Hydrogel coated Slides for Protein Microarrays

Technical product information

The challenge of multiplexed protein analysis

In the past years DNA microarrays have become a standard tool for the investigation and functional interpretation of sequence information. Protein microarrays - the methodological successor of this technology - have recently moved into the focus of scientific interest, as they appear to be a promising approach for the fast and simultaneous detection of proteins in biological samples. Theoretically, their sensitivity is expected be as low as a few femtograms per ml or less[1], which should be sufficient for most applications.

Although protein microarrays have the potential to open up numerous applications of high scientific and economic relevance, practice has shown that turning this promise into a reliably working technology is not as easy as initially thought. This is mainly due to the fact that proteins are much more sensitive towards denaturation than relatively robust DNA and tend to stick nonspecifically to most surfaces. Furthermore, protein structure and chemistry is widely diverse and expression level can vary as much as seven orders of magnitude[2]. Adapted chip substrates and detection technologies are thus needed to meet the specific requirements of multiplexed protein assays.

Demands on the substrate

As the immobilization and molecular interaction between receptor and mobile analyte proceeds at the interface between chip substrate and analyte matrix, it is clear that the surface matrix design is the key factor in determining the performance of a protein microarray.

When assessing potential substrate materials, the following requirements should be addressed[3]:

- High immobilization capacity. Proteins are usually considerably bigger than oligos and the density of attached labels is lower. Due to their three dimensional nature, they need more immobilization space on the chip surface to generate a comparable signal. Additionally, frequently low abundance of analyte molecules at equilibrium, results in a high fraction of unoccupied receptor sites; therefore, in order to achieve an acceptable signal at all, the absolute density of receptors should be as high as possible but always within the “ambient analyte” regime[1]. Further, the immobilization procedure should retain the substrate’s molecular activity and be suited not only for ‘standard’ proteins but also for other species such as peptides or low molecular weight compounds.
- Protein-friendly environment. Proteins are delicate species which easily unfold upon contact with low-energy interfaces, or in response to drying, ionic strengths that are too high or too low and/or extreme pH. Too often, this process is irreversible, leaving behind an inactive polymer which frequently induces non-specific interactions and turns valuable immobilization sites into increased background.
- Low level of non-specific binding. Proteins tend to adsorb irreversibly to many surface types[4], including plastic, metals, glass etc. This kind of ‘immobilization’ results in 92 – 95% inactive protein[5], and the resulting surface has to be additionally blocked. This is usually done with protein containing blocking buffers which further increase the background and can lead to unwanted crossreactivity of the analyte. This directly decreases signal-to-noise ratio and consequently lowers the assay sensitivity.
- Low inherent substrate noise. Neither the support nor the immobilization matrix (the coating) itself should add significantly to the background of a solid phase assay. Generally fluorescence detection is employed, so minimal autofluorescence of the chip substrate is desired.

- Maximal planarity and parallelity. The performance of most spotting systems, especially ring-pin spotters depends on the precise calibration of the pin/head – slide distance, which is often just a couple of µm. Deviation from the calibrated value can result in omitted spots or damage at pin and surface. Furthermore, many scanners employ confocal optics, which require a substrate planarity of < 5 µm for reproducible results. A tolerance of 20 µm, for example, causes signal shifts of 30% and more.
- Ease of handling during preparation procedures and high reproducibility. Despite increased complexity and sensitivity of the immobilizate and the entire assay, production protocols and experimental procedures shouldn't become too tedious and lengthy thereby increasing labour costs and the risk of operating errors.
- Sufficient storage stability of the slide substrate and the spotted microarrays. A protein-compatible surface should protect the immobilizate from denaturation and significantly enhance the shelf life and stability of immobilized proteins.

State of the art technology

Currently available surfaces for protein microarrays can be divided into two- and three - dimensional coatings, the latter further into thin and thick film products.

