doi:10.2533/chimia.2012.803

FH HES

Universities of Applied Sciences

Fachhochschulen – Hautes Ecoles Spécialisées

Construction of a Peptide Microarray for Auto-antibody Detection

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Abstract: Peptide and protein microarrays provide a multiplex approach to identification and quantification of protein–protein interactions (PPI), useful to study for instance antigen–antibody properties. Multivariate serology assays detecting multiple tumor auto-antibodies (TAA) is an emerging class of blood tests for cancer detection. Here we describe the efficient coupling of peptide baits derived from the BRCA1-associated RING domain protein 1 (BARD1) to a solid surface and detection of a commercially available anti-BARD1 antibody with this newly designed peptide microarray. Analytical sensitivity and specificity were shown to be comparable to a microtiter plate based enzyme-linked immunosorbent assay (ELISA).

Keywords: BARD1 · Click chemistry · Microarray · Peptide antigens · Tumor auto-antibodies

1. Introduction

There is increasing evidence for a humoral response to cancer in humans, as demonstrated by the identification of antibodies against a number of intracellular and surface antigens in patients with various tumors.[1–4] For instance, anti-tumor suppressor p53 auto-antibodies have been detected in patients with a variety of cancers including breast, gastrointestinal, lung, pancreas and prostate.[5,6] There is therefore interest in the potential screening and diagnostic utility of auto-antibodies and their corresponding antigens. As part of a project to develop a multivariate serology assay that detects tumor auto-antibodies (TAA) against specific isoforms of BRCA1-associated RING domain protein 1 (BARD1)[7,8] we were interested in investigating possible assay advantages when antigenic peptides were chemically linked to a solid surface as opposed to passively adsorbed to a microtiter plate. Relatively short peptides containing for instance 15-25 amino acids and depending on their polar or hydrophobic moieties and buffer/solvent composition as well isoelectric point (pI), may not adsorb well to solid surfaces of commercially available microtiter plates, come off during solvation and thus cannot be used for sensitive serological assays. On the other hand if short peptides remain physically attached they may not be present in solution in antibody recognizable conformations and thus also lead to poor diagnostic sensitivity.

Covalent immobilization of peptides on solid supports can be done for instance by classical amide bond formation or by reaction of hydrazine functionalized surfaces with aldehyde-functionalized peptides obtained by periodate oxidation of an N-terminal serine.[9] Recently, the Huisgen 1,3-dipolar cyclo addition reaction of azides with alkynes, also known as the click reaction gained a lot of attention in the modification and functionalization of biomolecules as it is straightforward to perform and compatible with many functional groups.[10]

Here we present a coupling chemistry that allows fairly rapid microarray solid-surface functionalization with peptide baits. Indeed, the BARD1 model microarray assay with little optimization work revealed similar sensitivity to anti-BARD1 antibodies as a microtiter plate-based ELISA. A robust and reproducible chemistry allows also construction of medium- or high-density microarrays for other applications such as epitope mapping and auto-antibody profiling.

2. Experimental

2.1 *Reagents*

6-Azidohexanoic acid was prepared according to literature.[11] ADIBO amine was purchased from Jena Bioscience (P/N: CLK-A103, Jena, Germany), (3-glycidyloxypropyl)trimethoxysilane was purchased from Sigma Aldrich (P/N: 440167, Sigma-Aldrich, MO, USA) all other chemicals and solvents were reagent grade and used as supplied. Immunoassay reagents were purchased from KPL (P/N: 072-01-18-06 Gaithersburg, MD, USA) for Cy3-labeled goat anti-mouse and from AbNova (P/N: H00000580-A01, Taipei City, Taiwan) for mouse anti-BARD1 antibodies.

