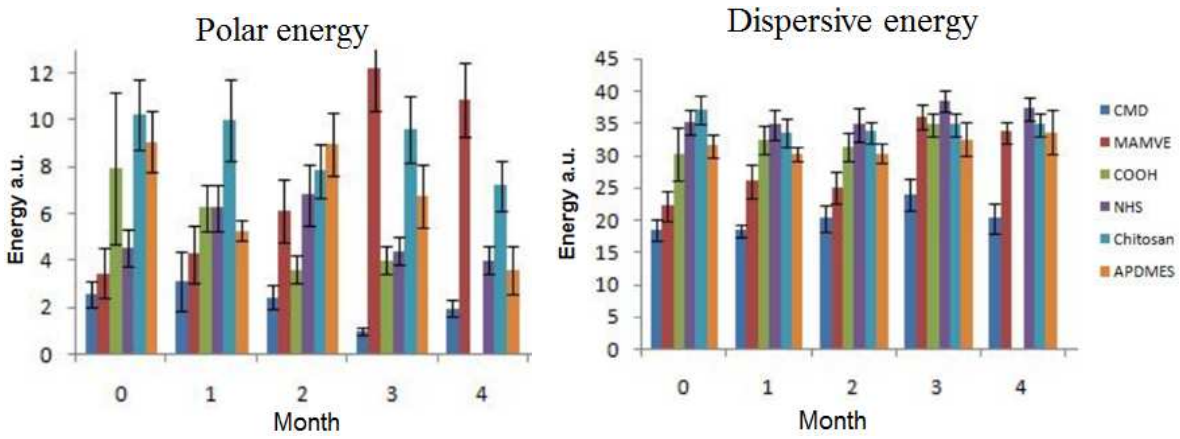
of MAMVE surface also increase but in a lower amount (Figure 15c). The hydrolysis of anhydride units leading to the opening of the cycle, more London interactions could be established between pendent chains.

Concerning APDMES surface, although total surface energy displayed very slight variation with time, its polar contribution clearly decreased (Figure 15(b)). Smith et al. [21] described the decrease of the aminosilane layer depth due to siloxane hydrolysis catalyzed by amino groups in the presence of water at 40°C during 1 or 2 days. In our case, the decrease of polar energy was observed after 3 months of storage. Thus, it is possible that the water contained in ambient atmosphere hydrolyzed slowly the siloxane link, leading to the partial loss of APDMES molecules, so to the decrease of polar energy.

At last, CMD surface displayed a slight decrease of its polar energy from month 3 suggesting a degradation of the surface. Indeed, we observed some blotches on the surface (Figure 16). Further characterizations will be needed in order to understand the phenomenon inducing these blotches and their composition.



(a)



(b) (c)

Figure 15 *Variation of total surface energy (a), polar energy (b) and dispersive energy (c) of the 6 surface chemistries developed for protein microarray with storage time under dry condition.*

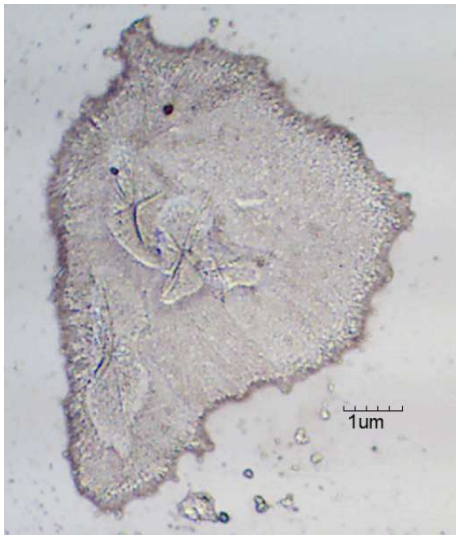


Figure 16 *Blotch observed on CMD surface after storage through optical microscope.*

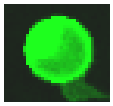
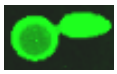
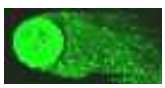
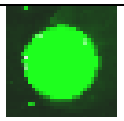
In conclusion, COOH, NHS and chitosan surfaces are the most stable following 4 months storage in ambient atmosphere. MAMVE, APDMES and CMD surfaces seem to be more sensitive to humidity present in ambient atmosphere. Therefore, for long time storage of chemically functionalized surfaces, sealed nitrogen conditions should be required in order to avoid degradation of the physico-chemical properties of the surfaces.

In addition, all chemically functionalized surfaces were evaluated for their ability to immobilize biological molecules as a function of time. For that purpose, 3 different fluorescent labeled molecules (oligonucleotide-Cy3 (ODN-Cy3), streptavidin-Cy3 (Strep-Cy3), Immunoglobulin-Cy3 (IgG-Cy3) were spotted at various time of storage (from 0 to 4

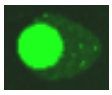
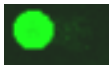
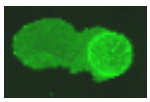
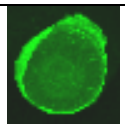
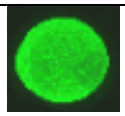
months) onto surfaces. Immobilization efficiency was analyzed in terms of spot quality (size, homogeneity) and in terms of amount (fluorescent intensity).

Table 5 showed the evolution of the quality of spots with time, for streptavidin-Cy3, IgG-cy3 and ODN-cy3 on chitosan and MAMVE surfaces. On chitosan surface (Table 5a), IgG and ODN spots showed good homogeneity and constant spot size. However, streptavidin spots, at both concentration tested, became less homogenous while spot size was constant. On MAMVE surface (Table 5b), the quality of all spots changed from month 3 in agreement with the modification of surface energy. Indeed, spots became larger indicating that the surface became more hydrophilic as its polar energy increased. Only streptavidin spots remained homogenous until 3 months of storage, IgG and ODN spots showing inhomogeneity. The same behavior was observed with CMD surface. From these observations we can conclude that modifications in the quality of spots are in agreement with variations in surface energy, but spots homogeneity is closely dependent on molecules and surface chemistry as already described in [22]. Thus, it will be very important to characterize at the molecular level the interactions between biomolecules and chemical groups grafted on solid support.

Table 5 *Fluorescent images of streptavidin-Cy3, IgG-cy3 and ODN-cy3 on chitosan surface (a) and MAMAVE surface (b) as a function of storage of time.*

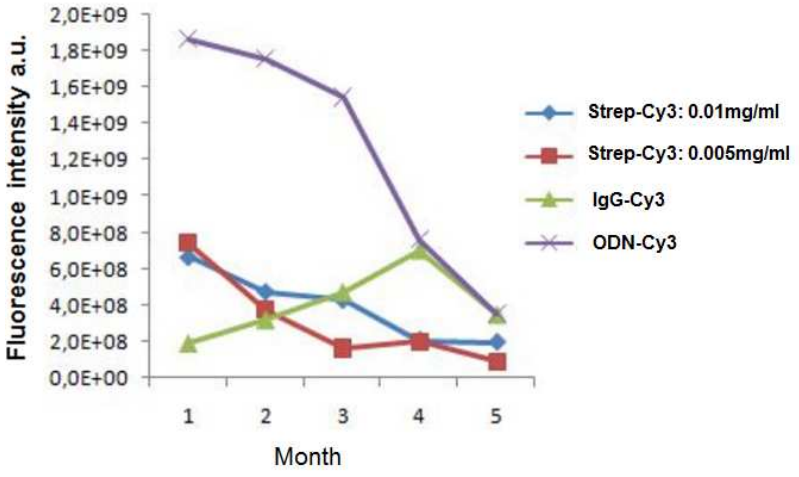
Molecules	No storage	1 month	2 months	3 months	4 months
Streptavidin-Cy3 0.01 mg/ml					
Streptavidin-Cy3 0.005 mg/ml					
IgG-Cy3					
ODN-Cy3					

(a)

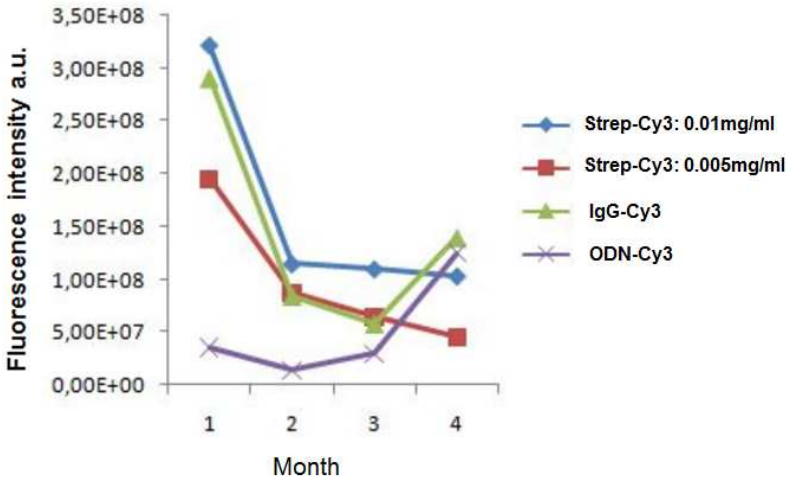
Molecules	No storage	1 month	2 months	3 months
Streptavidin-Cy3 0.01 mg/ml				
Streptavidin-Cy3 0.005 mg/ml				
IgG-Cy3				
ODN-Cy3				

(b)

In order to quantify the variation observed previously, the fluorescent intensity of each spotted molecule on each surface chemistry was analyzed with the time of storage. Figure 17 showed the results obtained on chitosan and MAMVE surfaces. On both surfaces, the fluorescent intensity of spotted molecules decreased with time, except for IgG on both surfaces and for ODN on MAMVE surface. On MAMVE surface, the main decrease was obtained during the first month of storage suggesting that major degradation of surface chemistry occurred during this period. For the other surfaces, the decrease is smoother with time. However, until 3 months of storage of chemically functionalized glass slides, the immobilization of biomolecules remained efficient enough in terms of surface density.



(a)



(b)

Figure 17 *Fluorescent intensity of streptavidin-Cy3, IgG-cy3 and ODN-cy3 spotted on chitosan surface (a) and MAMVE surface (b) as a function of storage time.*

In conclusion, all chemically functionalized glass slides used in this study for the elaboration of protein microarray were shown to be sensitive to storage in ambient atmosphere. Even though no significant difference in surface energy was observed between fresh and stored surfaces, their ability to immobilize molecules could change a lot indicating that properties at the molecular level could be affected with effect at macroscopic level. Indeed, modifications of macroscopic physico-chemical properties were confirmed at the molecular level. Functionalized glass slides could retain efficient reactivity until 3 months of storage in ambient atmosphere, but the storage time could probably be improved in sealed nitrogen environment. Thus, further analysis on the stability of surface chemistries in the least condition would be interesting as well as on biological activity of immobilized molecules.

4.3.2.2 Stability of spotted protein microarray

In order to reduce the number of microarray slides used and experiments, we divided this study in 2 parts. In each part we chose 2 surface chemistries, one allowing covalent binding of proteins (NHS or CMD surfaces) and one allowing physical adsorption of proteins (COOH or chitosan surfaces). The first part was devoted to rapidly identify the best storage conditions (under solution or dry, with or without blocking) of spotted protein microarray, and the second part aimed at studying biological activity of spotted protein as a function of storage time in the best condition defined previously.

So for the first part, P53 and HSPD1 were immobilized onto NHS and chitosan surfaces and stored under various conditions for 1 month. Then their biological activity was evaluated by their capacity to recognize their specific antibody. Storage conditions were selected according to the literature [12-15]: in 50% glycerol solution, in 5% trehalose solution, under nitrogen atmosphere, without blocking step, after blocking step with PBS/BSA solution. Results indicated that biological activity of immobilized antigens is better retained when protein microarray was stored after blocking with PBS/BSA solution than before, for both p53 (Figure 18 a) and HSPD1 (Figure 18 c) immobilized on NHS surface. This was caused by the lower unspecific binding of buffer after blocking process (Figure 18 b and d). The results of P53 and HSPD1 on chitosan surface led the same conclusion. The results were consistent with those obtained by Adarsh D. Radadia, they also found that spotted microarray had a higher signal after blocking process [23].

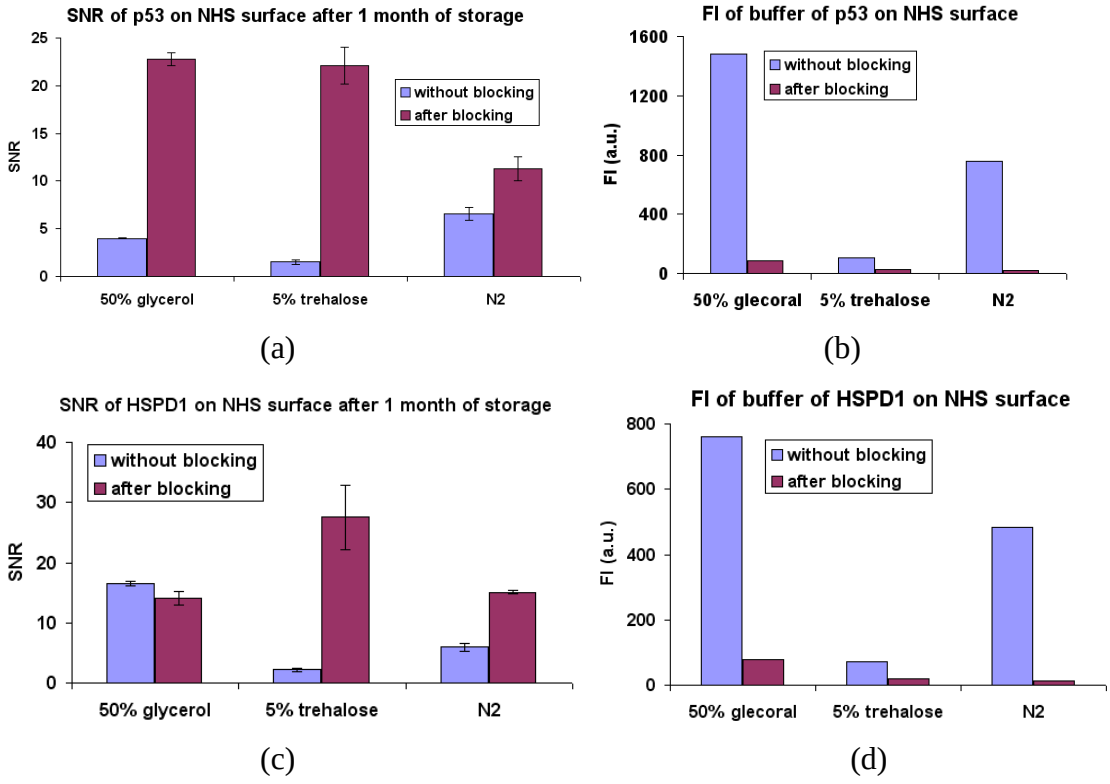
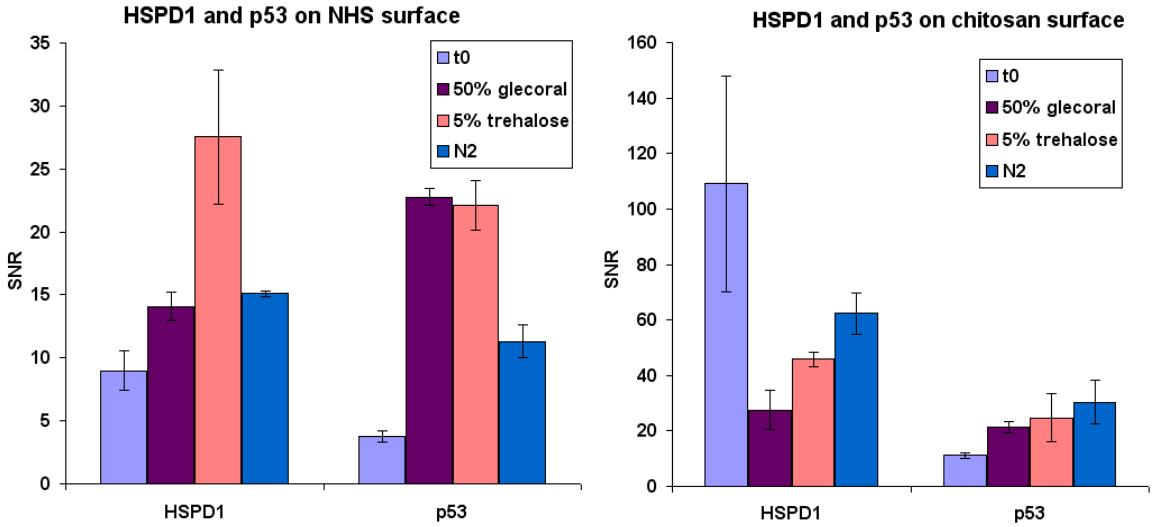


