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Layer-by-Layer Coated Digitally Encoded Microcarriers for Quantification of Proteins in Serum and Plasma

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The “layer-by-layer” (LbL) technology has been widely investigated for the coating of flat substrates and capsules with polyelectrolytes. In this study, LbL polyelectrolyte coatings applied at the surface of digitally encoded microcarriers were evaluated for the quantitative, sensitive, and simultaneous detection of proteins in complex biological samples like serum, plasma, and blood. LbL coated microcarriers were therefore coupled to capture antibodies, which were used as capture agents for the detection of tumor necrosis factor (TNF- α), P24, and follicle stimulating hormone (FSH). It was found that the LbL coatings did not disassemble upon incubating the microcarriers in serum and plasma. Also, nonspecific binding of target analytes to the LbL coating was not observed. We showed that the LbL coated microcarriers can reproducibly detect TNF- α , P24, and FSH down to the picogram per milliliter level, not only in buffer but also in serum and plasma samples. Microcarrier-to-microcarrier intratube variations were less than 30%, and inter-assay variations less than 8% were observed. This paper also shows evidence that the LbL coated digitally encoded microcarriers are ideally suited for assaying proteins in “whole” blood in microfluidic chips, which are of high interest for “point-of-care” diagnostics.

Immunoassays, like radioimmunoassays, immunoprecipitation assays, and enzyme-linked immunosorbent assays (ELISAs), are routinely used in medical diagnostics. ELISAs can be considered as the golden immunoassay assay to quantify soluble analytes (antigens) in human samples. As more and more protein disease markers are discovered, there is a growing need to analyze more types of (diagnostic) proteins. ELISAs, however, are not convenient to answer this growing need because (a) each protein marker has to be analyzed individually, (b) there is a high consumption of reagents and biological samples, and (c) it is a labor-intensive and time-consuming technique. Therefore, fast, inexpensive, accurate immunoassays with increased sensitivity using smaller sample volumes are under development.

Over the past decade, “multiplexing” immunoassays were developed.¹ While a “monoplex” immunoassay aims to measure the binding of *one* analyte (e.g., an antigen), present in the biological sample, to its receptor (e.g., an antibody), a multiplexing immunoassay aims to measure simultaneously the binding of *several* analytes in the biological sample to their respective receptors. This multiplex approach allows faster analysis of a high number of protein markers, and both the sample and reagent consumption are considerably reduced.

Multiplex immunoassay technologies are divided into, respectively, “flat surface arrays” and “suspension arrays”. To the first category belong the protein microarrays, which use the *x,y*-coordinates of the spots of capture probes (antibodies) on a glass plate to identify which targets (antigens) are present in a sample.^{2–4} Like DNA microarrays, protein microarrays, however, cope with localization problems of the capture antibodies upon miniaturization and slow reaction kinetics (as the diffusion of the antigens in the sample to the capture antibodies is time-consuming).^{5–7} The use of protein microarrays has also been limited by the high cost of both the microarray consumables and the instruments. Suspension arrays may have a number of advantages compared to the flat microarrays regarding, for instance, the reproducibility of the attachment of probes, the flexibility in surface chemistry, the flexibility in panel of tests, and improved kinetics.^{5,8,9} Suspension arrays use encoded micrometer-sized particles for multiplexing; the code allows knowing which capture antibody is bound to the surface of the microcarriers.^{8,10,11}

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Antigens present in the biological sample will bind to their corresponding microcarriers, which are added to the sample. Fluorescent labeling of the bound antigens can be obtained in different ways, e.g., “directly” by fluorescently labeled detection antibodies or “indirectly” by using fluorescently labeled or enzyme-labeled reporter molecules that bind to the detection antibodies.^{12–14} Decoding of the “positive” microcarriers (i.e., those microcarriers which show fluorescently labeled antigens at their surface) subsequently allows knowing which antigens are present in the sample. The microcarrier-based platforms are gaining popularity because they can detect antigens as sensitive and as reproducible as the traditional ELISAs.^{15–20} Current applications of microcarrier-based assays include detection of immunoglobulins,²¹ and cytokines,^{16,18–20,22,23} the analysis of single nucleotide polymorphisms,²⁴ DNA methylation profiling,²⁵ and gene expression.²⁶