2.2 *Microscopy Slide Functionalization*

The glass slides were functionalized in analogy to literature,[12] therefore micro glass slides 75×25 mm (P/N: 2948, Corning, NY, USA) were placed in a Pyrex beaker cooled with ice, and 60 ml H_2SO_4 96% was added followed by 20 ml H_2O_2 30% and then stirred. After 30 min. the slides were removed, washed with water, then rinsed with acetone and dried for about 2 h at 100 °C. The activated slides were then immediately immersed in a solution of (3-glycidyloxypropyl)trimethoxysilane (2 ml) and diisopropylethylamine (2 ml) in toluene (200 ml). After 16 h at r.t., the slides were removed and washed with methanol and acetone for 15 min. in a sonication bath and dried by a stream of dry N_2. Next the slides were immersed in a solution of ADIBO amine (100 mg) and diisopropylethylamine (2 ml) in dry DMF (200 ml) and incubated over night at r.t. Again the slides were removed and washed with methanol and acetone for 15 min. in a sonication bath and dried by a flux of dry N_2 and stored in a freezer. Slide functionalization was quality controlled *via* coupling reaction with a Cy5-azide reagent.

2.3 *Peptide Synthesis and Derivatization*

Positive control (15 a.a., P1) and negative control (11 a.a., P2) peptides were synthesized using Fmoc-protected amino acids and a standard coupling procedure using uronium-based coupling reagents on an Apex DCFWS 396 synthesizer (AAPPTec, Louisville, KY, USA). The last step, coupling the 6-azidohexanoic acid, was also done using the same chemistry. Both synthesized peptides were purified by preparative RP-HPLC, lyophilized and

doi:10.2533/chimia.2012.804

finally their molecular weights were verified by mass spectroscopy (Agilent qTOF 6530, Santa-Clara, CA, USA). Solutions of peptides were then prepared at an initial concentration of 0.8mM in water and then diluted for further experiments.

2.4 *Solid-surface Immobilization and BARD1 Assay*

One microliter of corresponding peptide solutions at concentrations of 0.4 mM to 2 μM were manually deposited with a pipette (Gilson Pipetman P2N, 0.2–2 μL) on activated slides to form spots of approximately 2 mm diameter. Once the spots had dried, slides were immersed in a bath of 50 mL azidohexanoic acid at a concentration of 0.05 μg/mL for 30 min. to cap all unreacted ADIBO groups. Alternatively, as a control step, azidohexanoic acid was replaced by Cy5-azide. Slides were subsequently washed in water, acetone and methanol baths to remove peptides that have not reacted covalently. Slides were dried under an air or N_2 flow and assembled into Nexterion IC-16 hybridization chambers. Wells were first blocked with a 5% BSA solution during 1 hour. Between assay steps wells were washed three times with a phosphate buffer (PBS 1×) to remove reagent excess. Mouse anti-BARD1 antibody solution was prepared with concentrations ranging from 6.25 to 200 ng/mL in a 1% BSA/PBS buffer solution and 100 μL were dispensed into the wells and incubated for 30 min. at room temperature. The last step involved 30 min. treatment with 100 μL of Cy3-labeled goat anti-mouse antibody at dilution ratios of 1/250 or 1/500, respectively. Slides were finally washed with PBS 1×, hybridization chambers were dismantled and the slides briefly rinsed with ultrapure water and then dried with a flow of N_2 or air before fluorescence measurements.

2.5 *Automated Microarray Pattern Printing*

Functionalized slides were prepared using a microdrop printing system developed at HEPIA and validated in a recent study.[13] It is designed to realize reproducible patterns of 6×4 spots of about 250 μm diameter on standard slides without contact. According to the assays, 3 μL solutions were dispatched in the 24 individual microreservoirs directly implemented in the printhead. The printing process is driven by an adjustable piezoelectric system that ejects roughly 1 nL microdrops. Optimal ejection parameters are determined specifically such as to allow correct simultaneous ejection of the 24 solutions with different viscosities.

2.6 *Microarray Spot Fluorescence Measurements*

Slides were measured with a non-confocal GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA) in simultaneous 2-channel scanning mode (excitation with λ = 532 nm, Cy3 compatible, emission range from 558 to 593 nm and 635 nm, Cy5 compatible, emission range from 660 to 690 nm). Pixel resolution set at 10 μm unless otherwise stated. Dual photomultipliers (PMTs) with manual gain adjusted for better dynamic range and data stored as a TIFF 16-bit files. Features were manually extracted and the averages for each spots were used for the analysis.