Figure 18 *Biological recognition activity of P53 (a), fluorescence intensity of buffer spots (b), biological recognition activity of HSPD1 (c), fluorescence intensity of buffer spots (d) immobilized on NHS surface after 1 month of storage in various conditions (50% glycerol solution, 5% trehalose solution, N_2 atmosphere).*

Then, if we compared biological activity of immobilized antigens before storage (T0) and after 1 month storage with blocking step, it appeared that the best storage condition depends on surface chemistry (Figure 19). Indeed, for both P53 and HSPD1 immobilized on NHS

surface, the best storage conditions seemed to be in solution (Figure 19a) whereas on chitosan surface, nitrogen atmosphere gave better results (Figure 19b).



(a) (b)

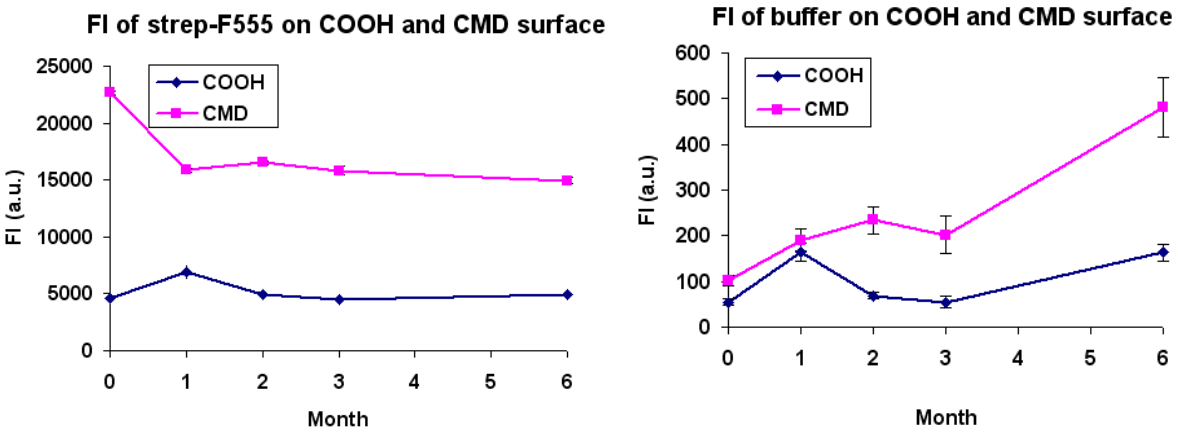
Figure 19 *Biological recognition activity of P53 and HSPD1 immobilized on NHS (a) and chitosan (b) surfaces after 1 month of storage after blocking in various conditions (50% glycerol solution, 5% trehalose solution, N_2 atmosphere).*

The immobilization strategy is different on the two surfaces studied. On NHS surface, proteins are immobilized through covalent binding while on chitosan surface, immobilization of proteins is achieved through physical adsorption. Under wet condition, covalently linked proteins are more stable and could be less released from the surface compared to physical adsorption. Thus, storage of protein microarray in solution is better when covalent immobilization strategy is used, whereas storage under nitrogen atmosphere should be better using physical adsorption strategy. Same results were obtained from [14].

Moreover, we can notice that for both immobilized antigens on both surfaces (except for HSPD1 on chitosan surface), there is an increase of the fluorescent signal (SNR) after 1 month of storage under all conditions. This was due to the decrease of the fluorescent intensity of buffer spots (data not shown). The same kind of phenomenon was observed by other researchers studying storage conditions for protein microarray [14, 15, 23]. Increase of the biological activity of immobilized protein after storage could be attributed to the favorable rearrangement of immobilized antigens on the surface, thus resulting in better accessibility of biological recognition sites with antibodies. Considering that immobilized proteins would

have higher possibility to change their conformation in solution than in gas atmosphere, we decided to store protein microarray in 50% glycerol solution for further experiment.

Then, in the second part, taking into account these results, we studied the stability of 5 proteins (streptavidin-F555, HSPD1, HSP70, HSP110 and HSPA5) immobilized on 2 different surface chemistries (COOH and CMD surfaces) after blocking and storage in 50% glycerol as a function of time. Two parameters were analyzed. The first one was the stability of immobilized protein depending on immobilization strategy. For that purpose, we immobilized fluorescent protein (streptavidin-F555) and followed its fluorescent signal with time on both surfaces. Results are presented in Figure 20a.

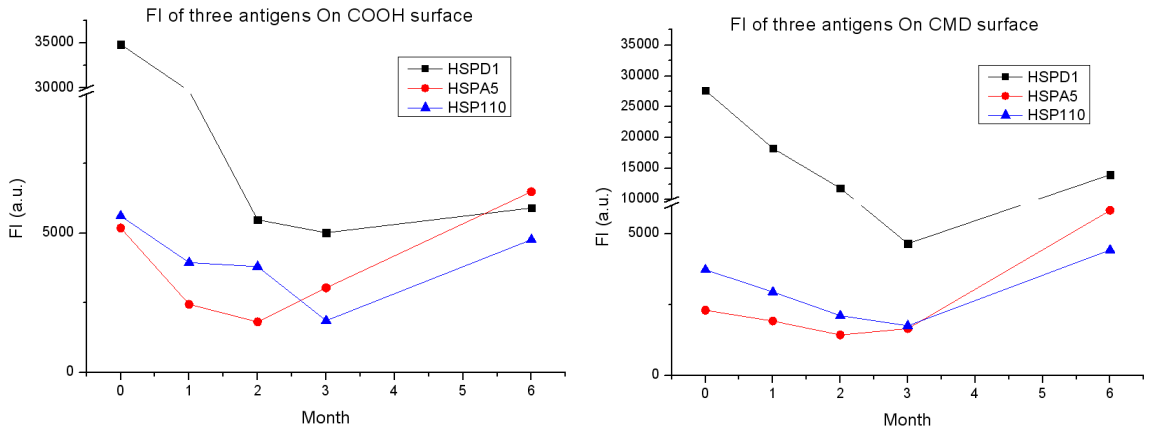


(a) (b)

Figure 20 *Fluorescence signal of immobilized streptavidin-F555 (a) and spotted buffer solution (b) on COOH and CMD surfaces after different time of storage in 50% glycerol solution.*

We can see that the fluorescence intensity of strep-F555 and buffer kept constant during storage on COOH surface. In contrast, the fluorescence intensity of strep-F555 decreased greatly on CMD surface after 1 month of storage, and then stayed constant; whereas the fluorescence intensity of buffer kept stable untill first 3 months and then increased greatly (Figure 20b). Considering that the fluorescence intensity of immobilized strep-F555 showed a high signal after 6 month of storage on both surfaces, we could conclude that the condition of 50% glycerol is efficient to store immobilized proteins.

The second parameter studied was the biological activity of spotted proteins. After immobilization of HSPD1, HSP70, HSP110 and HSPA5 onto COOH and CMD surfaces, their ability to be recognized by their specific antibodies was evaluated (Figure 21).



(a) (b)

Figure 21 *Evaluation of the biological activity of immobilized antigens (HSPD1, HSP110 and HSPA5) on COOH (a) and CMD (b) surfaces after storage in 50% glycerol solution after blocking, at different time; FI: fluorescence intensity.*

During the first month of storage, the fluorescence signal decreased greatly for all spotted proteins on both surfaces, especially for HSPD1. In total, fluorescence intensity of spotted proteins decreased about 20% to 50% on both surfaces suggesting that the biological activity of immobilized antigens was lost in the same amount. Then the fluorescence intensity showed slight change between 1 month and 3 months of storage. Unexpected sharp increase of fluorescence intensity was observed for all spotted proteins on both surfaces after 3 months of storage. This increase was due to strong unspecific binding of surface. Indeed, after 3 months of storage, biological activity of spotted proteins was not detectable. However, our protein microarrays were enough stable for 3 months of storage under 50% glycerol solution and after blocking step, to retain biological activity to sensitively recognize their corresponding antibody. These results are in agreement with those reported in the literature [12-15, 23].

4.3.3 Reproducibility of protein microarray

Reproducibility is a main challenge of protein microarray [24], in the view to replace ELISA. Thus, to evaluate the reproducibility of our protein microarray, the 8 antigens studied were spotted onto flat glass slides and microstructured glass slides functionalized with the different surface chemistries. Then after recognition with their antibody, inter-slides and intra-slide coefficient variation (CV) were calculated. Intra-slide CV evaluates the repeatability of spotting and biological interactions on the same slide. Inter-slides CV evaluates the

reproducibility between slides functionalized with the same surface chemistry, spotted with the same proteins and processed in the same way. Table 5 presents the results obtained for P53/anti-P53 system. We can see that the majority of intra- and inter-CV are lower than 25%, except on chitosan surface. Same tendency was obtained with the other antigen/antibody systems tested. Therefore, our protein microarrays displayed good repeatability and reproducibility to be use in clinical evaluation and routine experiments.

Table 6 *Repeatability and reproducibility of protein microarrays. Intra-slide and inter-slides coefficient of variation (CV) of P53/anti-P53 system studied onto flat and microstructured glass slides functionalized with COOH, NHS and chitosan.*

P53/surface	COOH		NHS		Chitosan	
	Flat	micro-structured	Flat	micro-structured	Flat	micro-structured
Intra CV	20%-22%	21%-24%	10%-13%	7%-13%	15%-29%	4%-12%
Inter CV	14%	5%	6%	21%	28%	9%

4.4 Conclusions

In this part, we have studied various experimental parameters involved in the performance of protein microarrays such as concentrations and incubation time of recognition and detection solutions, blocking time. Optimization of these parameters allowed reducing the time of processing protein microarray from 4 hours to 2 hours 30 minutes, and the cost by decreasing concentrations of biological solutions. Furthermore, we have shown that chemically functionalized glass slides could be store in ambient atmosphere up to 3 months, and may be more under sealed nitrogen atmosphere. For printed protein microarrays, they could be stored after blocking step in 50% glycerol, at 4°C, for 3 months. The biological activity of immobilized proteins decreased but remained sensitive enough for efficient antibody detection. At least, we evaluated the reproducibility and repeatability of our protein microarray and showed that they were in the same range as classical immunoassay such as ELISA.

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Conclusions

The aim of our study is to develop efficient protein microarray to screen biomarkers in breast cancer patients, thus providing diagnostic, prognostic and predictive value for each patient.

An overview of recent literature shows that a large amount of biomarkers (> 1200 molecules) are presented as candidates of high potential to develop molecular diagnosis for cancer detection. Thus, after a selection of numerous biomarkers involved in breast cancer, specific technology was developed to make customized microarrays based on microstructured glass slides. Two types of microarrays were elaborated.

We used antigen microarray to screen autoantibodies against heat shock proteins (HSPs) in breast cancer patients for providing diagnostic and prognostic value. In order to obtain efficient microarray performance, we firstly optimized various factors which influence the performances of protein microarray, including surface chemistry, spotting concentration, etc. Among the 6 surface chemistries tested, two of them (COOH and chitosan) showed good performances for the immobilization of HSPs; therefore, these two surfaces were selected for screening the antibodies against HSPs in breast cancer serum. In total, 50 breast cancer patients and 26 healthy controls were tested. Our results showed that combining multiplex detection of anti-HSPs antibodies could achieve AUC of 0.978. It could discriminate breast cancer patients from healthy controls with sensitivity 86% and specificity 100%. Compared with literature, our antibody panel showed better performance for discriminating breast cancer patients from healthy controls. Furthermore, our data analysis method is more complete and comprehensive. Various studies only provide data on sensitivity without specificity, which is not complete. In contrast, we analyzed the AUC of the performance of our antibody panel and we could provide both sensitivity and specificity of our antibody panel.

Secondly, we used antibody microarray to test the concentration of urokinase type plasminogen activator (uPA) and its main inhibitor plasminogen activator inhibitor 1 (PAI-1) in breast tumor tissue. High levels of uPA and PAI-1 are associated with high risk of recurrence and benefit of chemotherapy for breast cancer patients; therefore, they are good prognostic and predictive biomarkers for breast cancer. In order to obtain efficient antibody microarray, we firstly optimized the immobilization process. We have tested 3 antibodies against PAI-1; however, only one scFv antibody worked on one surface (COOH surface).

Therefore, we didn't quantify PAI-1 in tumor tissue extraction. For antibody against uPA, three surface chemistries (COOH, NHS and chitosan) performed well and they were selected for further experiments. In total, we have tested 16 cytosolic extracts of tumor tissue. Results showed that results obtained from our antibody microarray are surface dependent. For example, no difference was observed between results obtained from chitosan surface with ELISA. In contrast, results obtained on NHS are higher than ELISA and those obtained on COOH surface are lower than ELISA. These results are very promising. Firstly, our antibody microarray showed a higher sensitivity and a wider dynamic range compared to Femtelle ELISA kit. Secondly, considering that one of the main limitations of Femtelle kit is that this kit needs 100-300mg of fresh or frozen samples; our antibody microarray shows high potential as it consumes 25 times less sample volume if we take the dilution times into consideration.