Our group introduced the encoding of fluorescent polystyrene microspheres (of about 40 μm in size) with a digital barcode by means of “spatial selective photobleaching” (Figure 2B and C).²⁷ The thus encoded microspheres were called “memobeads”. To optimize the surface characteristics of memobeads, we recently proposed to coat their surface with polyelectrolytes by the “layer-by-layer” (LbL) approach.²⁸ As shown in Figure 1, LbL coating is based on the alternate adsorption of oppositely charged polyelectrolytes onto a charged substrate.^{29–32} The LbL coating of the surface of the memobeads was proven to be “multifunctional” in the sense that it (a) allows positioning of the memobeads for decoding, (b) does not optically interfere with the encoding and reading process, and (c) allows a high loading of the surface of

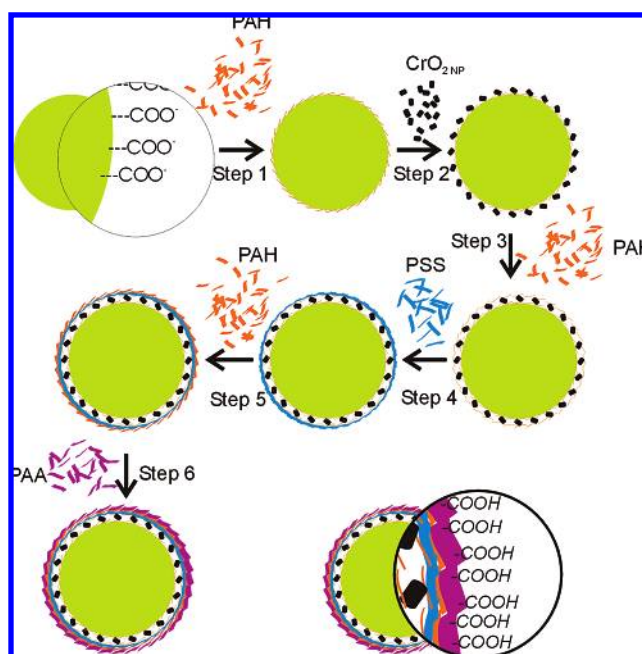


Figure 1. Schematic representation of the LbL coating of microcarriers. Oppositely charged polyelectrolytes are sequentially adsorbed on the negatively charged polystyrene microspheres (PAH, poly(allylamine hydrochloride); PSS, poly(styrenesulfonate); PAA, poly(acrylic acid)). Ferromagnetic chromium dioxide nanoparticles (CrO_2 NP, <450 nm) are added in between two PAH layers.

the microparticles with capture probes (like proteins and DNA molecules).²⁸

A major aim of the encoded memobeads is to use them in protein multiplexing. To our knowledge, it has never been evaluated whether LbL coatings are suitable to bind capture probes (antibodies) in such a way that they allow quantitative protein (antigen) analysis. Therefore, in this paper, we investigate whether LbL coated memobeads allow sensitive and accurate detection of proteins, not only in buffer but also in complex biological samples like serum and plasma. Clearly, for this purpose, the LbL coating should remain stable in the serum/plasma, which contains many types of charged compounds that may interfere with the LbL coating. Also, nonspecific binding to the LbL coating should be avoided. In addition, we investigate whether the LbL coated memobeads allow performing protein multiplexing in “whole” blood without “washing” (separating) the memobeads from the blood at the time of readout of the microcarriers. “Whole blood analysis” is a challenging objective in the field of diagnostics.