Two-dimensional surfaces can be either untreated or covered with a few nm thin monolayers bearing various functional groups. Since these monolayers follow the topology of the (mostly planar) substrate material, their immobilization capacity is characteristically limited to a monolayer. Examples for this kind of surface include silanized glass, functionalized with aldehyde, amino, poly-l-lysine, thiol, SCN, NHS or epoxy groups; untreated plastic substrate materials fall under this category as well. As all of these surfaces are more or less hydrophobic, protective substances have to be added to the spotting solution to prevent protein denaturation[6]. Nevertheless very often a large fraction of the immobilized proteins is inactive. Another consequence of this hydrophobicity is the higher degree of nonspecific protein adsorption, making blocking necessary and the analysis of complex samples difficult.
With respect to nonspecific binding and spot morphology, polyethylene glycol (PEG) coatings[6] fare better. However, due to the monofunctional nature of surface grafted PEG, the immobilization density cannot exceed a monolayer.

To solve the problem of limited immobilization capacities, several three-dimensional coatings have been developed. As nitrocellulose membranes are well established as excellent blotting substrates, the technology has also been adapted to the slide format (FAST Slides, Schleicher&Schuell). A similar approach employs macroscopic polyacrylamide hydrogel pads (HydroGel Slides, Perkin Elmer) having a thickness of approx. 10 µm which are attached to the glass surface. With both products the proteins are immobilized via adsorption and the surfaces are blocked afterwards. Very high binding capacities can be realized, especially with nitrocellulose. Disadvantages include: the previously mentioned (partial) immobilizate denaturation associated with adsorptive immobilization, high autofluorescence, and significant nonspecific binding which makes analysis of complex samples difficult[7]. Thick film polyacrylamide coatings suffer from high CVs of over 30%[3] and require long incubation times to reach thermodynamic equilibrium, especially for low abundance proteins[8].

Besides the above mentioned coatings with thicknesses in the µm range, a few thin film three-dimensional surfaces are available (Slide H, Schott and OptArray, AccelR8). These hydrogels have a thickness of 10 – 60 nm and are preactivated for covalent coupling of ligands through NH_2 groups. Due to the relatively small thickness of the hydrogel layer, the S/B (signal to background) enhancing effect is less than 50% compared to standard two dimensional epoxysilane surfaces. Although amine reactive groups must be deactivated prior to use, these surfaces do not have to be blocked.

In summary it may be said that three-dimensional coatings, especially hydrogels, provide a generally promising approach for the design of protein microarray coatings. Most problems associated with thick film matrices arise from thicknesses that are too high and are available in unfavorable material choices. On the other hand, the currently available thin film hydrogels are obviously too thin to exhibit significant effects. The optimal coating for protein microarray substrates should have a thickness between the thin and thick film type, and be made from a bioinert material with low autofluorescence which allows covalent or directed coupling of a variety of different species.

Next generation hydrogel surfaces: The HC series

Based on the above considerations and almost 10 years experience in surface manufacturing for biosensors, XanTec bioanalytics has designed a novel hydrogel coating with drastically enhanced protein immobilization capacity without showing the typical disadvantages of thick film coatings. Compared to standard planar surface coatings, the immobilization capacity of the HC-series' hydrogel matrix is 10 – 50 times higher; typical protein loadings are in the range between 10 - 100 ng/mm². As the polymer chains in this coating are oriented brush-like (perpendicular to the surface), diffusion through the < 5 µm thick layer is fast.