For optimizing the parameters of proteins microarray, we evaluated various factors including experimental duration, the concentration of incubation solutions, etc. Considering that protein microarray is a miniaturized system, it requires less reaction time and less sample volume. Our results showed that we could improve the performance of our customized protein microarray as well as become more economical. We also analyzed the storage condition of surfaces chemistry of protein microarray as well as spotted protein microarray. Results showed that our printed protein microarray could retain their biological activity for at least 3 month.

Consequently, our work demonstrated that our customized antigen and antibody microarray are efficient and powerful tools for rapid screening tumor biomarkers. Various factors influence the performance of protein microarray among which surface chemistry is critical. As observed from our results, several proteins completely lost their biological activity after immobilized on several surfaces. This was maybe caused by the change of structure after immobilization. We proved the necessity to adapt surface chemistry to each protein in order to improve performances of protein microarrays. Furthermore, due to the complex structure of proteins, there is no unique surface which will be suitable for all proteins.

Several aspects need to be improved in the future. 1) As our study was limited by sample size, therefore, further large scale investigation is needed to validate the real diagnostic performance of our customized antigen microarray. 2) Antibodies against HSPs were also over-expressed in other cancers; therefore, we could also test the diagnostic performance of

this panel in other cancers. Furthermore, considering the heterogeneity and complexity of tumor, we need to add other biomarkers in order to increase the sensitivity of the test, e.g. DNA, miRNA, etc. 3) In order to being a simple and powerful tool to be used in clinic like ELISA, the experimental processes of protein microarray need to be automated, like incubation process, data analysing, etc. Also, the process should be well controlled to obtain a good reproducibility. 4) Data analyzing methods should be standardized and complete. Compared with defining cutoff value as a certain value, analyzing data in AUC is better and more comprehensive. Only when data analyzing methods was standardized, could we compare the results from different studies.

Annexe

The receiver operating characteristic (ROC) curve is commonly used in medical decision making. ROC graph is two-dimensional graph in which sensitivity is plotted on the Y axis and 1-specificity is plotted on the X axis. We take the results obtained from HSPA5 immobilized on chitosan surface for an example to explain how we construct ROC curve. As shown in Table 1, the first column is the sample size. In total, we have tested 76 samples, including 26 healthy controls and 50 breast cancer patients. The second column is the value obtained from experiment; we also used these values as cut off value. The third column is the sample characteristic, either cancer or healthy control. Cancer patients were clinical diagnosed and we know this information before we tested. For the last two columns, one is the value of x axis: 1-specificity; the other is y axis: sensitivity.

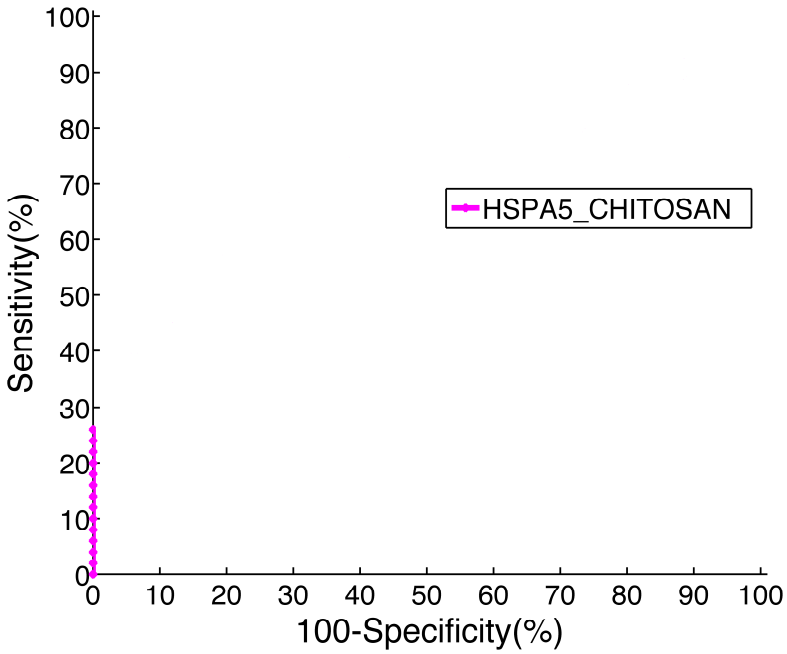
Table 1 Results of HSPA5 immobilized on chitosan surface for all samples

No. (26HC & 50 BC)	Value/ Cut off	Sample characteristic	X axis: 1- specificity	Y axis: Sensitivity
1	5.0	Cancer	1-(26/26)=0	1/50=2%
2	4.8	Cancer	1-(26/26)=0	2/50=4%
3	4.1	Cancer	1-(26/26)=0	3/50=6%
….	….	Cancer	1-(26/26)=0	….
13	2.3	Cancer	1-(26/26)=0	13/50=26%
14	2.2	Healthy control	1-(25/26)=4%	13/50=26%
15	2.1	Cancer	1-(25/26)=4%	17/50=34%
….	….	Cancer	….	….
20	1.8	Cancer	1-(25/26)=4%	20/50=40%
21	1.7	Healthy control	1-(24/26)=8%	20/50=40%
….	….	….	….	….
76	0.4	Healthy control	1-(0/26) =100%	50/50 = 100%

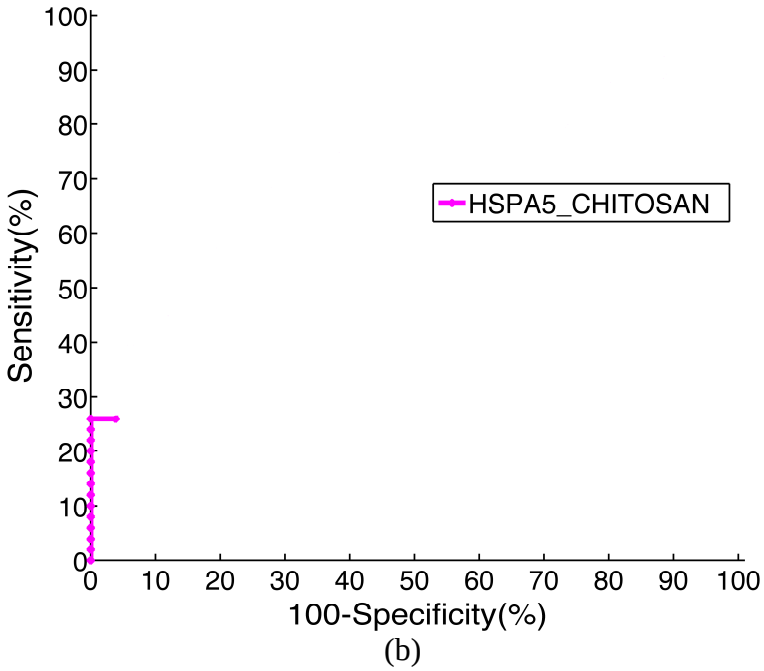
HC: Healthy controls; BC: breast cancer.

If we take the value of the first sample as the cut off value, it means that only one cancer was diagnosed as positive, therefore, the sensitivity is 2%; as there is no healthy control diagnosed as positive, then the specificity is 100%, yielding (2%, 0). Then we continued from No. 2 to No. 13, during which the sensitivity increased gradually while the specificity didn't change because no healthy control was diagnosed as positive. It corresponds to ROC curve as

shown in Figure 1 a. Then we continue No. 14. As it is a healthy control, so if we take its value as cut off value, no more cancers were diagnosed as positive, therefore, the sensitivity didn't change; however, as one healthy control was diagnosed as positive, the specificity decreased, yielding (4%, 26%), shown in Figure 1 b. Then we continued from No. 15 to No. 20, during this process, the specificity didn't change while the sensitivity increased, shown in Figure 1 c. Then we continued and we could obtain the complete ROC curve for HSPA5 immobilized on chitosan surface.



(a)



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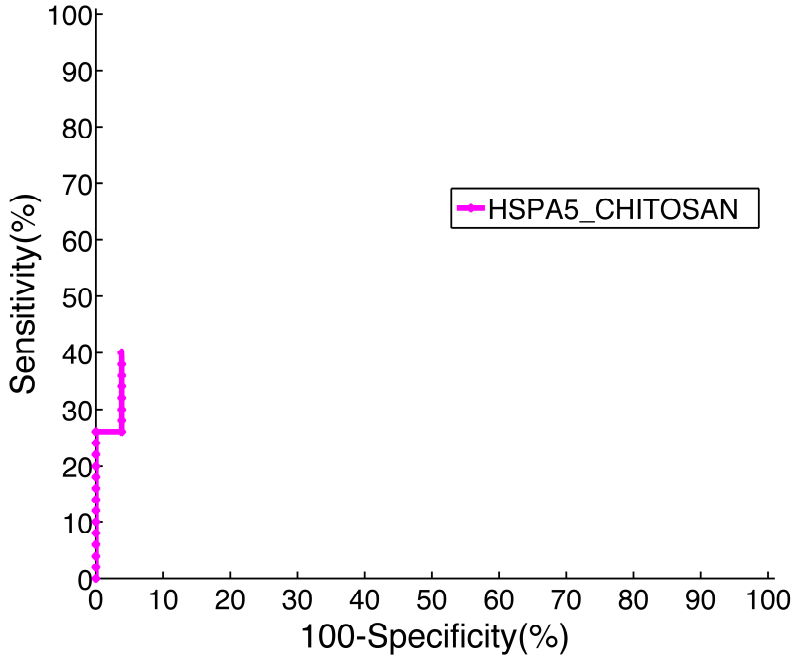
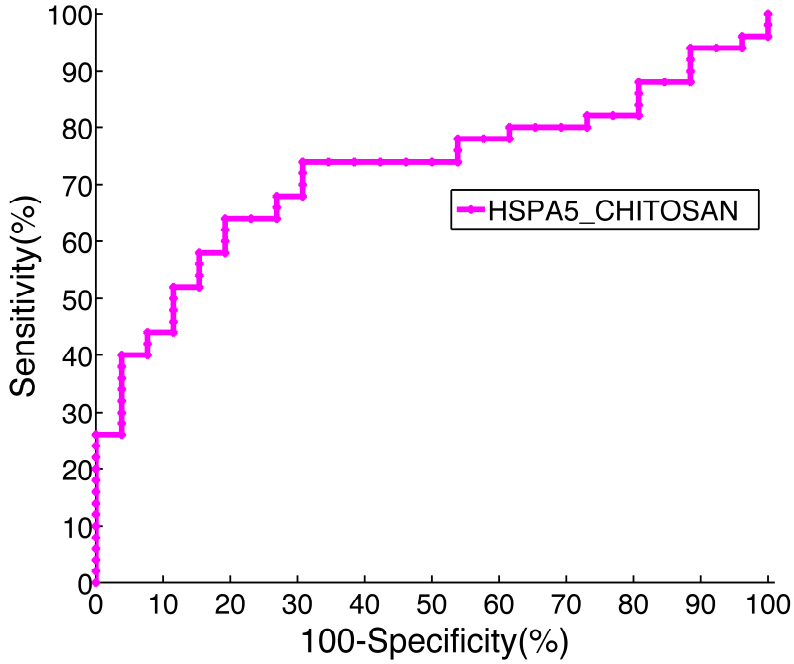


(c)



(d)

Figure 1 *Receiver operating characteristic (ROC) curve analysis of the detection of auto-antibody against HSPA5 on chitosan surface*

Résumé en français

1.1 Le cancer du sein : chiffres clés et techniques de détection

Le cancer du sein demeure un problème de santé publique majeure dans le monde. Selon l'Organisation Mondiale de la santé, 1.7 million de cancer du sein ont été diagnostiqué en 2012 et le taux augmente de plus de 20% depuis 2008 [1]. C'est le cancer le plus fréquemment diagnostiqué chez les femmes quelque soit la zone géographique et il représente maintenant 25% de l'ensemble des cancers chez la femme (voir Figure 1). Comparé au taux d'incidence, le taux de mortalité du cancer du sein est le plus faible reflétant probablement les progrès en termes de diagnostic précoce et de l'amélioration des traitements. [2].

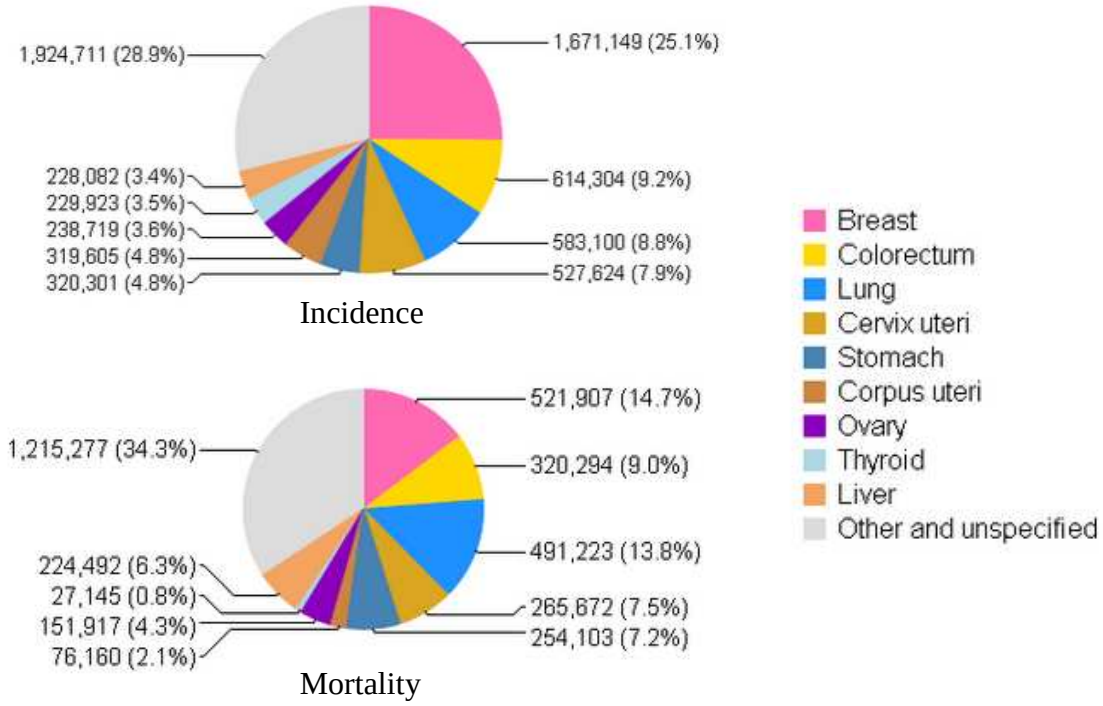


Figure 1. Incidence estimée et Taux de mortalité des cancers au niveau mondial en 2012 [2]

Des études récentes montrent qu'un diagnostic précoce augment les chances de survie des patients et facilitent la prise en charge du traitement dans un stade pré invasive et avant les métastases. Il est rapporté que la survie à 5 ans des patientes ayant un cancer du sein est fortement corrélée au stade de la tumeur. Ainsi, pour les stades précoces (stades 0 and I), le taux de survie à 5 ans est de 98%, il diminue à 85% pour le stade II, passe à 60% pour le stade III et est seulement de 20% pour le stade IV [3].