EXPERIMENTAL SECTION

Materials. Nonmagnetic fluorescent carboxylated microspheres (CFP-40052-100, i.d. 39 μm) were purchased from Sphero-tech (Libertyville, IL). Poly(allylamine hydrochloride) (PAH; 28,322-3), sodium poly(styrenesulfonate) (PSS; MW ~ 70 000; 24-305-1), and poly(acrylic acid) (PAA; MW ~ 45 000; 18,128-5) were obtained from Sigma Aldrich (Steinheim, Germany). The polymers were dissolved into 0.5 M sodium chloride (31434, Sigma Aldrich, Seelze, Germany). Bovine serum albumin (BSA; A-7906) and 2-[N-morpholino]ethanesulfonic acid (MES; M-8259) were purchased from Sigma (Bornem, Belgium), PBS Dulbecco’s (14190-094) was

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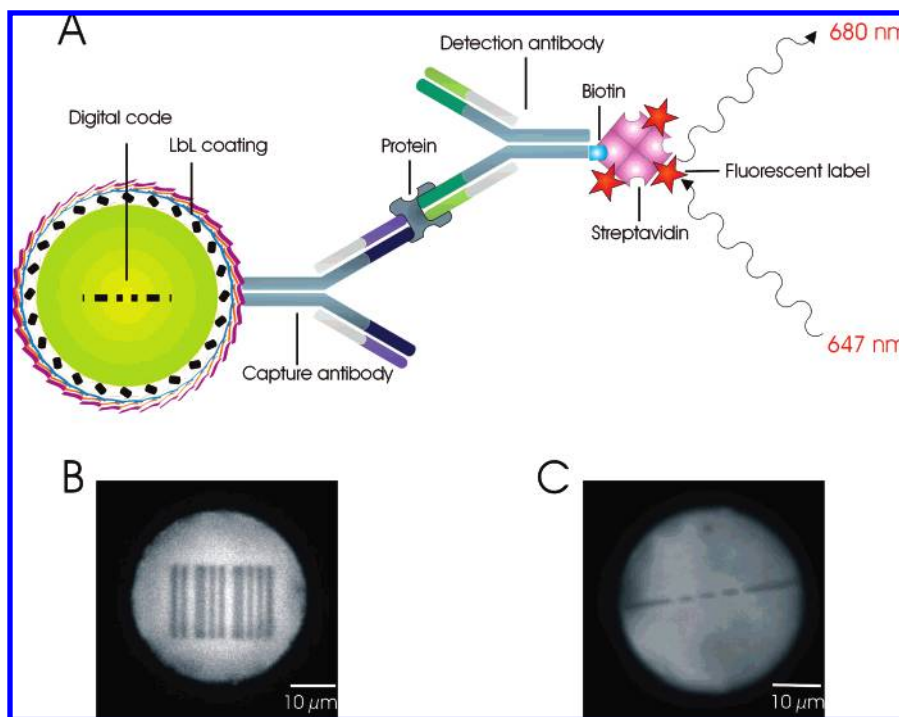


Figure 2. (A) Schematic representation of the detection of proteins with LbL coated memobeads. Proteins (antigens) are captured from the medium by means of capture antibodies bound to the surface of the encoded microcarriers. To detect the bound proteins, biotinylated detection antibodies are used. The detection antibodies are labeled with AlexaFluor 647 conjugated streptavidin. (B) Example of a 39- μm -sized microcarrier digitally encoded with a bar code. (C) Example of a 39- μm -sized microcarrier digitally encoded with a dot code.

from Gibco, and Tween-20 was (655204) from Calbiochem. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC; 22980) was obtained from Perbio Science (Erembodegem, Belgium), desiccated, and stored at $-20\text{ }^{\circ}\text{C}$. *N*-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS; 106627-54-7) was purchased from Sigma Aldrich (Steinheim, Germany), desiccated, and stored at $4\text{ }^{\circ}\text{C}$. Purified anti-human TNF α (551220), recombinant human TNF α (551838), and biotinylated mouse anti-human TNF α (554511) were purchased from BD Pharmingen (Erembodegem, Belgium). AlexaFluor 647-labeled streptavidin (S21374) was purchased from Molecular Probes (Inc. Invitrogen, Eugene, OR). Rat follicle stimulating hormone (FSH), purified anti-rat FSH antibody, biotinylated anti-rat FSH antibody, blanc and unknown serum samples, and ELISA buffer were a gift from Biocode-Hycl (Luik, Belgium), P24, purified anti-P24 antibody, biotinylated anti-P24 antibody, and blanc plasma samples were a gift from BioMaric (Ghent, Belgium).