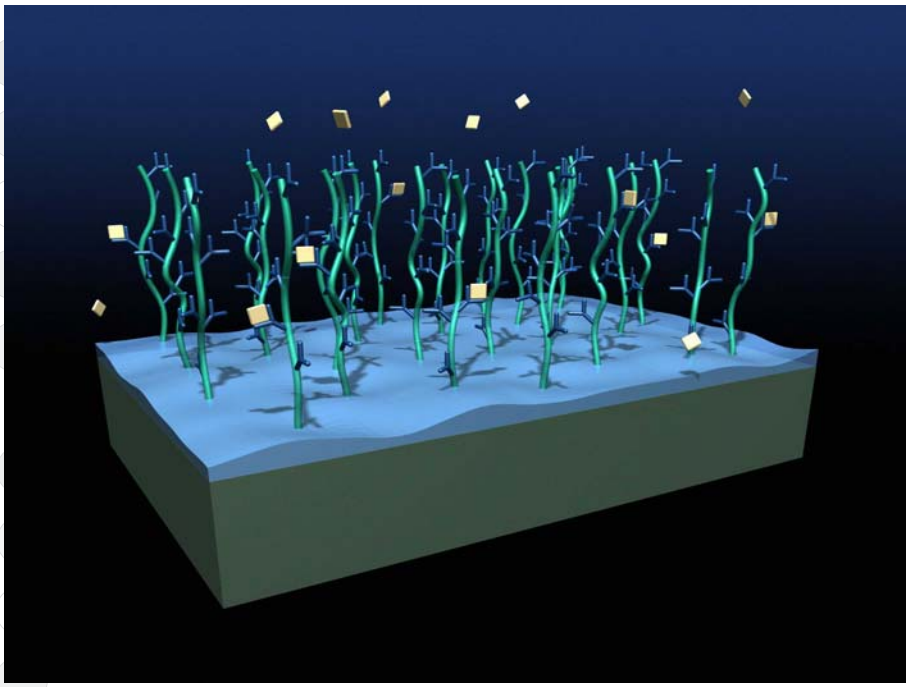


Fig. 1: Molecular structure of XanTec's HC hydrogel coating (not to scale). A several µm thick hydrophilic and bioinert polymer brush is attached to a passivation layer.

The biocompatible character of the HC hydrogels is similar to separation media used in protein purification and stabilizes even sensitive proteins, thus ensuring excellent long term storage stability. Another interesting feature is the screening of fluorophor markers in the dry state. Bleaching, dimerization and quenching phenomena are suppressed and the fluorophor signal is maximized.

A straightforward, robust spotting protocol and the efficient immobilization process yield consistently high S/B ratios already at low protein quantities, saving valuable biomaterial. In a typical spotting experiment, approximately 50% of the spotted protein is immobilized with a typical CV of < 10% over the slide surface. The contact angle of the activated surface is 50° - 55° which is a good prerequisite for homogeneous spot shapes. After quenching, the surface is very hydrophilic (θ=0°). Blocking steps are usually not required, significantly enhancing the biospecificity of the resulting protein arrays.

Due to the covalent immobilization, small ligands such as peptides, cytokines and other low MW compounds can also be immobilized in a stable manner, provided that they bear amino or other functionalities for covalent coupling.

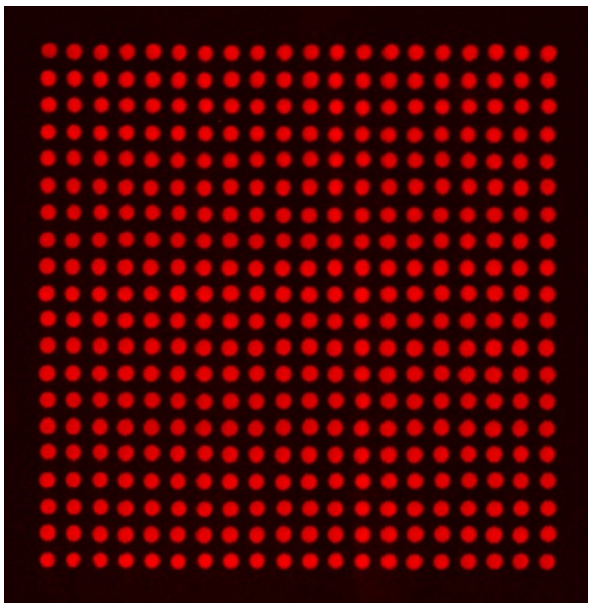


Fig. 2: Even spot shape and distribution of Oyster® 656 fluorophor labelled BSA spots on a HCX surface.