Les techniques conventionnelles de diagnostic du cancer du sein regroupent la mammographie, l'examen clinique du sein, l'autopalpation, et l'imagerie par résonnance magnétique (IRM) etc. Signalons que l'utilisation de cet arsenal d'outils présente certains inconvénients tels que les faux positifs, les biopsies inutiles, le surdiagnostic, le coût et la génération de l'anxiété chez les femmes etc… [4, 5].

La mammographie est l'outil de dépistage le plus étudié et un examen tous les 2 ans est fortement conseillé pour les femmes à partir de 50 ans [6]. Par contre, ce mode de dépistage systématique pour la tranche d'age 40 à 49 ans est contreversé, aucun élément d'évidence ne permet à l'heure actuelle de déterminer le rapport risque/bénéfice [7].

L'IRM, outil sensible n'est pas préconisé pour un dépistage systématique mais est particulièrement utile pour poser le diagnostic de cancer du sein et évaluer le degré de la tumeur. Il est cependant recommandé par la société américaine en cancérologie pour suivre la population à très forts risques telles que les femmes porteuses des mutations BRCA1 et BRCA2 [8, 9].

Actuellement, le dépistage de marqueurs tumoraux est une nouvelle approche d'intérêt grandissant. Ces marqueurs sont associés à la genèse de la tumeur. Leur dépistage est donc une aide précieuse pour établir un diagnostic précoce et fournir une aide à la décision thérapeutique.

De nos jours, on parle de plus en plus de médecine personnalisée, qui consiste idéalement à donner le bon traitement au bon patient au bon moment. De ce concept, il est attendu une forte amélioration de la prise en charge des patients, une plus grande efficacité des traitements et une diminution globale des coûts de santé [10, 11].

Une des clefs de la médecine personnalisée tient dans l'utilisation massive des biomarqueurs et en parallèle, le développement d'outils de classification haut débit. Qu'ils soient issus de tissus ou de sera, les biomarqueurs doivent fournir une valeur prédictive ou de pronostic fiable, ou permettre de pouvoir estimer le risque de récidive ou l'efficacité d'un traitement donné sur un patient donné. Ainsi, dans le cas des cancers du sein, l'objectif ultime serait par exemple de pouvoir classer les patients selon le risque plus ou moins élevé de taux de récidive, de leur administrer la thérapie la mieux adaptée et ainsi d'éviter des sur-traitements et ainsi augmenter les succès de la thérapie [12, 13].

Dans ce contexte, grâce à leur capacité d'analyse haut débit et le faible volume d'échantillon nécessaire, les puces à protéine (protein microarray) présentent de nombreux avantages pour le criblage des biomarqueurs et la mise au point d'outils de diagnostic et de pronostic à visée de médecine personnalisée. De nombreux biomarqueurs du cancer du sein, qu'ils soient sériques ou tissulaires, sont décrits dans la littérature avec des valeurs diagnostiques et pronostiques faibles lorsqu'ils sont pris indépendamment. Un des challenges est donc d'identifier les combinaisons de biomarqueurs permettant d'atteindre des valeurs diagnostiques et pronostiques élevées. Ceci constitue un des objectifs de cette thèse. Pour ce faire, nous avons élaboré des puces à antigènes à façon, afin d'optimiser leurs performances. La chimie de surface, les conditions d'immobilisation des antigènes sondes ainsi que les conditions de reconnaissance avec leurs anticorps ont été optimisés et validés par l'étude d'une cohorte de 50 patientes. Un autre objectif de cette thèse était de développer une puce à anticorps pour le dosage des marqueurs tissulaires du cancer du sein, uPA et PAI-1, permettant une décision thérapeutique pour les malades. Les performances de notre test miniaturisé ont été comparées à celles du test commercial ELISA sur 16 échantillons biologiques. Le manuscrit est donc présenté sous forme de 4 chapitres.

Le chapitre 1 dresse **un état de l'art** des biomarqueurs sérologiques et tissulaires décrits dans le cancer du sein ainsi que les récents développements d'outils de criblage comme les puces à protéines.

Le chapitre 2 est consacré à **l'élaboration des puces à antigènes** pour l'analyse des profils d'expression d'anticorps anti-heat shock protein (anti-hsps) chez des patientes atteintes de cancer du sein.

Le chapitre 3 concerne l'**élaboration d'un immunoassay miniaturisé pour le dosage des protéines** uPA et PAI-1 à partir d'extraits cytosoliques de tissus tumoraux de cancer du sein.

Le chapitre 4 traite de l'**optimisation** des différentes étapes d'élaboration et d'utilisation **des puces à protéines.**

Chapitre 1 : Etat de l'art

Au cours de la génèse tumorale, les cellules cancéreuses ou d'autres cellules du corps vont produire des substances en réponse aux nouvelles conditions induites par la progression du cancer. Ces substances appelées marqueurs tumoraux peuvent avoir des niveaux d'expression différents et se trouvent dans le sang, les urines, les tissus tumoraux ou autres fluides des patients. Dépister ces marqueurs peut aider non seulement à la détection précoce de cancer mais aussi à choisir l'option la plus pertinente parmi les différents traitements disponibles. Le suivi de certains marqueurs surexprimés doit permettre de vérifier et valider le bénéfice du traitement choisi [14, 15].

La problématique actuelle n'est pas le manque de candidats biomarqueurs mais plutôt la validation de leur pertinence. Ainsi, plus de 1200 protéines candidates ont été décrites dans la littérature comme biomarqueurs potentiels mais seulement 9 antigènes tumoraux ont été approuvés par la FDA (US Food and Drug Administration). Le taux d'introduction de nouvelles protéines approuvées par la FDA stagne à environ une par an sur les 15 dernières années pour l'ensemble des maladies.

Dans la suite, nous nous focaliserons sur les marqueurs sériques et ceux des tissus recommandés par l'ASCO (American Society of Clinical Oncology) et l'EGTM (European Group on Tumor Markers Recommandations) pour une utilisation en routine.

Les performances des biomarqueurs en qualité de tests diagnostiques sont évaluées à l'aide de plusieurs indicateurs tels que les positifs, les négatifs, les faux positifs, les faux négatifs, la sensibilité, la spécificité, la valeur prédictive positive et la valeur prédictive négative. Les méthodes pour calculer ces paramètres sont issues de la méthodologie biostatistique utilisées en Epidemiologie [16] avec notamment la détermination de la courbe de caractéristique de performance d'un test (courbe ROC - Receiver Operating Characteristic), l'aire sous cette courbe notée AUC (area under the ROC curve) et la valeur p qui représente la valeur de significativité de l'hypothèse. En épidémiologie, deux niveaux de significativité sont généralement utilisés ($P < 0.05$ and $P < 0.01$) [17].

Les courbes ROC sont particulièrement utilisées en statistiques lorsque le seuil de discrimination varie. Cela est le cas en biologie moléculaire compte tenu d'une part de la complexité des fluides et d'autre part de la grande variabilité des niveaux de seuil d'un individu à l'autre.

Biomarqueurs sériques dans les tests cliniques

Les marqueurs tumoraux sériques sont des molécules solubles dans le sang qui peuvent être détectées à l'aide d'anticorps monoclonaux. Ces marqueurs sont produits par les cellules tumorales ou par les autres cellules en réponse à la présence de cellules tumorales [18, 19]. A l'heure actuelle, les tests cliniques utilisant des biomarqueurs sériques pour le cancer du sein contiennent des glycoprotéines mucines (MUC-1) et des antigènes carci embryonnaires (CEA) [20], recommandé par l' ASCO et l' EGTM. Ces marqueurs peuvent être utilisés soit pour le dépistage et le diagnostic de la maladie, soit pour la détection précoce de la récidive, soit pour le suivi thérapeutique. Selon l'objectif du test, leur mesure peut être plus ou moins pertinente.

La famille des MUC- 1 est impliquée dans les différentes voies du processus complexe de la génèse tumorale (signalisation des recepteurs de la tyrosine kinase, prolifération et mort des cellules..) et inclus des antigènes de cancer tels que CA 15-3, CA 27-29, CA 549, largement utilisés pour le diagnostic de cancer du sein. [21].

CEA est une glycoprotéine oncofétale également utilisée pour les cancers du sein. Leurs taux sont généralement moins élevés que ceux de MUC-1. Mais les mesures de CEA peuvent donner des informations complémentaires, c'est pourquoi, la combinaison de ces 2 types de biomarqueurs semble pertinent pour suivre les patientes atteintes d'un cancer du sein [22].

Cependant, cette combinaison n'est pas recommandée pour un dépistage systématique ou un diagnostic précoce du fait de leur faible sensibilité et spécificité dans les premiers stades de la tumeur [22, 23]. Par contre, la sensibilité de MUC-1 augmente fortement avec l'avancée de la tumeur passant 10–15% à 20– 25% puis 30–35% pour les stades I, II, et III, respectivement [24].

Dans le cas de la détection d'une récidive, deux études bien construites ont montré qu'après un traitement thérapeutique, des niveaux élevés de MUC-1 et CEA sont corrélés avec une récidive. Ils permettent de prédire une récidive en moyenne 6 mois plus tôt que d'autres symptômes ou tests [25, 26]. Cependant, l'ASCO et l' EGTM sont trés prudents dans leur recommandations d'utilisation car il n'est pas encore suffisamment démontré qu'une détection précoce de métastases a une incidence importante sur le taux de survie et la qualité de vie du patient… [22, 23]. Ainsi, si le suivi n'est pas recommandé par l'ASCO, l'EGTM préconise tout de même de suivre les femmes asymptomatiques avec ce panel tous les 2–4

mois Durant les 5 premières années après le diagnostic , puis chaque 6 moiss dans les trois années suivantes puis ensuite une fois par an.

En ce qui concerne le suivi thérapeutique, selon l'ASCO, les données actuelles sont insuffisantes pour préconiser un contrôle avec cet unique panel de biomarqueurs.

L'exemple illustré par MUC-1 et CEA montre que le repérage de nouveaux biomarqueurs est loin d'être suffisant pour qu'ils soient validés et approuvés. Afin de diminuer le gap entre la découverte d'un biomarqueur potentiel et sa validation en tant que tel, il est nécessaire de poursuivre de vastes études en gardant en tête plusieurs points :

1) les échantillons biologiques doivent être soigneusement choisis avec des procédures bien établis de la banque de patients et des contrôles.

2) les objectifs des études doivent être clairement définis et les résultats doivent être rapportés de manière claire [27].

3) les études doivent être menées à grande échelle, ce qui nécessite le développement d'outils haut débit.

Comparés aux analyses actuelles par simple immunoassays, les systèmes multiplexes présentent d'indéniables avantages tels que l'augmentation de l'efficacité, la réduction des coûts, un plus grand nombre de paramètres mesurés pour un même volume d'échantillons et le traitement en parallèle d'un très grand nombre d'échantillons.

Ainsi, les immunoassays multiplexes, les immunoassays planaires (tels que les microarrays à proteines) peuvent s'avérer comme des outils efficace et simple pour mener des études à grande échelle permettant de transférer plus rapidement la découverte de nouveaux biomarqueurs dans les évaluations cliniques et ceci à moindre coût [28].

Biomarqueurs tissulaires dans les tests cliniques

Dans cette partie, on se focalisera sur les biomarqueurs des tissus déjà utilisés en tests cliniques pour les cancers du sein. Ces biomarqueurs sont **ER** (récepteurs estrogène), **PR** (les récepteurs progestérone), **HER-2 (**récepteurs 2 du facteur de croissance épidermique humain), **uPA** (activateur du plasminogène de type urokinase) et son inhibiteur principal (**PAI-1).**

ER et PR sont des facteurs de transcription qui régulent les actions des estrogènes et de la progesterone respectivement. [18]. Actuellement la détermination des ER et PR est rendue obligatoire pour toutes les patientes atteintes d'un cancer du sein selon les recommandations

de l'EGTM [23] et l'ASCO [22]. En général, les patientes positives en ER ont un meilleur pronostic que celles négatives. Cette différence s'atténue au bout de 4-5 ans. Une limitation en tant que facteur pronostique des ER est qu'il est de faible valeur pour des cas non ganglionnaires. Les patientes avec des tumeurs exprimant PR ont aussi tendance à avoir un meilleur pronostique que celles qui manquent de ce récepteur [18, 29]. En plus de leur valeur pronostique, ER et PR sont considérés comme d'importants indicateurs pour l'analyse de la réponse aux thérapies hormonales. Ainsi, pour les malades à un stade précoce comme à un stade avancé, la présence de récepteurs hormonaux donne une probabilité de réponse à la thérapie hormonale beaucoup plus importante que pour les patientes manquant de ces récepteurs.

Notons qu'il existe 3 types de tests bien établis pour mesurer ces récepteurs hormonaux : par liaison de ligand, ELISA ou immunohistochimie (IHC). Seul ce dernier test est recommandé par l'EGTM [23]. Il faut toutefois souligner qu'environ 20% de détermination de taux de ER/PR par IHC serait inopérante (faux positif ou faux négatif) au niveau mondial du fait de variations des variables pré-analytiques, des seuils de positivité, de l'utilisation d'anticorps relativement inefficaces, et des critères d'interprétation. [30].

L'oncoprotéine HER2 est surexprimée dans environ 15% des cancers du sein [31]. Toutes les patientes positives aux récepteurs HER2 doivent être traitées par immunothérapie avec l'Herceptin® (trastuzumab). [18]. 3 méthodes permettent de déterminer le taux d'HER2: l'immunohistochimie (IHC), l'hybridation in situ par fluorescence (FISH), et l'hybridation in situ chromogénique (CISH). Mais, seule l' IHC est recommandée l' ASCO [18, 32, 33].

L'activateur du plasminogène de type urokinase uPA est une protéase dégradant la matrice extracellulaire et impliquée dans l'invasion du cancer et des métastases. uPA interagit aussi avec son inhibiteur (PAI-1). Ainsi, les deux (PAI-1 et uPA) favorisent la progression de la tumeur et des métastases. [34]. La présence de uPA et PAI-1 est un fort indicateur de dissémination du cancer pour des patientes avec ou sans envahissement ganglionnaire. Des taux élevés sont associés à un faible taux de survie sans rechute et même à un faible taux de survie globale. Par contre, des études montrent que des niveaux bas de uPA and PAI-1 sont associés à des risques de récidive suffisamment faibles qui font qu'une chimiothérapie n'ajoute pas un bénéfice substantiel [35]. Ces deux marqueurs sont donc considérés comme particulièrement pertinents par l'American Society of Clinical Oncology (ASCO) [22] et l'European Group on Tumor Markers (EGTM) [23].