Layer-by-Layer Coating of the Microspheres. One milliliter of the (stock) suspension of (green fluorescent, carboxylated, 39 μm) nonmagnetic microspheres ($\pm 400\,000$ microspheres/mL) was centrifuged at 450 rpm for 1 min. The supernatant was aspirated, and the microspheres were washed with a 0.05% Tween-20 solution (in deionized water). After resuspension of the microspheres, the centrifugation and washing procedure was repeated.

As illustrated in Figure 1, the microspheres were LbL coated by suspending them in 1 mL (2 mg/mL) of PAH solution (prepared in 0.5 M NaCl); the suspension was continuously vortexed (1000 rpm, $25\text{ }^{\circ}\text{C}$) for 15 min. The nonadsorbed PAH was removed by repeated centrifugation and washing. Subsequently, the microspheres were dispersed in deionized water

containing sub-500-nm ferromagnetic chromium dioxide nanoparticles (CrO_2 NP; The CrO_2 NP were used to position the microspheres in a magnetic field to allow reading of the code, as described in detail elsewhere.^{27,28} The nanoparticles were obtained by filtration through a 0.45- μm -pore filter. The size of the CrO_2 NP was measured with a Zetasizer Nano ZS (Malvern, Worcestershire, UK). The microsphere dispersion was continuously shaken for 15 min, and the excess of CrO_2 NP was removed by repeated centrifugation/washing steps. The third and fourth polyelectrolyte layers were applied in a similar way as the first layer. A 2 mg/mL PSS and a 1 mg/mL PAA solution (both in 0.5 M NaCl) were used. Finally, the microspheres became coated with 6 layers in the following order: PAH/sub-500-nm CrO_2 NP/PAH/PSS/PAH/PAA. The thus obtained LbL coated microspheres were resuspended in 1 mL of ultrapure demineralized water ($\pm 400\,000$ microspheres/mL) and subsequently encoded (see below).

Encoding of the Microspheres. The LbL coated microspheres were encoded by spatial selective photobleaching as previously described.²⁷ An in-house-developed encoding device was used, being a microscopy platform equipped with an Aerotech ALS3600 scanning stage, a SpectraPhysics 2060 argon laser, and an Acousto-Optic Modulator (AA.MQ/A0.5-VIS, A.A-Opto-Electronique, Orsay Cedex, France). The encoding process consists of two steps, a writing step (i.e., the photobleaching process) and a magnetizing step, during which the CrO_2 loaded microspheres are exposed to an external magnetic field sufficient to provide them with a magnetic memory. The microspheres were fixed on a grid during the encoding process to avoid rotation between the two steps. An example of an LbL coated microsphere encoded with a bar code and a dot code is respectively shown in Figure 2B and C.

Coupling of Capture Antibodies to the LbL Coated Microspheres. "Capture" antibodies were covalently attached to the (PAA) carboxyl groups at the surface of the microspheres by the two-step carbodiimide method.³³ In brief, ~10 000 microspheres (in 80 μ L of "activation buffer": 0.1 M Na₂HPO₄/NaH₂PO₄, 0.05% Tween-20, pH 6.3) were activated with 10 μ L of EDC (50 mg/mL); at the same time, the active intermediate was stabilized with 10 μ L of sulfo-NHS (50 mg/mL). Note that the storage and handling of EDC has to be done under proper conditions.³⁴ The microspheres were then washed twice with 0.05 M MES buffer (0.05% Tween-20, pH 5) and centrifuged (4000 rpm, 30 s). Subsequently, the antibodies were coupled by incubating the microspheres in 30 μ L of antibody solution (83 μ g/mL) for 2 h in an Eppendorf Thermomixer (250 rpm). Finally, the microspheres were washed twice with "assay buffer" (1% BSA and 0.05% Tween-20 in PBS) to avoid nonspecific binding to unreacted coupling places later on ("blocking step"). The microspheres were stored in 200 μ L of "assay buffer" (\pm 50 000 microspheres/mL) at 4 °C.