3. Results and Discussion

In preliminary work on peptide immobilization on glass slides we evaluated the hydrazine/aldehyde protocol and the copper-catalyzed Huisgen azido/alkyne click reaction. The functionalization of the glass slides with a hydrazine group and the coupling with an aldehyde-peptide worked well, but the preparation of the aldehyde-peptide by periodate oxidation of a N-terminal serine adds additional steps and has the disadvantage when one is working with oxidation-sensitive peptides. On the other hand, the copper-catalyzed click reaction of azido-functionalized peptides

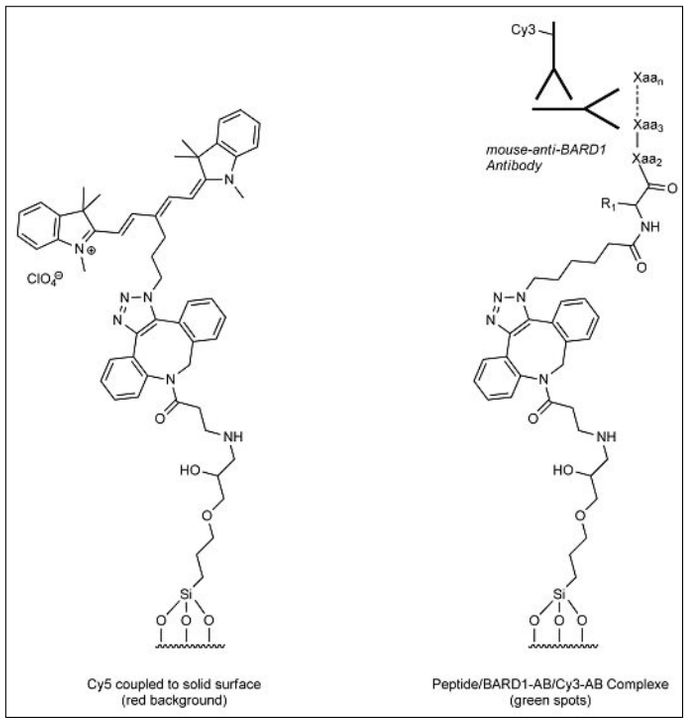

Fig. 1. Cy5 coupled to ADIBO-functionalized slides gives a red background (left). Antigenic peptide sequences covalently attached to slides are recognized by mouse anti-BARD1 antibodies and made visible as green spots with Cy3-labeled goat anti-mouse detection antibody (right).

with a terminal acetylene-functionalized glass slide was rather sluggish and gave no reproducible results.

We therefore evaluated the copper-free click reaction of strained cyclic alkynes and selected aza-dibenzocyclooctyne (ADIBO) as reagent as it can be easily prepared[12] and it is also commercially available. Functionalization of the glass slides was done by activation of the glass slides with *Piranha* solution followed by reaction with (3-glycidyloxypropyl)trimethoxysilane to prepare epoxy-functionalized glass slides, which then reacted with the ADIBO-amine to furnish the ADIBO-functionalized glass slides as shown in Fig. 1. First tests with TAMRA-azide confirmed proper and uniform functionalization of the glass slides and fast reaction times – approximately 5 min. was sufficient to obtain maximum fluorescence intensity. The glass slides can be easily stored under dry conditions in a freezer for at least two weeks, however further stability studies are ongoing.

To verify covalent attachment of azide derivatized peptides after spotting, the slides were treated with Cy5-azide to cap free ADIBO groups. As illustrated in Fig. 2, panel A, black spots reveal sites unable to react with Cy5-azide, but capable of being specifically recognized *via* the mouse anti-BARD1/goat anti-mouse antibody assay format. A negative control (NC) peptide sequence is not detected even when spotted at higher concentrations (panel B). At peptide concentrations of 50 μM or less free ADIBO-sites presumably remain available for reaction with the Cy5-azide, which explains disappearance of the black spots. However, there is a sufficient amount of immobilized positive control (PC) peptide molecules available to be detected without any loss in fluorescence intensity (panel C).