Actuellement, le test ELISA est la seule méthode préconisée par l'ASCO. Il existe un test disponible commercialement : le ELISA test (Femtelle ®) développé par Sekisui Diagnostics. Ce Kit assure une bonne qualité et est largement utilisé en clinique. Cependant, le test ELISA nécessite un minimum de 300 mg de tissu frais ou congelé de cancer du sein, ce qui peut être problématique notamment dans le cas de tumeurs de très petites tailles [36]. Cependant, en recherche, comparés aux tests ELISA, les microarrays à protéines présentent certains avantages tels qu'une bonne sensibilité avec de très faibles volumes d'échantillons nécessaires [37].

En 1960, Robert W. Baldwin démontra que le système immunitaire était impliqué dans le développement tumoral. En effet, au cours du développement tumoral, des protéines intracellulaires mutées, modifiées ou exprimées de manière aberrante dans les cellules tumorales, appelées TAA (tumor-associated antigens), peuvent être la cible du système immunitaire conduisant alors à la production d'auto-anticorps (AAb) contre ces TAAs. [38]. Ces auto-anticorps peuvent être utilisés pour des marqueurs de diagnostics précoces de cancer [39]. Par exemple, Lubin et al. ont détecté des anticorps p53 spécifiques environ 18 mois avant qu'un diagnostic de cancer du poumon soit établi cliniquement [40]. Le système immunitaire permet une amplification biologique efficace conduisant à une concentration élevée d'auto-anticorps permettant une détection indirecte de très faibles quantités d'antigènes tumoraux. De plus , ces auto-anticorps sont très stables dans les sérums et ont une durée de vie relativement longue (T1/2 entre 7 and 30 jours, selon la sous classe d'immunoglobuline) [41-46].

Ces dernières années, plusieurs études ont été menées sur différents AAbs contre des TAAs en évaluant leurs valeurs diagnostiques et pronostiques notamment pour les cancers du sein. En particulier, un vif intérêt a été porté sur des anticorps dirigés contre des protéines hsp (heat shock proteins). Les Hsp sont des protéines de forte conservation classées en 6 familles selon leur poids moléculaire (MW): hsp110, hsp90, hsp70, hsp60, hsp40 et un ensemble de petites protéines hsp (dans la gamme de 13-42kD) incluant hsp27 and hsp10. [47]. Les protéines hsp sont surexprimées dans une grande partie des cancers humains. Cette surexpression élevée des hsp dans les cellules malignes joue un rôle important de protection des cellules contre l'apoptose spontanée induite par la malignité [48].

Dans notre étude bibliographique, nous avons trouvé 6 rapports décrivant l'utilisation de tests unitaires d'hsps dirigés contre les auto-anticorps (AAbs) afin de discriminer les patientes

atteintes d'un cancer du sein des contrôles sains. En utilisant des tests ELISA, Conroy *et al*. ont mené en 1995 la première étude pour identifier la présence d'auto-anticorps anti-hsp90 sur des patientes ayant un cancer du sein diagnostiqué. Ils ont trouvé que les anticorps ciblés étaient détectables dans 46 cas sur 125 (36.8%) patientes atteintes d'un cancer mais pas dans le cas d'individus sains ou ayant une tumeur bénigne. De plus, la présence de ces anticorps était corrélée avec le développement de métastases même sur les personnes non atteintes aux ganglions lymphatiques axillaires [49].

Le tableau suivant regroupe les résultats publiés.

		Taille de l'échantillon (N)			AAb frequence %				
Hsp	méthode	cancer	sain	benin	cancer	sain	benin	P valeur	reference
Hsp27	ELISA	579	53	-	37.8%	1.9%	-	p<0.001	[50]
Hsp70	ELISA	369	53	-	40.9%	35.9%	-	-	[50]
Hsp90	ELISA	125	-	-	36.8%	-	-	-	[49]
Hsp60	WB	40	42	-	47.5%	4.7%	-	p < 0.01	[51]
Hsp60	ELISA	107	93	-	31.8%	4.3%	-	p<0.0001	[52]
Hsp90	ELISA	13	22	10	8%	0	0	-	[53]

Ainsi pris indépendamment, le potentiel diagnostique ou pronostique de chaque anti-hsp est très faible. Cependant, l'utilisation de nouveaux outils de détection multiplexée telle que les microarrays permet d'une part des études à grande échelle (large panel de biomarqueurs, cohorte importante) et d'autre part d'augmenter considérablement la sensibilité et la spécificité du diagnostic jusqu'à des valeurs de plus de 80% [20].

Au delà des hsps, d'autres antigènes tumoraux ont été dirigés contre les auto-anticorps. [54]. Le tableau suivant recense un certain nombre d'études visant à identifier les auto-anticorps tumoraux dans les sera de cancer du sein, mais un petit nombre seulement (anti-p53, anti-Her2/neu, anti-MUC1) ont fait l'objet d'études plus détaillées.

L'antigène tumoral p53 est surexprimé dans les cellules cancéreuses et induit la production auto anticorps anti-p-53. In 2000, Soussi compulse la bibliographie de 1979 à 1999 concernant les auto-anticorps anti-p53 AAbs dans les sera de patients de tout type de cancer. Une quinzaine d'études a permis d'identifier l'anti-p53 dans les cancers du sein avec une fréquence allant de 2.8% à 47.5%. Si l'on considère l'ensemble de ces études, l'anti-p53 AAbs a été détecté dans 14.7% de patientes (296/2006) avec une différence significative des sujets sains ($P < 0.0001$) [55]. Ces études montrent que les anticorps anti-p53 ont une forte spécificité (supérieur à 95%) mais une faible sensibilité (en moyenne 20.8%). Aussi, il est impératif de combiner la recherche d'anticorps anti- avec d'autres biomarqueurs pour

augmenter la sensibilité sans réduire la spécificité du test. D'autre part, les anticorps anti-p53 circulant sont associés à un mauvais pronostic avec une survie courte.

TAA	Taille de l'échantillon (N)		AAb frequence %	Reference/année
	cases	Sains		
p53	101	-	7.9%	[56] 1999
	2006	-	14.7%	[55] 2000
	158	-	19%	[57] 2003
	71	205	18.3%	[58] 2003
	144	242	21.5%	[59] 2005
	50	436	34%	[60] 2006
	25	879	16%	[61] 2009
	61	20	35%	[62] 2010
HER2	20	-	55%	[63] 1994
	107	200	11.2%	[64] 1997
	37	157	7%	[65] 2000
MUC1	24	-	8.3%	[66] 1994
	40[a]		37.5%	
	140[b]		25.7%	
	61[c]	96	18%	[67] 1996
c-myb	72	49	43%	[68] 1991
fibulin	20	20	75%	[69] 2002
RPA32	801	65	10.9%	[70] 2002
	74		27%	
lipophilin B	35 [c]	20	37.1%	[71] 2003
cyclin B1	7	27	42.8%	[72] 2005
survivin			23.9%	
livin	46	10	32.6%	[73] 2005
	36 [b]		66.6%	
endostatin	59 [c]	24	42.4%	[74] 2006
GIPC1	22	10	77%	[75] 2007
IGFBP2	80	200	5%	[76] 2008
AHSG	81	73	79.1%	[77] 2009
SPAG9	100	50	80%	[78] 2009
	282		18.4%	
SOX2	78 [a]	194	6.4%	[79] 2012
p90/CIP2A	168	88	19.1%	[80] 2014

Tableau de Fréquence des auto-anticorps dans les patients du cancer du sein : [a] tumeurs bénines, [b] carcinoma premier stade, [c] cancer en stade avancé

HER2 est un récepteur de facteur de croissance épidermique (EGFR) qui est amplifié et surexprimé dans 20%–30% des cancers du sein. Un test positif à HER2 est associé à un mauvais pronostic dû à la forte incidence des métastases et à la résistance aux chimiothérapies conventionnelles ou endocriniennes [81].

Les Anti-HER2 aussi sont détectés chez les patientes atteintes de cancers du sein. Une étude conduite sur une période de 6 ans (de 1994 à 2000) indique une présence significative des anticorps anti-HER2 chez les patientes au premier stade des cancers du sein comparée à des sujets sains [63, 64] et un niveau beaucoup plus élevé est noté pour des stades avancés [65]. Ces études suggèrent qu'une réponse immune humorale aux HER2 pourrait jouer un rôle dans la limitation de la progression tumorale.

Les Mucines (MUC) sont des glycoprotéines de fort poids moléculaire exprimées à la surface cellulaire. MUC1 a été trouvé de manière abondante dans les cancers du sein mais aucune corrélation avec le stade de la maladie n'a pu être faite [66]. Par contre, les anticorps Anti-MUC1 ont été détectés beaucoup plus souvent pour des tumeurs bénignes que pour des cancers du sein. Ainsi, une corrélation négative a été observée entre la présence d'anti-MUC1 et le développement de la maladie. Ceci suggère qu'une réponse immune humorale naturelle aux MUC1 serait protectrice d'une progression de la maladie tandis qu'un manque de réponse immune serait associé à un pronostic défavorable [67].

A coté des 3 auto anticorps présentés ci dessus, d'autres molécules telles que c-myb, fibulin, RPA32, lipophilin B, cyclin B1, survivin, livin, endostatin, GIPC-1, insulin-like growth factor binding protein 2 (IGFBP-2), AHSG, SPAG9, SOX2 and p90/CIP2A etc… sont également impliquées dans les cancers du sein à travers les différents mécanismes de la cancérisation. Mais la fréquence de leur détection varie fortement (de 5% à 80%). Ces grandes variations pourraient résulter de plusieurs facteurs tells que l'hétérogénéité de la tumeur, la taille, la qualité et l'origine des échantillons des méthodes et protéines utilisées… etc.

Un résumé des travaux basés sur des tests ELISA et utilisant des panels plus ou moins importants (de 2 à 10 biomarqueurs) est présenté dans le tableau ci-dessous.

panel	malades	sains	AUC	SN/SP	Reference/année
IMP1, p62, Koc, p53, cMYC, cyclin B1, survivin	64	346	-	92%/85%	[82, 83]2003
survivin and livin	46	10	-	52.2%/-	[73] 2005
p16, p53, and c-myc	41	82	-	43.9%/97.6%	[84] 2006
p53, c-Myc, HER2, NY-ESO-1, BRCA1, BRCA2, MUC1	97[a]		-	64%/85%	
	40[b]	94	-	45%/85%	[85] 2007
MUC1, HER2, p53, IGFBP2			-	31%/-	
p53, HER2, IGFBP-2, TOPO2α	184[c]	134	0.63	-	[46] 2008
ASB-9, SERAC1, and RELT	87	87	0.861	77%/82.8%	[86] 2008
FKBP52, PPIA, PRDX2, hsp60 and MUC1	60[a]		0.73	55.2/87.9%	
	82[b]	93	0.80	72.2%/72.6%	[87] 2009
RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1	59[a]	61[b]	0.749	86.1%/75%	[88] 2012
GAL3, PAK2, PHB2, RACK1 and RUVBL1	114	68	0.81	66%/87%	[89] 2013
p62, p53, c-myc, survivin, p16, cyclin B1, cyclin D1 CDK2	41	82	-	61%/89%	[90] 2013
FTH1 and hnRNPF	150	150	0.816	91.1%/72%	[91] 2013

Etudes d'un ensemble d' anti-TAA AAbs sur des cohortes de cancers du sein et patients sains
[a] cancer primaire , [b] patients premier stade (DCIS), [c]stade avancé, SN: sensibilité,
SP: spécificité, AUC aire sous la courbe ROC

Parmi tous les anti-TAAs étudiés, 9 présentent un intérêt accru et sont regroupés dans le tableau suivant. Soulignons cependant la dispersion des résultats qui peut avoir plusieurs origines : 1) la diversité des populations étudiées, 2) la définition de la valeur seuil qui est un facteur important déterminant la performance du test (un seuil bas induit une forte sensibilité mais une faible spécificité et *vice versa*.)

antigène tumoral	Nombre d'études	Echantillonage (N)		Gamme de sensitivité	reference
		Patients malades	Patients sains		
p53	14	25-2006	82-346	7.9%-35%	[46, 55-62, 82-85, 90]
HER2	6	20-144	157-242	7%-55%	[46, 59, 63-65, 85]
MUC1	5	24-241	93-134	8.3%-37.5%	[46, 66, 67, 85, 87]
c-myc	4	41-137	82-346	13%-22%	[73, 82-85, 90]
Survivin	3	41-64	10-346	7.8%-23.9%	[72, 82, 83, 90]
cyclin B1	3	7-64	27-346	4.7%-42.8%	[82, 83, 90]
P16	2	41	82	12.2%	[84, 90]
P62	2	41-64	82-346	7.8%-12.2%	[82, 83, 90]
IGFBP2	2	80-184	134-200	5%-7%	[46, 72]

Ainsi les besoins d'études à grande échelle et avec un large panel d'auto-anticorps nécessitent une transition urgente entre les méthodes classiques ELISA et les systèmes de criblage (screening) multiplexes tels que les microarrays à protéines.

Microarray à protéines

La technologie microarray fait référence à la miniaturisation de centaines de tests rassemblés sur une seule plaque. Différentes protéines (antigènes ou anticorps) sont fixées sur un support solide de manière bien ordonné pour un repérage aisé des potentielles interactions étudiées. Le microarray est ensuite incubé avec un échantillon contenant une grande diversité de protéines. Après cette incubation, les interactions éventuelles peuvent être détectées soit par des techniques de lecture dites avec marquage (fluorescence, chimiluminescence…) soit par des modes de lectures sans marquage (spectrométrie de masse, résonance de plasmon de surface, etc.) [92].

Généralement, le support principal utilisé est en verre à cause de ses propriétés de transparence et de faible bruit de fluorescence. Mais le support est fonctionnalisé avec un

grand nombre de chimies de surface pour pouvoir ensuite immobiliser de manière robuste les protéines tout en conservant leur intégrité, leur conformation originelle et leur fonction biologique [93]. [94].

Du point de vue commercial, on trouve une large variété de lames disponibles avec des chimies de surface différentes telles que des lames en nitrocellulose FAST, hydrogel, SuperAldehyde and epoxy-silane ES, des poly- L-lysine, des surfaces aldehyde , polyacrylamide, PolyEthylenGlycol-epoxy or dendrimères …

Il existe une littérature foisonnante concernant les stratégies d'attachement des protéines sur une surface, cette étape clé déterminant les propriétés du microarrays. Le tableau ci-dessous rassemble les différentes modalités d'interactions surface/protéines et les avantages/inconvénients des différents modes d'immobilisation [95-97].