As described above, the microspheres were incubated in a 83 μ g/mL antibody solution. This seemed to be the optimal antibody concentration as a higher antibody concentration did not result in a higher loading of the microspheres with antibodies (as observed from measurements using AlexaFluor 647 labeled antibodies; data not shown). All of the coupling reactions described above were performed at room temperature.

Assay Procedure. As described below in detail, proteins (antigens) were captured on the LbL coated microspheres carrying the capture antibodies. The bound proteins were detected by means of biotinylated detection antibodies and AlexaFluor 647 (AF647) conjugated streptavidin, as illustrated in Figure 2A. The concentration of biotinylated detection antibody and AF647 conjugated streptavidin used for detecting the bound proteins was optimized (data not shown): lower concentrations resulted in a decrease of the fluorescence intensity, while higher concentrations did not further increase the signal. Note that incubations with AF647 conjugated streptavidin were performed protected from light. All of the following steps were performed at room temperature.

For the detection of TNF- α and P24 in a monoplex assay, ~100 microspheres, coated with capture antibody, were incubated for 1 h in 100 μ L of standard dilutions spiked with, respectively, TNF- α and P24 (Eppendorf Thermomixer, 250 rpm, 25 °C). The dilutions were made in, respectively, assay buffer, serum, or a plasma/buffer mixture. After 1-h incubation, the microspheres were washed with assay buffer. As illustrated in Figure 2, the microspheres were subsequently incubated for 1 h in 100 μ L of a biotinylated detection antibody solution (8 μ g/mL in assay buffer). Finally, the microspheres were washed three times with assay buffer. The labeling of the bound detection antibodies with AF647 conjugated streptavidin occurred as described below.

For the simultaneous detection of TNF- α and P24 in the duplex assay, ~300 (encoded) microspheres were used; one-third of the microspheres (bearing a dot code encoding for TNF- α) was coated with capture antibody against TNF- α , one-third (bearing a dot code encoding for P24) with capture antibody against P24, and one-

third of the microspheres (bearing a dot code encoding for a control) did not bear antibodies ("control microspheres"). The rest of the procedure occurred as described above.

For the detection of FSH, ~100 microspheres, coated with capture antibodies, were incubated for 2 h in, respectively, 25 μ L of standard dilutions spiked with FSH (in ELISA buffer) or 25 μ L of unknown serum samples. A total of 100 μ L of biotinylated detection antibodies (8 μ g/mL in assay buffer) was added. The microspheres were subsequently washed three times with assay buffer.

Labeling of the bound detection antibodies was always done with AF647 conjugated streptavidin. The microspheres were incubated for 1 h in the dark in 100 μ L of AF647 conjugated streptavidin solution (8 μ g/mL). Next, the microspheres were washed three times with 100 μ L of PBS.

Calibration Curves. Lyophilized TNF- α , P24, and FSH were reconstituted in, respectively, assay buffer, ELISA buffer, and serum or plasma. For each protein, 6–9 serial dilutions were made.

The calibration curves were calculated by the GraphPad Prism software. The 4-parameter logistic eq (4-PL) was used to obtain the most accurate determination of the protein concentration:³⁵

$$y = a + \frac{b - a}{1 + 10^{[(d-x)*c]}}$$

where x is the logarithmic of the protein concentration, y is the response (i.e., the red fluorescence at the surface of the microcarriers), a is the estimated response at zero protein concentration (i.e., the "zero calibrator"), b is the estimated response at infinite protein concentration, c is the slope of the tangent midpoint, and d is the logarithmic of the midrange protein concentration.