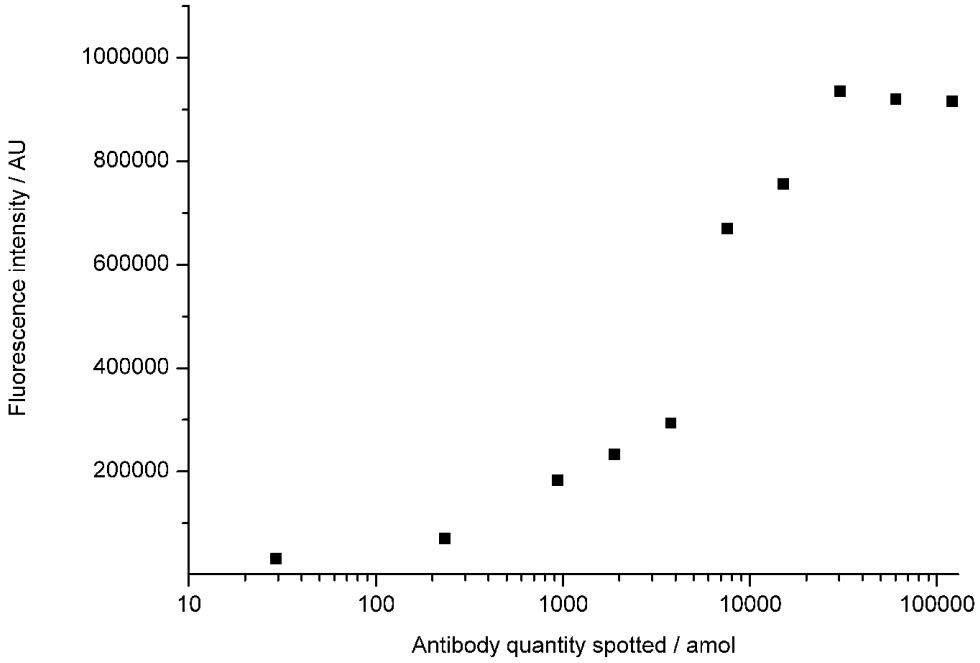
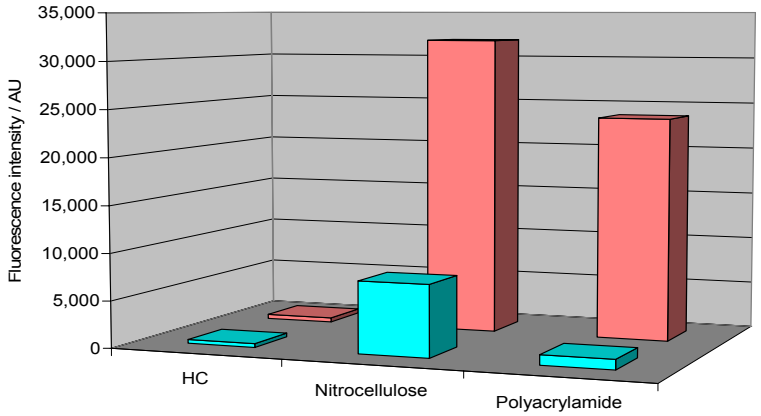


Fig. 3: Evaluation of immobilisation efficiency according to literature procedures[3]: Different quantities of α-CRP* antibody were spotted on NHS activated carboxymethyldextran (similar to HC surface) slides followed by quenching and overnight incubation with 10 µg Cy5-CRP / ml PBS. Scanning was performed with a GMS 418 scanner. Spots above the upper detection limit at 65.000 AU were scanned at lower gains and recalculated accordingly. The average background noise was around 1500 AU. The lowest concentration (27 amol/spot) yielded a signal of 31.000 AU and thus a signal to baseline (S/B) ratio of 20. At around 30 fmol / spot the maximum immobilisation capacity was reached with a corresponding signal of 850.000 AU (S/B: 570).

* CRP: C-reactive protein



	HC	Nitrocellulose	Polyacrylamide
Autofluorescence, Cy 5 channel	403	7,582	1,057
Autofluorescence, Cy 3 channel	461	31,955	23,758

Fig. 4: Comparison of different 3D substrate autofluorescence measured at 550 (Cy3 channel) and 650 nm (Cy5 channel).