Type d'immobilisation	Chimie de surface	site d'attachment	Avantages	Inconvenients
Adsorption	Nitrocellulose, Poly-L-lysine, agarose, etc.	interactions électrostatiques, liaison hydrogène interactions de Van der Waals	Immobilisation la plus simple	Orientation aléatoire Fort bruit de fond
Liaison Covalente	Maleimide, hydrazine, succinimidyl ester, epoxide, aldehyde, etc	Thiol, carbohydrate, amine	immobilisation robuste	Perte potentielle de l'activité biologique de la proteine immobilisée
Liaison par affinité	Proteine A ou G, streptavidine, glutathione, etc.	Fc region, biotine, GST tag, etc	immobilisation orientée	Prétraitement des proteines spottées

Les différentes stratégies d'immobilisation de protéines sur un support

Jusqu'à présent; il est impossible de comparer les études issues de différents laboratoires compte tenu des différences dans les protocoles expérimentaux et les protéines utilisées. Cependant, de la revue bibliographique, un point parait évident : il n'y a pas une surface unique parfaitement adaptée à l'immobilisation de toutes les protéines compte tenu de la complexité de leur structure. Chaque type de protéine a un comportement différent sur chacune des surfaces. Aussi, il est nécessaire de sélectionner finement les microarrays avec les surfaces les mieux appropriées.

En plus de la chimie de surface, de nombreux facteurs influencent les performances des microarrays tels que (a) la composition de la solution de spotting , (b) le taux d'humidité Durant le spotting (c) la concentration des cAbs, (d) le temps d'incubation et le séchage de la solution de spotting (e) la composition du tampon de blocage (f) le temps de blocage, (g) le tampon utilisé pour la dilution des échantillons, (h) le temps d'incubation des échantillons (i) la température, (j) le niveau d' agitation et de mélange durant l' incubation, (k) la composition du tampon de rinçage (l) l'agitation durant le rinçage, (m) la composition du tampon de détection, (n) la concentration des dAbs, (o) le temps d'incubation des dAbs, (p) l'affinité et la stabilité des dAbs, (q) la concentration des marqueurs de détection (e.g., Ab secondaire ou la streptavidine marquée, (r) la composition du tampon buffer, (s) le temps de l'incubation (t) la nature du marqueur de détection (fluorescent ou enzyme), etc… [98]. Un enjeu important pour le développement futur des microarrays multiplexes est d'identifier les facteurs critiques et de les optimiser.

Aujourd'hui, plusieurs sociétés ont commercialisé des microarrays à protéines pour la détection et l'analyse de protéines dans des échantillons humains tels que le serum, l'urine, le tissu, etc…Le tableau suivant recense ces différents dispositifs et listent leurs caractéristiques.

Categorie	Société	Produits	Printed proteins	Replicat/ proteines	Prix/lame	Sample tested/ slide	Surface	Reference
Cytokine test	Whatman	FAST Quant TH1/TH2 arrays	Antibodies against 9 cytokines	3	525 €	16	Nitro cellulose	[99, 100]
		FAST Quant angiogenesis arrays	Antibodies against 9 cytokines	3	525 €	16	Nitro cellulose	[100]
	R&D system	Human Cytokine	Antibodies against 36	2	128 €	1	Nitro cellulose	http://www.rndsystems.

		Array Panel A	cytokines					com/Products/ary005/Citations
		Human XL Cytokine Array	Antibodies against 102 cytokines	2	186 €	1	Nitro cellulose	No
	RayBiotech	Human Quantibod® Cytokine Arrays Q1	Antibodies against 20 cytokines	4	698 €	16	N.A	[101-104]
		Human Quantibod® Cytokine Arrays Q440	Antibodies against 440 cytokines	4	12900 €	16	N.A	[105]
Protein profiling	Invitrogen	ProtoArray® Human Protein Microarray	9,000 unique human proteins	2	1180 €	1	Nitro cellulose	http://www.lifetechnologies.com/fr/fr/home/life-science/protein-biology/protein-assays-analysis/protein-microarrays/technical-resources/literature-citations.html
	Sigma-Aldrich	Panorama® Antibody Microarray - Cell Signaling Kit	224 anticorps	2	discontinued	1	Nitro cellulose	http://www.sigmaaldrich.com/catalog/product/sigma/
Cancer biomarker screening	RayBiotech	Array Q1 Cancer gastrique	Anticorps dirigés contre 5 biomarqueurs de cancer	4	221 €	16	N.A	No

			gastrique					
	Arrayit	OvaDx® Test Diagnostique du cancer des ovaires	N.A	N.A	N.A	N.A	N.A	No
Allergy microarrays	Thermo Fisher Scientific	ImmunoCAP ISAC	103 allergens	3	N.A	4	Polymer	[106-109]
	Arrayit	Allergy microarrays	123 allergens to IgE 101 allergens to IgG	N.A	264$	1	N.A	No

Table 10. Les microarrays commercialisés

Soulignons par exemple l'intérêt du microarray ProtoArray® Human Protein Microarray développé par Invitrogen qui contient plus de 9 000 protéines humaines . Selon leur site, plus de 110 publications utilisent ce produit pour tester des maladies différentes comme les transplantations [110-112], différents cancers [113-119], et des maladies auto immunes [120-122]. Cependant, des dispersions sont observées lorqu'on compare les résultats issus de différents laboratoires mais utilisant les mêmes produits. Aussi, le ProtoArray® Human Protein Microarray est un outil puissant pour faire un premier screening de biomarqueurs mais une validation est ensuite nécessaire avec des méthodes traditionnelles comme l'ELISA**.**

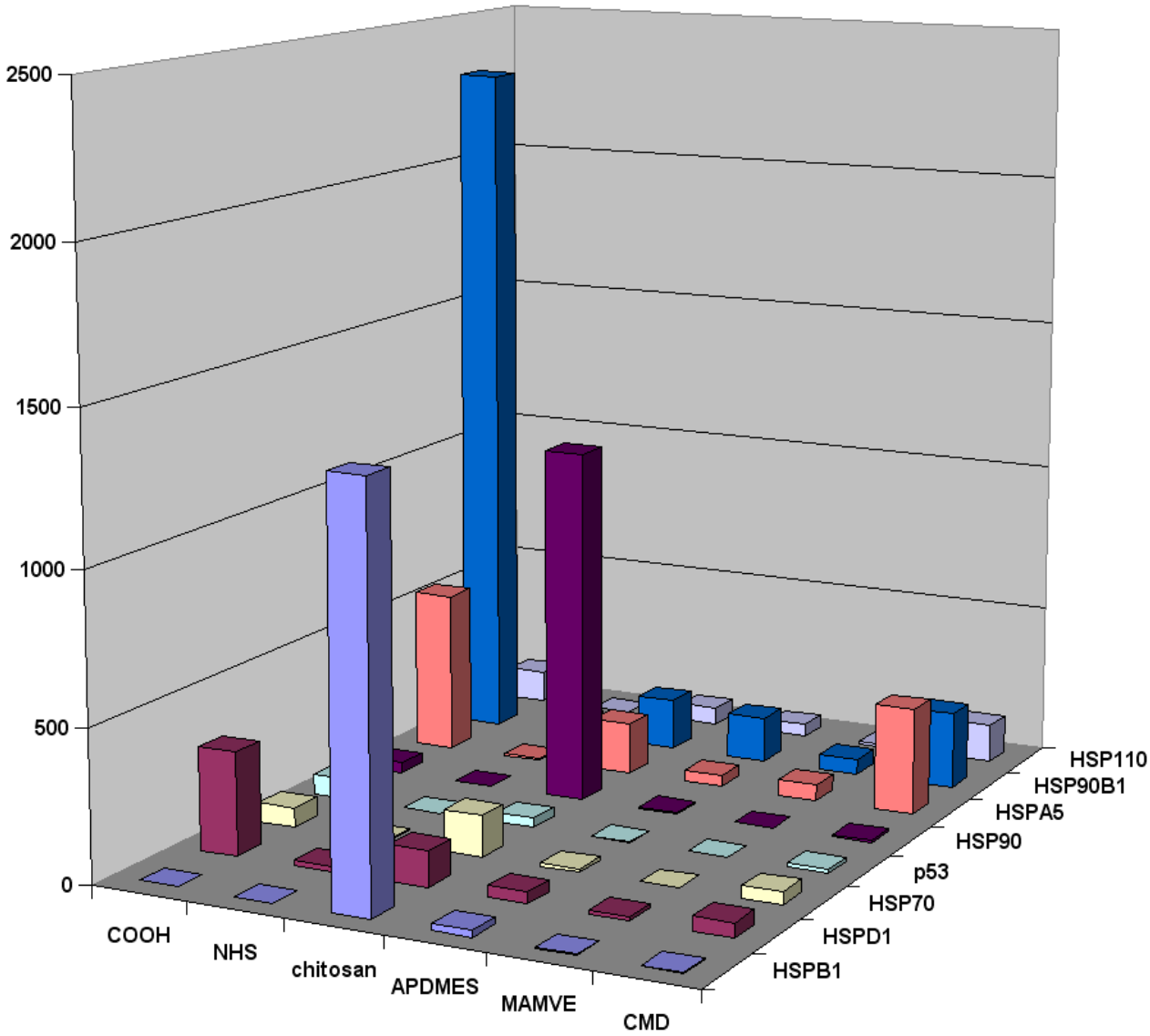
Sigma-Aldrich a aussi développé une puce Panorama® Antibody Arrays pour analyser les profil d'expression des protéines [123]. Celle-ci a permis d'identifier de nouveaux biomarqueurs potentiels aussi bien dans le cancer du sein [124-126], le cancer colorectal [127], le cancer de la prostate [128], le cancer du poumon [129]. Les protéines d'interêt ont aussi été validées par Western Blot. Malheureusement, ce microarray n'est plus disponible à cause du manque de ventes.

Chapitre 2 : Criblage d'autoanticorps Anti HSP dans les sera de cancer du sein à l'aide de microarrays à protéines faits à façon

Le chapitre 2 est consacré à l'élaboration des puces à antigènes pour l'analyse des profils d'expression d'anticorps anti-heat shock protein (anti-hsps) chez des patientes atteintes de cancer du sein. En effet, de nombreuses études ont mis en évidence des taux élevés d'anticorps anti-hsps dans le sérum de patientes atteintes de cancer du sein, certains d'entre-eux étant associés à la progression de la maladie. Toutefois ces études ont porté sur l'analyse d'un ou deux anticorps anti-hsps simultanément, ce qui n'a pas permis de dégager un réel intérêt diagnostique ou pronostique de tels marqueurs. Ainsi, en collaboration avec le CHU de Montpellier, nous avons sélectionné 7 protéines appartenant à la famille des « heat shock proteins » (hsp27, hsp60, hsp70, hsp90, hsp110, grp78, grp94) comme antigènes sondes pour l'élaboration des puces à antigène. Nous avons également inclus dans ce panel, la protéine p53 largement décrite pour induire la production d'anticorps anti-p53 associés à un mauvais pronostic du cancer du sein.

Dans un premier temps, nous avons étudié l'influence de la chimie de surface et des conditions d'immobilisation des antigènes sondes sur les performances de la reconnaissance antigène-anticorps afin de définir les conditions optimales pour le criblage des sérums de patientes. En effet, lors d'une étude précédente, 6 chimies de surfaces différentes ont été développées dans l'équipe pour l'immobilisation covalente ou non de protéines sur support de verre. Il s'agit de surfaces fonctionnalisées avec un silane carboxylé (surface COOH), avec le silane carboxylé activé (surface NHS), avec du chitosan (surface chitosan), avec un silane aminé (surface APDMES), avec un carboxyméthyl dextran (surface CMD), ou encore avec un polymère d'anhydride maléique (surface MAMVE).

Quatre concentrations de dépôt d'antigène sonde (de 0.005 mg/mL à 0.1 mg/mL) ont été testées pour leur capacité à reconnaitre de façon spécifique et sensible les anticorps correspondants. La figure suivante montre les rapports signal sur bruit (SNR) obtenus après la reconnaissance entre antigènes sondes immobilisées à 0.1 mg/ml et les anticorps purifiés incubés sur les 6 chimies de surface étudiées.

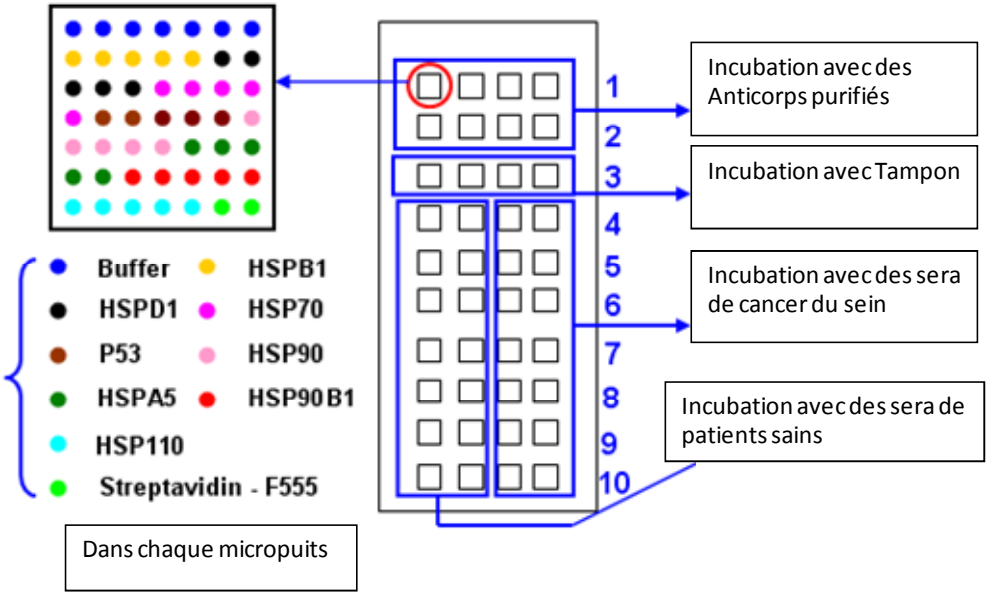


Parmi les 6 chimies de surface évaluées, 3 surfaces (COOH, chitosan et CMD) permettent une détection efficace des 8 anticorps testés et ceci même pour une concentration de 0,05 mg/mL en antigène sonde déposée.

En tenant compte de ces conditions, 50 sérums de patientes atteintes de cancer du sein et 26 sérums de donneurs sains ont été évalués pour la présence des anti-hsps et de l'anti-p53. Des lames de verres micro-structurées et fonctionnalisées avec les chimies de surface COOH, chitosan et CMD ont été utilisées.