The R^2 value of the calibration curves together with back-calculations of the standards were used to measure the "goodness of fit". The latter is done by calculating the concentration of each standard and then comparing it to the expected concentration using the following formula: calculated concentration/expected concentration * 100.

The lower limit of detection (LOD) of a protein was defined as the concentration corresponding to the median fluorescence intensity plus 3 times the standard deviation of the "zero calibrator". The interassay variability was defined as the coefficient of variation (CV; in %) on the average value of repeated measurements on identical samples performed at different days.

Microscopy on the Microspheres. The LbL coated encoded microspheres were decoded using a pseudoconfocal Perkin-Elmer CSU-10 scanning unit mounted to an inverted Zeiss microscope equipped with an objective 40 \times lens (NA 0.6) and a cooled Hamamatsu ORCA-ER charge coupled device (CCD) camera. Excitation occurred with laser light (488 nm). Imaging Technology PC-DIG framegrabber boards took care of image transfer from the CCD camera to the computer.

The (red) AlexaFluor 647 fluorescence at the surface of the microcarriers was measured with the same instrument, but excitation occurred with a halogen lamp. A 647-nm LP filter was put in front of the lamp to obtain the optimal wavelength. To

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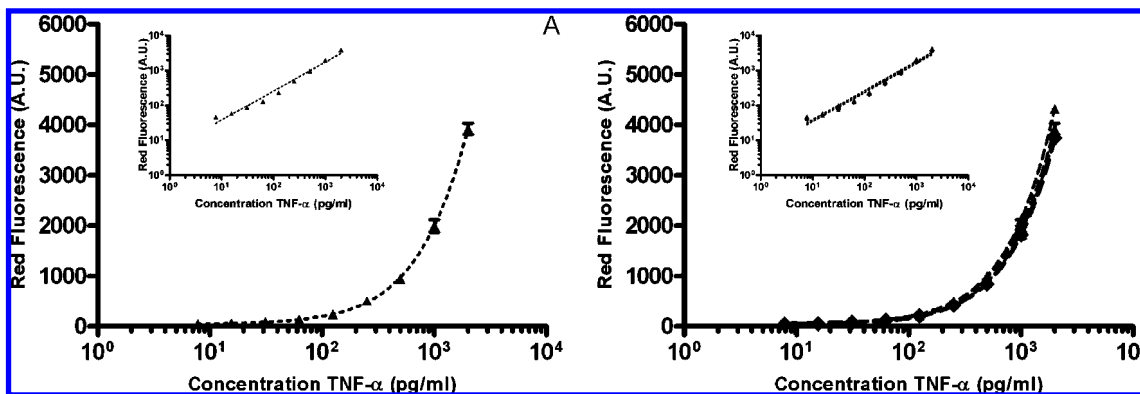


Figure 3. Quantitative analysis of TNF- α spiked in buffer. (A) Red fluorescence at the surface of the LbL coated microcarriers as a function of the TNF- α concentration (AU, arbitrary units). Each data point is the average value of the red (surface) fluorescence of ~ 20 LbL coated microcarriers (CVs varied between 4 and 26%). A nonlinear four-parameter plot accurately fits the data. The lower LOD equalled 10 pg/mL. (B) The red fluorescence of the microspheres was measured respectively immediately (\blacktriangle), 5 (\blacklozenge), 10 (\blacksquare), and 20 days (\bullet) after the assay. R^2 values of the nonlinear four-parameter plots range from 0.97 to 0.99 and standard recovery was between 70 and 130% for all standards. Inset: representation on a log–log plot. F -tests on the slopes ($p = 0.9992$) and Y -intercepts ($p = 0.549$) of the 4 fitted linear curves yielded no significant differences between the curves measured at the different time points. The pooled slope equalled 0.90, and the pooled Y -intercept equalled 3.72.

determine the red fluorescence of a microcarrier, a region of interest (ROI) was drawn around the microcarrier and the red fluorescence within the ROI was measured using the Matlab 7.1 version equipped with homemade imaging processing software. The red fluorescence of each microsphere was defined as the mean of the intensity of all pixels within the ROI.

