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Single step, direct fluorescence immunoassays based on metal enhanced fluorescence (MEF-FIA) applicable as micro plate-, array-, multiplexing- or point of care-format

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ABSTRACT

Although Enzyme Linked Immuno Sorbent Assay (ELISA) technology is approaching it's 45th year of existence since first described in 1971, it is still the main diagnostic tool in clinical research and routine diagnostics.

However, despite its broad usage it suffers from some drawbacks, limiting its use especially in more advanced assay formats like multiplexing platforms, point of care devices or protein arrays. Those limitations result from the need for an enzyme label, a soluble enzyme substrate, washing steps (multiplexing, point care, arrays) and in some cases also insufficient sensitivity, because the majority of circulating proteins and thus potential biomarkers may be found in lower sub-picomolar concentrations.

We hereby present a new assay platform based on metal enhanced fluorescence (MEF), that remedies these problems since it eliminates the need for washing steps, for using enzyme labels and allows detection of analytes down to sub-picomolar concentrations. In addition this technology is fully compatible to standard fluorescence reader equipment as it is found in many laboratories nowadays.

Since our present work is focused on single biomarker evaluation, we chose a 96 well plate format for convenience, but any other formate like antibody arrays, strip-like point of care devices etc. is feasible too.

Introduction

Although Enzyme Linked Immuno Sorbent Assay (ELISA) technology is approaching it's 45th year of existence since first described by Engvall and Perlman [1] in 1971, it is still the main diagnostic tool in clinical research and routine diagnostics.

However, despite its broad usage it suffers from some drawbacks, limiting its use especially in more advanced assay formats like multiplexing platforms, point of care devices or protein arrays.

Those limitations result from the need for an enzyme label, a soluble enzyme substrate and washing steps (multiplexing, point care, arrays) and in some cases also insufficient sensitivity, because the majority of circulating proteins and thus potential biomarkers may be found in low- or sub-picomolar concentrations [2].

We hereby present a new assay platform based on metal enhanced fluorescence (MEF), that remedies these problems since it eliminates the need for washing steps, for using enzyme labels (Fig. 1) and allows detection of analytes down to sub-picomolar concentrations.

In addition this technology is fully compatible to standard fluorescence reader equipment as it is found in many laboratories nowadays.

Since our present work is focused on single biomarker evaluation, we chose a 96 well plate format for convenience, but any other format like antibody arrays, strip-like point of care devices etc. is feasible due to the generality of the concept.

Material and methods

Reagents

All chemicals and biologicals were used as received from the respective suppliers.

Metal enhanced fluorescence measurements and substrates

The principles of metal enhanced fluorescence are well understood and described in literature [3–5]. Briefly, the fluorescence of molecules approaching a surface with metallic nano-structures can be greatly enhanced, if those nano-structures have the right shape, size and distance to each other [6]. Silver and gold are the preferred metals used and there are a growing number of academic papers investigating

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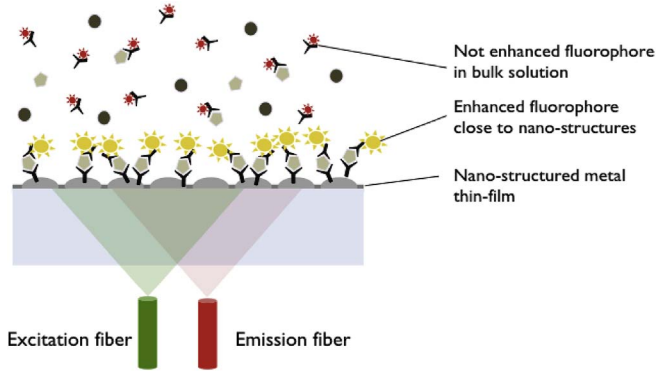

Fig. 1. Principle of Metal Enhanced Fluorescence: Only the fluorescence of molecules (yellow stars) close to the nanostructures is enhanced. Those further away (> 50 nm distance) do not increase in emission. This discrimination between bound/unbound molecules (e.g. fluorescent labeled antibodies) allows detection in the presence of the bulk solution, thus eliminating the need for washing steps. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

different aspects of MEF.

So far industrial application of MEF in life sciences was hindered by issues like stability of the metal structures in aqueous solution, reproducibility of the metallic nano-structures or insufficient fluorescence enhancement.

The MEF-structures used for the experiments in this paper were developed in cooperation with our partner STRATEC Consumables GmbH (former Sony DADC BioSciences GmbH) using proprietary manufacturing techniques derived from Blu-Ray production.

Briefly the manufacturing process can be described as a standard injection molding process but instead of creating macroscopic structures, micro-to nano-sized patterns are generated (Fig. 2).