La figure suivante montre la conception d'une lame sur laquelle sont gravées 40 micropuits. Dans chaque micropuits, sont spottées 8 protéines différentes (7 hsp et P53) en 5 réplicas dans leur concentrations optimales et avec le tampon adéquat qui dépend de la chimie de surface faite préalablement sur la surface ; ainsi sur des surfaces COOH, on utilise un tampon acetate (pH= 4.5) tandis que sur des surfaces de chitosan, on utilise un tampon

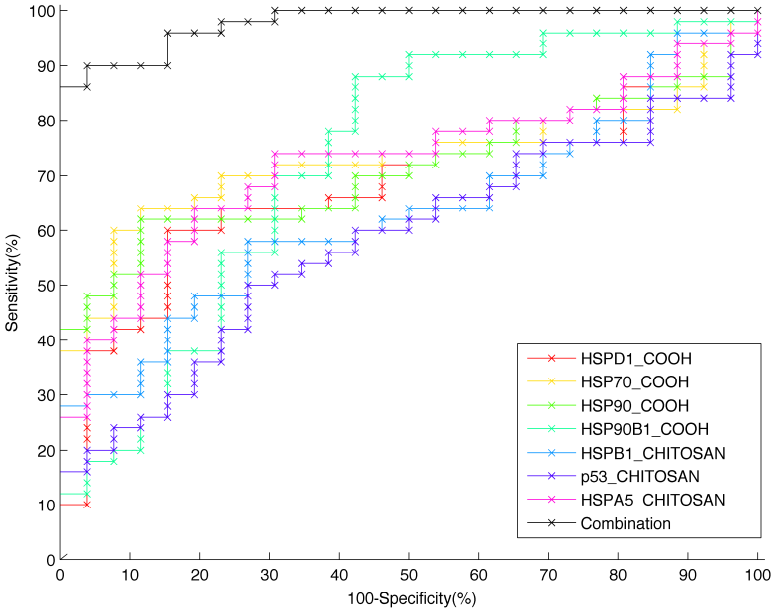
carbonate de pH 9.6. Enfin, des plots contenant uniquement de la solution tampon ou de la streptavidine-F555 sont réalisés pour les contrôles négatifs et de qualité du microarray. Après le spotting, les lames sont laissées une nuit à 4°C sous hygrométrie contrôlée pour laisser la réaction d'immobilisation se faire. Les lames sont ensuite rincées soigneusement dans du PBS puis une étape de blocage avec une solution de 10% BSA/PBS-T 0.1% à température ambiante est réalisée pendant 2 heures afin de limiter ultérieurement les phénomènes d'adsorption non spécifique. Enfin les lames sont rincées 3X5 minutes dans du PBS-T 0.1% et séchées 3 minutes par centrifugation à 1300 tours par minute (rpm).



En présence de sérum, les lames fonctionnalisées avec le CMD présentaient un bruit de fond très fort, empêchant leur exploitation. En revanche, la surface COOH permet de discriminer de façon significative ($p<0,05$) les sérums cancéreux des sérums sains vis-à-vis de la présence des anticorps anti-hsp60, anti-hsp70, anti-hsp90 et anti-grp94. Les taux d'anticorps anti-hsp27 et anti-grp78 sont significativement différents entre les sérums cancéreux et les sérums sains ($p< 0,05$) lorsqu'ils sont évalués sur la surface fonctionnalisée avec le chitosan. Par contre, aucune des surfaces testées ne permet de discriminer les 2 populations de sérums par rapport aux anticorps anti-hsp110 et anti-p53. A partir de ces résultats, nous avons construit les courbes ROC (Receiver Operating Characteristic) et calculer l'aire sous la courbe correspondante (AUC) pour chaque anticorps dans ses meilleures conditions de détection et de discrimination. Pour qu'un biomarqueur soit utilisable en clinique pour du diagnostic ou du pronostic, il faut que l'AUC soit supérieur à 0,75. Ainsi, si on considère chaque anticorps anti-hsp indépendamment, les valeurs des AUC varient de 0,576 à 0,731, indiquant que la détection unique d'un anti-hsp n'a pas de valeur clinique. Toutefois, si on considère la détection simultanée des 7 anticorps (anti-hsp27, anti-

hsp60, anti-hsp70, anti-hsp90, anti-grp78, anti-grp94 et anti-p53) dans les conditions optimales, la valeur AUC obtenue est de 0,912. Ceci confirme que la détection d'un panel de biomarqueurs dans des conditions optimales permet d'augmenter significativement la valeur diagnostique et/ou pronostique du test. Aucune corrélation n'a pu être mise en évidence entre la présence des anticorps anti-hsp et anti-p53 et le stade de la maladie.

Courbes ROC des autoanticorps pris individuellement et en noir pour une combinaison de 7 autoanticorps et table rassemblant les valeurs de AUC extraites des courbes afin de discriminer les patientes ayant un cancer du sein des contrôles sains



Anti-HSP antibody	AUC (95% CI)
Anti-HSPB1 antibody*	0.631 (0.468-0.739)
Anti-HSPD1 antibody**	0.683 (0.592-0.781)
Anti-HSP70 antibody**	0.732 (0.619-0.801)
Anti-p53 antibody*	0.581 (0.478-0.710)
Anti-HSP90 antibody**	0.710 (0.625-0.848)
Anti-HSPA5 antibody*	0.723 (0.672-0.806)
Anti-HSPB1 antibody**	0.728 (0.627-0.820)
Combination of 7 antibodies	0.978 (0.911-1.012)

Seulement 4 des autoanticorps anti-HSPs étudiés dans notre travail ont été évalués dans d'autres travaux. Le tableau suivant donne un premier aperçu de nos résultats comparés à ces travaux. Cependant, la taille de notre échantillonnage (50 patientes atteintes de cancer et 26

donneurs sains) est trop limitée pour pouvoir conclure définitivement. Il sera donc nécessaire d'évaluer ces paramètres sur un échantillonnage incluant plusieurs centaines de personnes.

Table des Fréquences de chaque auto-anticorps anti-HSP détecté sur les cancers du sein et contrôles sains.

HSPs	Méthodes	Echantillonnage		AAb fréquence %		p-value	Reference
		cancer	sain	cancer	sain		
HSPB1	ELISA	579	53	37.8%	1.9%	p<0.001	[50]
	Microarray	50	26	8%	0	0.049*	Our study
HSPD1	Western B	40	42	47.5%	4.7%	p<0.01	[51]
	ELISA	107	93	31.8%	4.3%	p<0.0001	[52]
	Microarray	50	26	14%	3.8%	0.01**	Our study
HSP70	ELISA	369	53	40.9%	35.9%	PE	[50]
	Microarray	50	26	34%	0	0.002**	Our study
HSP90	ELISA	125	PE	36.8%	PE	PE	[49]
	ELISA	13	22	7.7%	PE	PE	[53]
	Microarray	50	26	4%	0	0.002**	Our study

*PE:pas exploitable; * Résultats obtenus sur chitosan, ** Résultats obtenus sur COOH*

En conclusion, cette étude a permis de démontrer que les supports de microarrays micro-structurés développés au laboratoire, sont un outil de criblage très puissant. En effet, ils ont permis de déterminer rapidement les meilleures conditions d'immobilisation (chimie de surface, concentration) d'antigènes sondes pour la détection d'anticorps. Ces supports micro-structurés ont également permis d'élaborer des puces à antigènes à façon (sélection des meilleures conditions pour chaque antigène sonde) afin d'établir les profils d'expression d'anticorps anti-hsps présents dans le sérum de patientes atteintes du cancer du sein. Nous avons alors montré que la détection multiplexe de 7 biomarqueurs permet d'augmenter significativement la sensibilité et la spécificité d'un test clinique à valeur diagnostique.

Chapitre 3 : Elaboration d'un microarray à anticorps pour le dosage des biomarqueurs tissulaires uPA et PAI-1 dans les tumeurs du sein

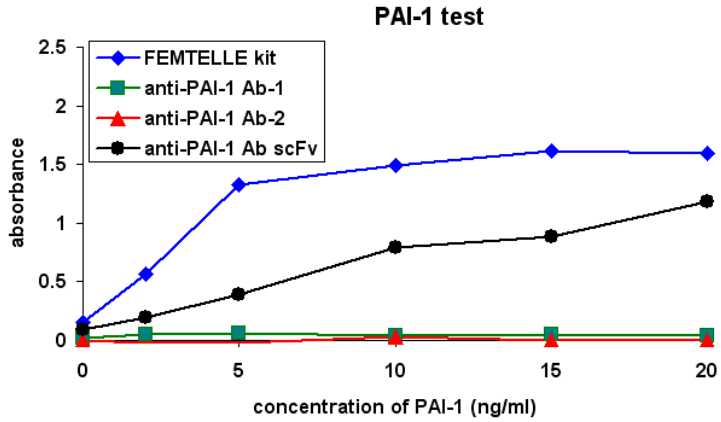
Le chapitre 3 concerne l'élaboration d'un immunoassay miniaturisé pour le dosage des protéines uPA et PAI-1 à partir d'extraits cytosoliques de tissus tumoraux de cancer du sein. En effet, uPA (urokinase Plasminogen Activator) et PAI-1 (Plasminogen Activator Inhibitor-1) sont 2 biomarqueurs tissulaires reconnus du développement du cancer du sein et de sa dissémination métastatique. Leur niveau d'expression dans les tumeurs du sein permet de classifier les patientes et de leur apporter un traitement adapté. De faibles taux d'uPA (< 3 ng/mg de protéines totales) et de PAI-1 (< 14 ng/mg de protéines totales) sont de bon pronostique et permettent d'éviter un traitement lourd sans réel bénéfice pour les patientes. Alors que de forts taux d'uPA et de PAI-1 sont corrélés à un haut risque de récidive, un traitement de chimiothérapie adjuvante est nécessaire afin de réduire ce risque.

A l'heure actuelle, un seul test (Femtelle®, Sekisui Diagnostics) basé sur une méthode immunologique (ELISA) permet de doser ces 2 biomarqueurs dans des extraits cytosoliques obtenus à partir de tissus tumoraux frais ou congelés. Cependant, cela nécessite de disposer au minimum de 300 mg de tissu tumoral, ce qui est une limitation importante notamment pour des tumeurs à un stade très précoce. Une solution proposée est donc de développer un test immunologique miniaturisé basé sur la technologie des biopuces à protéine, et permettant de doser les 2 biomarqueurs considérés à partir de quelques milligrammes de tissu tumoral. Ce travail a été réalisé en collaboration avec l'Institut de Cancer de Montpellier.

Les études précédentes menées dans l'équipe ont permis de développer des chimies de surface adaptées à l'immobilisation des protéines. Elles ont également mis en évidence l'importance d'adapter la chimie de surface et les conditions d'immobilisation (concentration, solution tampon de dépôt) à la protéine à immobiliser afin de conserver au maximum son activité biologique. Ainsi, dans un premier temps, nous avons optimisé les conditions d'immobilisation des anticorps anti-uPA et anti-PAI-1 de façon à avoir une détection sensible des marqueurs uPA et PAI-1. Puis nous avons réalisé, dans les conditions optimales définies, le dosage de uPA dans des extraits cytosoliques de tissus tumoraux de cancer du sein, fournis par le Centre de Ressources Biologiques de Montpellier. Nous avons alors comparés nos résultats avec ceux obtenus par le kit Femtelle.

Pour mener à bien cette étude, nous avons étudié l'activité biologique de 2 anticorps anti-uPA, 2 anticorps et 1 scFv anti-PAI-1, immobilisés sur les 6 chimies de surfaces développées dans l'équipe dans des conditions variables (concentration, solution tampon de dépôt). Parmi les 6 chimies de surface testées, 3 permettent de conserver une bonne activité biologique des anticorps anti-uPA et du scFv anti-PAI-1. Il s'agit des surfaces fonctionnalisées avec un silane carboxylé (surface COOH), avec le silane carboxylé activé (surface NHS), avec du chitosan (surface chitosan).

Cependant les anticorps anti-PAI-1 ont totalement perdu leur activité biologique suite à leur immobilisation sur les surfaces, et ce quel que soit les conditions utilisées. Aucune activité biologique de ces anticorps n'a également pu être détectée en test ELISA classique. Les conditions optimales définies pour le scFv anti-PAI-1 sont une concentration de dépôt à 10 µM en tampon PBS 1X (pH=7.4) permettant d'obtenir une limite de détection (LOD) de PAI-1 de 2 ng/mL.



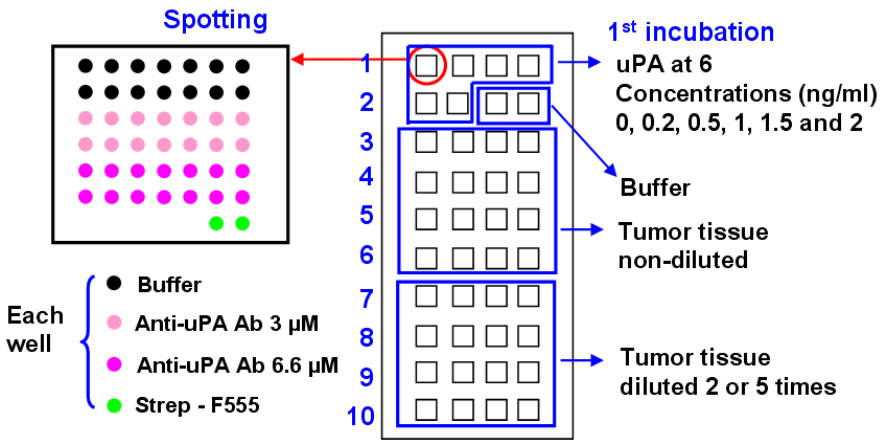
En ce qui concerne les anticorps anti-uPA, la concentration de dépôt est de 6.6 µM en tampon PBS 1X (pH=7.4). La LOD de uPA obtenue dans ces conditions est de 0.2 ng/mL avec une gamme dynamique de 0.2 – 2 ng/mL. La concentration de l'anticorps de détection anti-uPA biotinylé, préparé à partir du kit Femtelle, a également été optimisée.

Compte-tenu de ces résultats, des biopuces à anticorps anti-uPA ont été élaborées sur les surfaces COOH, NHS et chitosan, dans les conditions optimales, et évaluées pour la détection et le dosage de uPA dans des extraits cytosoliques de tissus tumoraux de cancer du sein. 16 extraits cytosoliques, préalablement dosés par le kit Femtelle et présentant des taux de uPA compris entre 0.4 et 8 ng/mL, ont été testés.

Une des difficultés de ce test est liée à l'aspect quantification. En effet, chaque biopuce doit intégrer une gamme étalon permettant le dosage de uPA. Pour cela, on a sélectionné 6 concentrations, en espérant avoir une bonne gamme dynamique de la courbe d'étalonnage. Au regard des gammes d'étalonnage de uPA sur les 3 chimies de surface testées, seule la surface NHS permet d'obtenir la gamme dynamique la plus étendue avec un coefficient de régression de 0,9795.