High immobilization densities with efficient conservation of immobilizate's activity combined with low nonspecific binding exhibiting negligible autofluorescence, result in remarkably high dynamics and low detection limits in microspot based assays performed on HC coatings (Fig. 5). This easily allows detecting proteins in low concentrations as well as drastically reducing the need for dilution steps.

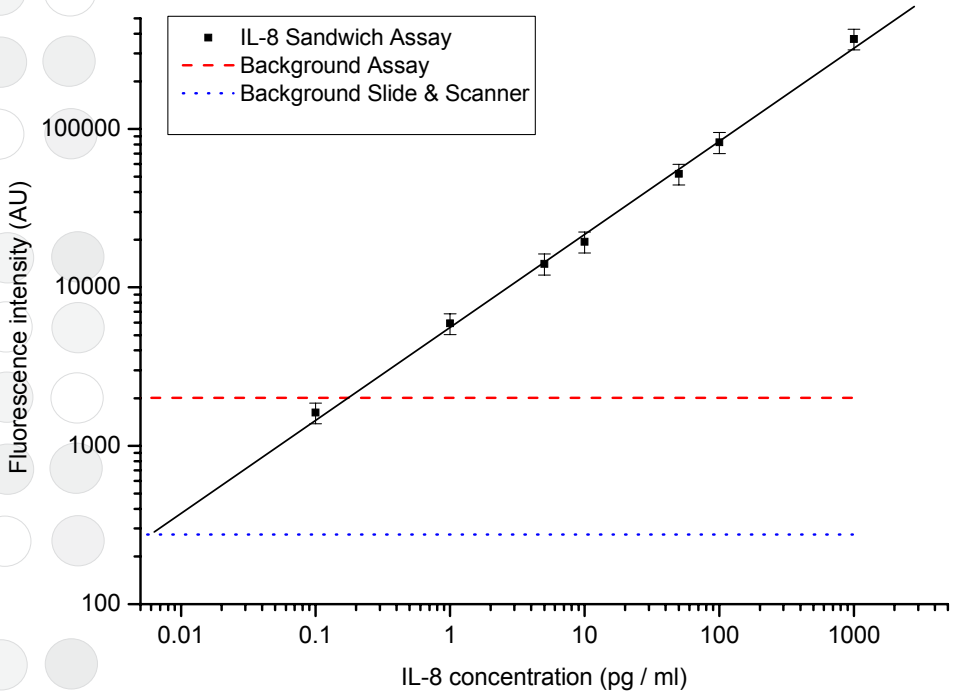


Fig. 5: Calibration curve of a cytokine assay in classic sandwich format. The upper dashed line represents the background produced by nonspecific protein-protein binding of the Oyster®650 fluorophor labelled polyclonal secondary detector antibody to the immobilized primary antibody which limits the detection level to ~300 fg / ml. The lower line is the sum of the NSB of the slide / coating and the noise of the scanner.

Application example I: Cytokine quantification

30 pg (200 amol) monoclonal α-IL-8 antibody / spot were spotted on HCX slides. After quenching, the slides were incubated with different IL-8 concentrations, rinsed with buffer and again incubated with Oyster®650 fluorophor labeled polyclonal secondary detector antibody. After washing with buffer and water, the slides were dried and scanned at laser power 100%, gain 100% (GMS 418 scanner). At spot intensities above 65.000, the slides were scanned at lower gains and the values recalculated accordingly.
The resulting calibration curve is shown in figure 5; table 1 compares detection limits of this assay with the corresponding data of kits manufactured with other substrates (manufacturer's data) and typical ELISA values. Please note that in contrast to all other reported cytokine assays with comparable sensitivity, amplification steps are not required on HC surfaces.