The realized nanostructures consist of pit-like structure arranged in periodic hexagonal array. The pit have diameters below 500 nm, an aspect ratio of about 2, and a spacing of several hundred nanometers. The master was manufactured by laser lithography, copied into a Nickel - stamp by electroforming, and replicated in a disc format by a variothermal injection compression process. The process is similar to the production optical disc (CD, DVD or BD). . As injection material a cyclic olefin polymer was used because of its a high transparency and its low level of background fluorescence in the visible region.

Subsequently, the polymer substrates were coated with a thin homogeneous Ag layer by a site directed DC sputtering process to form metal coated spots, that will become the bottom of microplates. After milling out the MEF-structures in microscope slide format, they are laser-welded to a 96 well standard microplate frame thus forming the bottom of the microplate (Fig. 3).

The determining factors in terms of the order of enhancement (up to 300 fold) have been the distance (period) of the pit-like structures, the thickness of the silver layer and the type of fluorescent dye used [7].

The MEF-MTP format allowed fluorescence measurements to be easily performed by using a standard microplate reader (TECAN F200 pro) equipped with suitable fluorescence filters and a Xenon flash lamp by simply adding the fluorophore containing solutions under investigation to the wells of the plate (MEF-MTP). Measurements were done either in bottom (excitation and emission detection from below the plate) or top configuration (excitation and emission detection from above the plate) which are built in features of the reader used. To fully exploit bulk fluorescence suppression by MEF, bottom measurements were applied during assays without washing. If assays were washed, the measurement mode (bottom/top) had no impact on results. Standard measurement parameters were 25 flashes per well of the Xenon lamp with an integration time of 20 µs. Gain (setting 1–255) of the built in photomultiplier tube was set to achieve at least 10000 fluorescence units (F.U.) between the lowest and highest result. For FITC, CY3, CY5 and AlexaFluor680 detection, filters with (Ex/Em) 485/535 nm, 535/590 nm, 635/680 nm and 670/720 nm having a bandwidth of 20 nm were used. The obtained enhancement of a specific assay was assessed by performing the identical assay on standard microplates from Greiner BioOne (Immuno-Strips, F-Form, 1 × 8, High Binding, VWR 737-0195) or on the MEF-MTP plates without silver coating.

Adsorptive binding of fluorescence labeled antibodies to MEF-MTPs to assess basic system properties

Antibodies against rabbit IgG raised in goat and labeled with FITC, Cy3, Cy5 and AlexaFluor680 were purchased from Thermo Fisher Scientific, diluted in buffered solutions and incubated on MEF-MTPs, Greiner ImmunoStrips HB and MEF-MTPs without silver coatings. Binding of the antibodies was monitored in bottom configuration for a period of 2 h. Then plates were washed 3 times with 250 µl sodium phosphate buffer (50mM/150 mM NaCl) pH 7.0 containing 0.1% (v/v) Triton X100 (Sigma-Aldrich, T9284) and measured again in bottom and top configuration.

Chromogenic detection of adsorbed rabbit IgG to determine coating efficiency

In order to compare the antibody coating efficiency of the developed MEF-surfaces to standard microplates from Greiner (see 2.3.) and Costar (1 × 8 Strip Well High Binding Plates, Sigma-Aldrich, CLS2592),

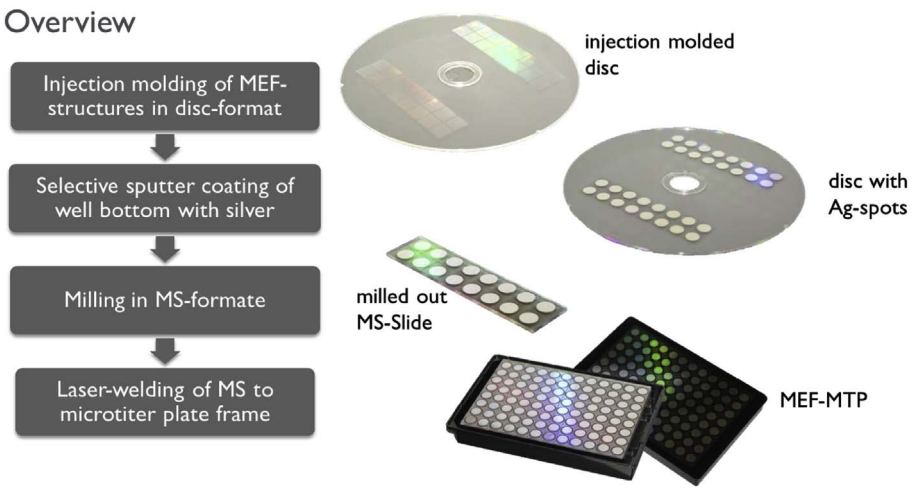

Fig. 2. Manufacturing process of MEF –MTPs.

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