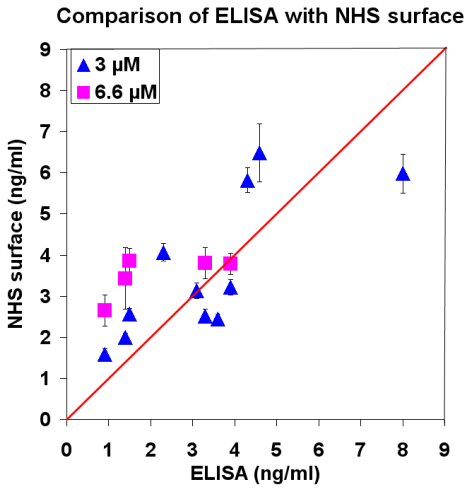
Anti-uPA Antibody spotted concentration	Surface	LOD	Dynamic range of uPA	Range of SNR
3 µM	COOH	0.2 ng/ml	0.2 – 1.5ng/ml	5.5-14.9
6.6 µM	COOH	0.2 ng/ml	0.2 – 1.5ng/ml	5.5-16.8
3 µM	NHS	0.2 ng/ml	0.2 – 2ng/ml	2.1-6.3
6.6 µM	NHS	0.2 ng/ml	0.2 – 2ng/ml	2.1-10.3
3 µM	chitosan	0.2 ng/ml	0.2 – 1ng/ml	1.1-1.8
6.6 µM	chitosan	0.2 ng/ml	0.2 – 1.5ng/ml	1.4-4.5

Compte tenu à priori de la quantité de uPA présents dans les échantillons à tester, l'analyse des 16 échantillons a été faite à 2 dilutions différentes (non dilués, dilués 5 fois) afin d'entrer dans la gamme dynamique de la courbe d'étalonnage.



Parmi les 16 échantillons cytosoliques testés, 11 d'entre-eux ont pu être correctement dosés pour uPA sur notre microarray. Les résultats obtenus sont en accord avec les dosages réalisés avec le kit Femtelle, et sont donc très encourageants pour l'utilisation des microarrays en clinique pour l'aide à la décision thérapeutique. De plus, les limites de détection atteintes avec notre microarray sont très inférieures à celles obtenues avec le kit commercial. En effet,

des quantités 10 fois inférieures en uPA, et 6 fois inférieures en PAI-1 peuvent être détectées et dosées par notre système, et ceci en consommant 100 fois moins d'échantillon biologique. Il reste cependant à évaluer les performances de notre microarray pour le dosage de PAI-1 en échantillons cytosoliques, puis à évaluer le dosage des 2 biomarqueurs dans des échantillons issus de tissus parafinés.



Chapitre 4 : Optimisation de l'élaboration des microarrays à protéines et de leurs conditions d'utilisation

Le chapitre 4 traite de l'optimisation des différentes étapes d'élaboration et d'utilisation des puces à protéines. En effet, dans un but d'utilisation en clinique, il est essentiel de contrôler, d'optimiser et de stabiliser chaque étape de fabrication des puces depuis la fonctionnalisation chimique de la surface jusqu'à l'étape de détection de l'évènement de reconnaissance biologique.

La stabilité des 6 chimies de surface utilisées a donc été évaluée au cours du temps (tous les mois pendant 6 mois) après stockage dans une enceinte contenant un desséchant. Cette évaluation a été réalisée par mesure de l'angle de contact et calcul de l'énergie de surface, ainsi que leur capacité à immobiliser des protéines marquées par un fluorophore. En effet, l'énergie de surface est directement corrélée à l'état de la surface et à sa composition chimique. Toute variation de l'énergie de surface implique donc une modification de la composition chimique de surface. Les résultats obtenus mettent en évidence la grande stabilité des chimies de surface COOH, NHS, chitosan, APDMES et CMD dans ces conditions. Seule la chimie de surface MAMVE présente une forte augmentation de son énergie de surface au cours du temps, notamment de la contribution polaire. Ceci peut s'expliquer par l'hydrolyse

des fonctions anhydride maléique suite à l'exposition à l'humidité ambiante, conduisant à la formation de groupements carboxyliques à caractère plus polaire. Par ailleurs, l'immobilisation de différentes molécules marquées (streptavidine-Cy3, anticorps-Cy3, ADN-Cy3) sur les 6 chimies de surface, diminue de façon plus ou moins importante selon les molécules et les surfaces après 3 mois de stockage. Ces résultats combinés aux précédents suggèrent que les chimies de surface développées au laboratoire pour l'élaboration de puces à protéines sont stables jusqu'à 3 mois de stockage dans une enceinte en présence de desséchant.

Nous avons également évalué la stabilité des protéines sondes (hsp60, hsp70, hsp110, grp78 et p53) immobilisées sur les puces (surfaces COOH, NHS, chitosan et CMD) après stockage dans différentes conditions (sous azote, dans une solution à 50% de glycérol, dans une solution à 5% de tréhalose, avant « capping » de la surface ou après). Cette étude a été réalisée par mesure de l'activité biologique des protéines immobilisées, c'est-à-dire leur capacité à reconnaitre leur anticorps spécifique. Les résultats indiquent que la stabilité des protéines sondes immobilisées est meilleure lorsqu'elles sont stockées après « capping » dans une solution à 50% de glycérol. Cependant leur activité biologique décroit avec le temps de stockage, mais permet une détection sensible des anticorps jusqu'à 3 mois de stockage. Ceci est en accord avec les résultats précédents concernant la stabilité des chimies de surface.

Nous avons ensuite optimisé les temps d'incubation relatifs aux différentes étapes de traitement des puces à protéines. Ainsi, après le dépôt des protéines sondes, un blocage de la surface avec une solution 10% BSA/PBS 1X pendant 1 heure est suffisant pour saturer tous les sites non spécifiques. Puis l'anticorps de détection biotinylé doit être incubé 1 heure à une concentration de 0,1 µM au lieu de 0,5 µM pour avoir une détection sensible de la reconnaissance antigène-anticorps. Enfin, il est possible de réduire de moitié le temps d'incubation avec la streptavidine-Cy3 (soit 30 minutes au lieu d'1 heure), et la concentration d'un facteur 4 (soit 0,05 µM au lieu de 0,2 µM). Ainsi, l'utilisation de systèmes miniaturisés tels que les puces à protéines pour étudier les interactions antigène-anticorps permet de réduire d'une part le temps d'analyse et d'autre part le coût par rapport à des systèmes classiques tels l'ELISA.

Conclusion

De ce travail, il ressort que les microarrays microstructurés sont des outils puissants permettant de cribler rapidement un très grand nombre de biomarqueurs et qui ne nécessitent

qu'un faible volume de prélèvement biologique. Selon le contenu biologique des microarrays faits à façon (c'est-à-dire en fonction des sondes immobilisées sur la surface) on peut développer des outils soit pour le diagnostic, soit pour le suivi thérapeutique des cancers du sein. Notons également que cet outil générique peut être adapté à d'autres types de cancer. L'enjeu des prochaines études étant de valider des panels de biomarqueurs pertinents pour chaque type de tumeurs.

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Résumé en Français

Le cancer du sein demeure un problème de santé publique majeure dans le monde. Afin d'améliorer les chances de survie et la qualité de vie des femmes, il est nécessaire d'effectuer le diagnostic à un stade précoce et d'appliquer le traitement. Dans ce contexte, un des objectifs de cette thèse est de développer des puces à protéines pour le diagnostic et le pronostic du cancer du sein. Parmi les nombreux marqueurs biologiques potentiels, des recherches récentes ont montré que des anticorps anti-heat shock proteins (anti-HSPs) sont associés à la genèse tumorale. Ces anticorps seraient donc de bons biomarqueurs diagnostiques et pronostiques pour le cancer du sein. Par conséquent, nous avons élaboré une puce à antigènes afin de détecter les anticorps anti-HSP dans le sérum de 50 patients atteints de cancer du sein et de 26 témoins sains. Nos résultats indiquent clairement que la la détection multiplex d'une combinaison d'anticorps anti-HSP permet de discriminer les patients atteints de cancer du sein des témoins sains avec une sensibilité de 86% et une spécificité de 100%. Ensuite, nous avons élaboré une puce à anticorps pour doser la concentration de l'activateur du plasminogène de type urokinase (uPA) et de son inhibiteur principal (PAI-1) dans 16 extraits cytosoliques de tissus tumoraux. uPA et PAI-1 sont décrits comme étant de bons biomarqueurs pronostiques et prédictifs du cancer du sein. De faibles taux de uPA (≤3 ng / mg de protéine) et PAI-1 (≤14 ng / mg de protéine) sont associés à un faible risque de récidive et pas de bénéfice d'une chimiothérapie pour les patients atteints de cancer du sein. Les résultats obtenus à partir de puces à anticorps étaient surface dépendante par rapport aux résultats obtenus sous forme ELISA. En outre, l'utilisation de nos puces à anticorps nécessite 25 fois moins de volume d'échantillon par rapport à un dosage ELISA, résolvant ainsi les principales limites de la méthode ELISA. Enfin, nous avons déterminé et optimisé les paramètres influençant les performances des puces à protéines, comme par exemple la chimie de surface, la durée expérimentale, la concentration des solutions, etc. Nous avons également étudié les conditions de stockage à la fois pour des surfaces chimiquement fonctionnalisées et pour les puces à protéines. Les résultats ont montré que les puces à protéines conservent leur activité biologique jusqu'à trois mois de stockage.

Mots clés: puces à protéines, anticorps, le diagnostic de cancer du sein, biomarqueurs prédictifs, le stockage

Curriculum vitae

Education

2012.09-2015.09	**PhD** of chemistry and nanotechnology**,** Ecole Centrale de Lyon (ECL), Lyon, France
2010.09-2012.06	**Master** on biology, Beijing Jiaotong University, Beijing, China
2006-2010	**Bachelor** on biology, Southwest Forestry University, Kunming, China

Research experience

2012.09- Present	**PhD subject**: Using protein microarray for aiding early diagnosis and providing personalized therapy for breast cancer patients
	Missions: We use protein microarray to identify biomarkers in breast cancer patients for aiding early diagnosis, prognosis and therapy monitoring, thus providing personalized medicine for each patient.
	Competences: protein microarray, ELISA, surface chemistry
2010.09-2012.06	**Master thesis:** Analyzing the function of LINE-1 in liver cancer cells
	Competences: cell culture, clone, qPCR, immunohistochemistry, western blot
2010.07-2010.08	**Internship**: Beijing University Shenzhen Hospital, Shenzhen, China
	Subject: Check pregnant women' chromosome to verify whether their babies are healthy
2010	**Bachelor** on biology

Publications

Submitted manuscripts

1. ***L. Shi***, T. Gehin, Y. Chevolot, E. Souteyrand, A. Mangé, J. Solassol and E. Laurenceau. Anti-heat shock proteins antibodies profiling in breast cancer using customized protein microarray. Analytical and bioanalytical chemistry (submitted), 2015.

2. ***L. Shi***, Y. Chevolot, E. Souteyrand and E. Laurenceau. Autoantibodies against heat shock proteins as biomarkers for the diagnosis and prognosis of cancer (review). Cell Stress and Chaperones (submitted), 2015.

Scientific meeting communications (7 oral presentations and 6 posters)

Oral Presentations

1. ***L. Shi***, T. Gehin, Y. Chevolot, E. Souteyrand, A. Mangé, J. Solassol, E. Laurenceau. Multiplex detection of anti-heat shock protein autoantibodies in breast cancer sera. 8th Entretiens pour la Recherche Beihang-GEC, Marseilles, France, 21-22 May 2015.

2. ***L. Shi***, T. Gehin, Y. Chevolot, E. Souteyrand, A. Mangé, J. Solassol, E. Laurenceau. Protein Microarray for the Diagnosis of Breast Cancer. PhD day of INL, Lyon, France, 2-3 October 2014.

3. ***L. Shi***, T. Gehin, Y. Ataman-Onal, A. Mangé, J. Sollassol, Y. Chevolot, E. Souteyrand, E. Laurenceau. 3D-Protein chip for the multiplex detection of cancer biomarkers. CMOS Emerging Technology Research, Grenoble, France, 6-8 July 2014.

4. ***L. Shi***, E. Laurenceau, Y. Chevolot, Y. Ataman-Onal, G. Choquet-Katylevsky, E. Souteyrand, "Protein microarray for the diagnosis of breast cancer", 20e Colloque de Recherche Inter Ecoles Centrales (CRIEC), Lyon, France, 2-6 June 2014.

5. ***L. Shi***, Z. Yang, Y. Chevolot, Y. Ataman-Onal, J. Solassol, A. Mange, E. Souteyrand, E. Laurenceau, "Customized multiplex immunoassay on 3D-protein chip for cancer biomarkers screening", 7th Annual World Cancer Congress, Nanjing, Chine, 16-18 May 2014.

6. YF. Zhu, ***L. Shi***, L. Lou, G. Li, L. Yu, M. Hu, J. Yu, W. Yue, Q. Zhou. "L1ORF1p via interacting with Ago2 participates a dsRNA-mediated regulation to promoter activity of L1-5'UTR", 7th Annual World Cancer Congress, Nanjing, Chine, 16-18 May 2014.

7. ***L. Shi***, E. Laurenceau, Y. Chevolot, Y. Ataman-Onal, G. Choquet-Katylevsky, E. Souteyrand, "A novel 3D protein chip to detect tumor associated antigen for cancer diagnosis", 19e Colloque de Recherche Inter Ecoles Centrales (CRIEC), Nantes, France, 24-28 June 2013.

Poster presentations

1. ***L. Shi***, Y. Chevolot, E. Souteyrand, and E. Laurenceau. Surface functionalization and characterization for protein microarray. School theme: chemistry and surface molecular engineering, la Londe les Maures, France, 8-12 June 2015.

2. ***L. Shi***, T. Gehin, Y. Chevolot, E. Souteyrand, A. Mangé, J. Solassol, E. Laurenceau. Heat shock proteins microarray for the serological profiling of breast cancer: identification of diagnostic and prognostic biomarker panels. 4th International Conference on Biosensor technology, Lisbon, Portugal, 10-13 May 2015.

3. ***L. Shi***, Y. Chevolot, J. Solassol, A. Mange, T. Gehin, E. Souteyrand, E. Laurenceau. Customized Tumor Antigen Microarray for the Diagnosis and prognosis of Breast Cancer, Forum de la Recherche en Cancérologie Rhône-Alpes Auvergne, Lyon, France, 7-8 Avril 2015.

4. ***L. Shi***, Y. Chevolot, J. Solassol, A. Mange, T. Gehin, E. Souteyrand, E. Laurenceau. Customized Protein Microarray for the Diagnosis of Breast Cancer, 6ème colloque UMI-LN2, Allevard les Bains, France, 9-10 July 2014.

5. ***L. Shi***, Z. Yang, Y. Chevolot, Y. Ataman-Onal, G. Choquet-Katylevsky, J. Solassol, A. Mange, E. Souteyrand, E. Laurenceau. Biomarker Screening Using Customized Multiplex Immunoassay on 3D-Protein Microarray, Entretiens Jacques Cartier, Santé, Biologie, Micronanosystèmes, INSA de Lyon, Villeurbanne, France, 25-26 November 2013.

6. ***L. Shi***, Eliane Souteyrand, Yann Chevolot, Emmanuelle Laurenceau. Tumor antigen microarray for the diagnosis and prognosis of breast cancer. PhD day of INL, Lyon, France, 17-18 October 2013.