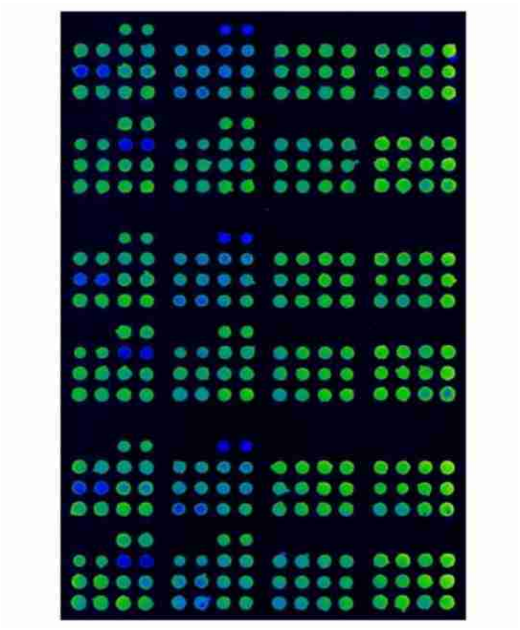
Product	XanTec HC	Competitor 1	Competitor 2	ELISA
Detection range (pg/ml)	0.3 – 3.000	4 – 10.000	5 – 2.500	3,5 – 3.000
CV	< 10%	15%	< 20%	8%

Tab. 1: Dynamic range and CV of cytokine assays on different substrate materials and with ELISA. The physiologic concentration range is 0.5 – 20 pg/ml.

The HC slides lower detection limit of 300 fg/ml is determined by the moderate selectivity of the polyclonal detector antibody used in this experiment which causes a background of 2.000 AU on the capture antibody spots. The NSB to the hydrogel matrix plus background of slide substrate and scanner alone accounts for ~ 400 AU only.

Application example II: On-spot purification of cell lysates

This protein array was manufactured by spotting different cell lysates containing one expressed biotinylated protein each on a Neutravidin derivatized CM dextran slide. The chromatographic properties of the surface, i.e. very low level of non-specific binding, allow the on-spot purification and selective immobilization of the biotinylated proteins from the spotted crude cell lysate. The elimination of purification steps prior to spotting results in an economic way of protein array manufacturing.
Source: Sense Proteomic Ltd., Cambridge, U.K.



Available coatings

To match different coupling strategies, the following derivatives of the HC surfaces are recommended for use in protein microarrays:

- **HC** Carboxylated bioinert hydrogel. Not activated.
- **HCX** NHS activated hydrogel. Standard surface for direct covalent coupling of proteins, peptides, amino modified DNA and other amine bearing molecules.
- **HHC** Hydrazide derivatized hydrogel. Couples aldehyde and keto functionalities and can be used to immobilize carbohydrates.
- **NiHC** Ni^{2+} complex derivatized hydrogel matrix. For immobilization of His_6-tagged molecules.
- **SHC** Streptavidin (density approx. 50 ng/mm²) immobilized in a bioinert hydrogel matrix. For immobilization of biotin conjugates.
- **BHC** Biotin immobilized in a bioinert hydrogel matrix. For immobilization of streptavidin conjugates
- **THC** Thiol derivatized hydrogel. Allows covalent immobilization of proteins via disulfide exchange.

In addition, the following dextran based surfaces are available, which are useful supports when adapting protocols developed for the immobilization of receptors to affinity chromatography media, or to CM dextran coated SPR sensorchips:

- **D** Dextran hydrogel. Can be acitivated using similar protocols as for Sephadex®. Suitable methods include epoxy, tresyl or bromocyan activation or periodate oxidation.
- **CMD** Carboxymethyldextran. Standard surface for efficient covalent coupling of proteins after NHS activation.

Coatings for manufacturing of DNA microarrays are described in the separate product information ‘Activated Slides for DNA Microarrays’.

Beside the above mentioned standard surfaces, numerous custom coatings with other chemistries and/or different hydrogel thicknesses are available. These products are listed in the separate brochure ‘Custom coatings’. Please consult our technical service prior to ordering surfaces from this section.

Technical data HC series slides

Glass type	Borosilicate glass with low autofluorescence
Dimensions	76.0 x 25.0 mm ± 0.2 mm
Thickness	1.00 mm ± 10 µm
Flatness	< 2 µm
Parallelism	< 5 arcsec
Surface treatment	Optically polished
Edge specification	Cut, slightly seamed
Coating	< 5 µm (swollen) hydrogel on both sides
Spottable area	60 x 21 mm

Literature

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