As mentioned above one objective was to investigate the benefit of chemical coupling of a peptide target sequences over passive adsorption in the context of sensitive serological assays. We thus conducted a head-to-head comparison between a micro titer plate and microarray-based BARD1 assay utilizing essentially identical reagents. As can be seen in Fig. 3 analytical performances were similar. The limit of detection (LOD) for the mouse

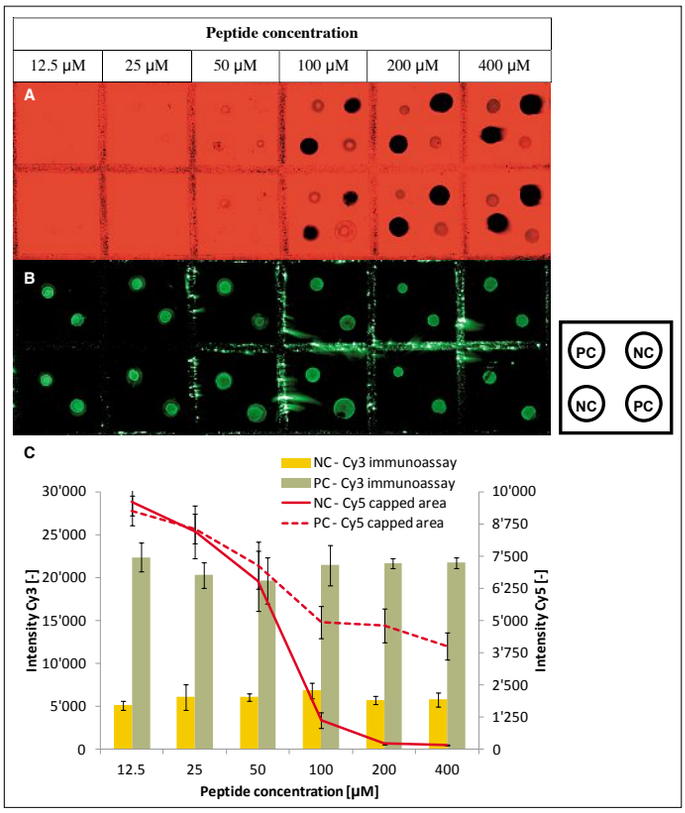

Fig. 2. Proof-of-concept experiments. Positive control (PC) and negative control (NC) peptide sequences were spotted as duplicates at various concentrations (90 min., diluted in water). A) At higher peptide concentrations no blocking of free ADIBO groups with Cy5-azide (12.5 ng/mL) is observed (black spots), whereas at lower concentrations strong fluorescence is 'illuminating' dark spot areas. B) The same slide after the immunoassay experiment clearly reveals presence of the PC peptide (green spots). Note that the NC peptide at even the highest concentrations does not react with the antibodies used in the experiments.

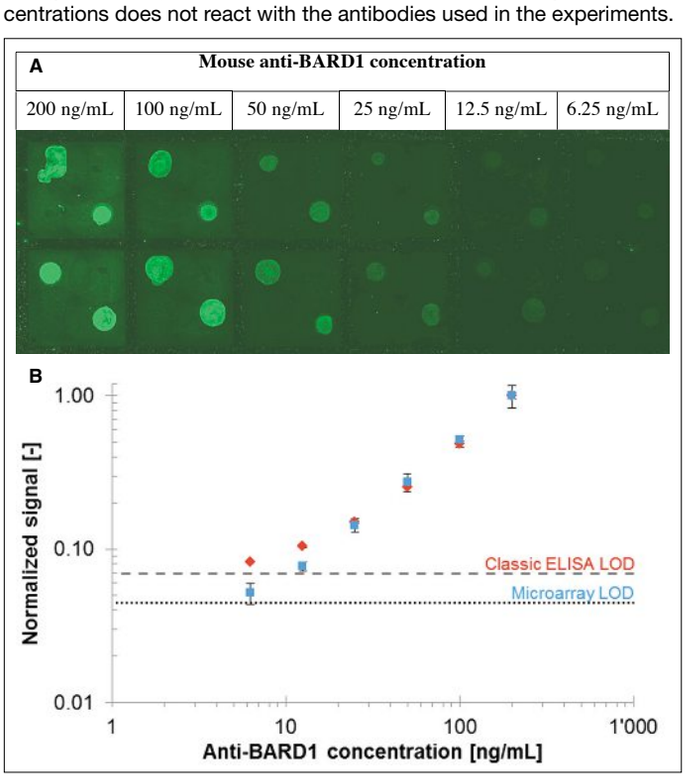

Fig. 3. Mouse anti-BARD1 antibody titration experiment to assess assay limit of detection (LOD). Dilution series: From 200 to 6.25 ng/mL, left to right as quadruplicated spots (Cy3-labeled detection antibody at 1:500 dilution). As indicated in graph both classic ELISA and peptide microarray reveal similar analytical sensitivity performance.

anti-BARD1 antibody in the not fully optimized peptide microarray assay was at 6 ng/mL. Work is in progress to, for instance, evaluate blocking reagents other than 6-azidohexanoic acid to further reduce non-specific binding and thus increase signal-to-noise ratio.