RESULTS

Detection of TNF- α in Buffer by LbL Coated Microcarriers. LbL coated microspheres, carrying TNF- α (capture) antibodies, were incubated in different TNF- α standard solutions, prepared in buffer. Following the procedure as depicted in Figure 2, they were further incubated with biotinylated detection antibodies and AlexaFluor 647 conjugated streptavidin. This is the preferred detection method in bead-based assays as (a) biotinylation of antibodies is well-known chemistry,^{36,37} (b) approximately three dyes per streptavidin molecule are present, which results in a (small) amplification of the signal, compared to the use of fluorochrome conjugated antibodies, and (c) conjugates with streptavidin give much lower background than those with NeutrAvidin–avidin.³⁸

As Figure 3A shows, the red fluorescence at the surface of the LbL coated microspheres is proportional to the TNF- α concentration in the sample. A nonlinear four-parameter logistic plot was proven to accurately fit the values. Other immunodetection methods reveal the same type of trend in the relation between the signal and the antigen concentration.³⁹ The inset in Figure 3A shows a linear fitting applied on the data.

In Figure 3A, the red fluorescence at the surface of the microspheres was measured immediately after the assay. To test the stability of the LbL coating and the “capture antibody/TNF- α /detection antibody/AlexaFluor 647 conjugated streptavidin” construct at the surface of the microcarriers, the red fluorescence of the microcarriers was also measured at 5, 10, and 20 days after

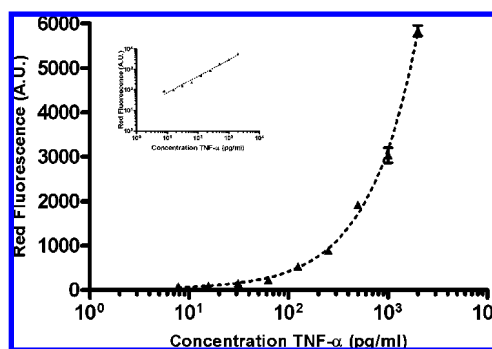


Figure 4. Quantitative analysis of TNF- α spiked in serum. Each data point is the average value of the red (surface) fluorescence of 4–13 LbL coated microcarriers (CVs between 6 and 28%). A nonlinear four-parameter plot accurately fits the data ($R^2 = 0.98$ and standard recovery was between 77 and 117% for all standards). The LOD equalled 16 pg/mL. Inset: representation on a log–log plot.

the assay (instead of immediate readout as in Figure 3A). Figure 3B shows that the microcarriers keep their red fluorescence, indirectly indicating that the antibody–antigen construct remains stable at the surface of the microcarriers for at least 20 days. It also indicates that the LbL coating does not disassemble within this time interval, which is not surprising since LbL coatings have been reported to be very stable.⁴⁰

Detection of TNF- α in Serum and Plasma by LbL Coated Microcarriers. For diagnostic purposes, the sensitivity and stability of the LbL coated microcarriers in more complex media like serum and plasma is important. Figure 4 shows the outcome of TNF- α measurements by LbL coated microcarriers in not-diluted sera spiked with TNF- α . A nonlinear four-parameter logistic plot was again proven to accurately fit the values ($R^2 = 0.98$). As expected, a slightly lower sensitivity was observed than in buffer; the LOD in serum equalled 16 pg/mL compared to 10 pg/mL in buffer, because serum contents generally suppress the antibody–antigen interaction.⁴¹