Assay miniaturization is a key requirement in the development of small point-of-care (POC) medical devices while high content screening is often pivotal in diagnostic biomarker discovery. For these reasons we were interested to see whether the click chemistry-based immobilization reaction kinetics were compatible with an automated nanoliter scale spotting process. Depicted in Fig. 4 is a microcopy slide patterned with 40×24 = 960 peptide and quality control spots. As also shown before in Fig. 2 the positive control (PC) peptide is clearly detected while the negative control (NC) peptide is undetectable. Spot size, positional accuracy, homogeneity and repeatability depend upon multiple factors and have to be further optimized to meet both manufacturing and assay quality requirements.

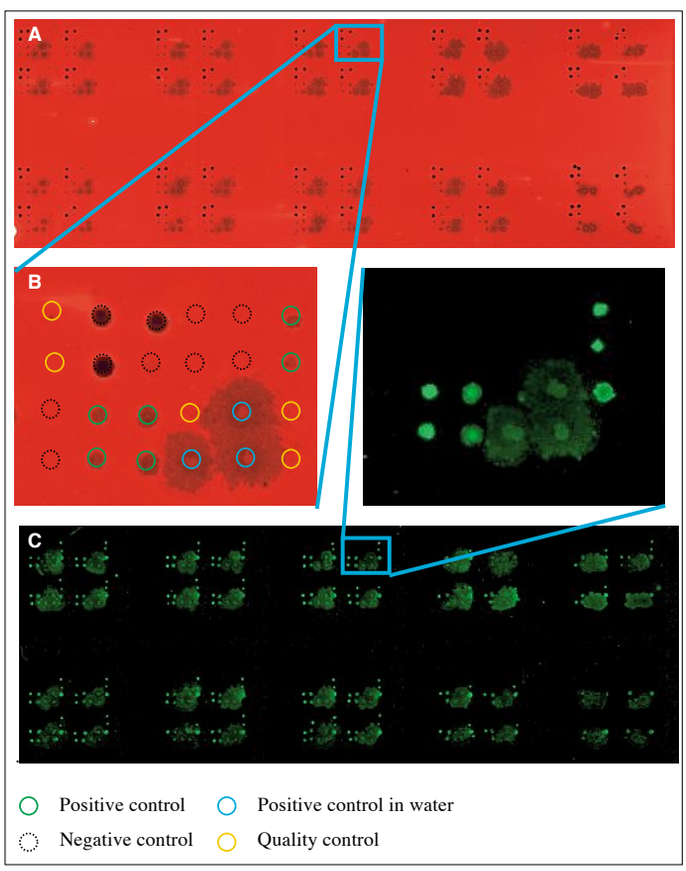

Fig. 4. Similar approach to Fig. 2, but nanoliter amounts deposited with automated device developed at HEPIA, 40 replicates of 24 spots each (spot diameter ~250 µm). PC peptide solubilized in water (without DMSO) leads to significant spot diffusion (blue circles). Yellow circled spots are quality controls features. Different peptide concentrations (0.1 to 0.4 mM) and solvent conditions (0% to 75% DMSO in water) were tested on this slide (more details can be obtained from the corresponding author).

In conclusion, the copper-free click reaction of ADIBO-functionalized slides with azide derivatized peptides has been shown to be reliable and efficient for the preparation of peptide microarrays useful for sensitive immunological tests.

Acknowledgements

This work was financially supported by HES-SO RCSO 'RealTech'. We are grateful to Prof. Marc Mathieu and Ms. Vanessa Gaillard (HES-

doi:10.2533/chimia.2012.806

SO Valais, 'DEBIOPharm Laboratory') for peptide synthesis and purification support and Mr. Lionel Chappuis for preliminary work on copper-free click chemistry.

Received: August 31, 2012

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