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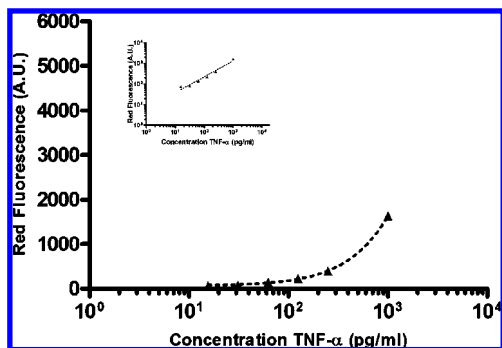


Figure 5. Quantitative analysis of TNF- α spiked in plasma/buffer matrix (50/50; v/v). Each data point is the average value of the red (surface) fluorescence of 9–16 microspheres (CVs between 7 and 29%); so there seems no significant difference in intra-tube variation whether the LbL coated microbeads are used in buffer, serum, or plasma. A nonlinear four-parameter logistic plots accurately fits the data ($R^2 = 0.98$ and standard recovery was between 70 and 130% for all standards). The LOD equalled 23 pg/mL. Inset: representation on a log–log plot.

It is well-known that during the separation of serum from blood one may lose some proteins of interest, which should obviously be avoided in the case of low-abundant proteins. It is therefore preferred to analyze plasma (or even “whole” blood) in stead of serum. Figure 5 shows the outcome of TNF- α measurements by LbL coated microcarriers in a plasma/buffer mixture (50/50; v/v) spiked with TNF- α . Clearly, compared to buffer and serum (respectively, Figures 3 and 4), in plasma the red fluorescence measured at the surface of the microcarriers is lower while the sensitivity is also not as high as in buffer and serum (the LOD equals 23 pg/mL). Indeed, plasma compounds may nonspecifically interfere with the antibody–antigen binding, a phenomenon that was also observed with other cytokines and in other bead-based assays.

As can be concluded from the low LOD values, there is almost no red fluorescence on the LbL coated microspheres (carrying TNF- α capture antibodies) incubated in the “zero calibrator”, indicating that biotinylated antibodies and AlexaFluor 647 labeled streptavidin do not bind aspecifically to the surface of the microcarriers. Also, control experiments in which microcarriers without capture antibodies were dispersed in TNF- α spiked in, respectively, buffer, serum, and plasma all showed the same neglectable red fluorescence after biotinylated TNF- α antibodies and AlexaFluor 647 labeled streptavidin were added (data not shown). It indicates that, even in serum and plasma, the LbL coating itself does not aspecifically bind detection antibodies or fluorescently labeled streptavidin molecules.

Detection of P24 in Plasma/Buffer Mixtures by LbL Coated Microcarriers. P24 is a major core protein of the human immunodeficiency virus encoded by the HIV gag gene. It is of interest to detect P24 at a very early stage of the infection in order to start drug therapy. P24 is currently analyzed by ELISA.⁴² Figure 6 shows the outcome of P24 measurements in plasma/buffer mixtures (50/50; v/v) spiked with P24. As was the case for TNF- α , the red fluorescence at the surface of the LbL coated microspheres is proportional to the P24 concentration in the sample. A

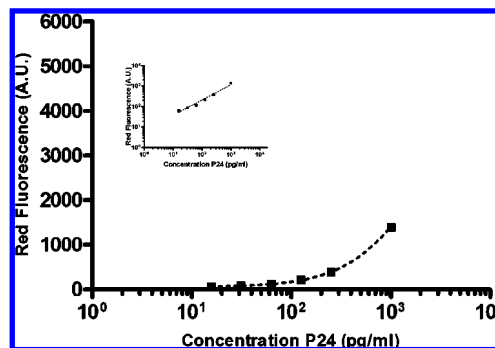


Figure 6. Quantitative analysis of P24 spiked in plasma/buffer matrix (50/50; v/v). Each data point is the average value of the red (surface) fluorescence of 11–14 microspheres (CVs between 12 and 29%); this means that the variation seems target independent. A nonlinear four-parameter logistic plot accurately fits the data (R^2 value = 0.95 and standard recovery was between 92 and 106% for all standards). The LOD equalled 34 pg/mL. Inset: representation on a log–log plot.

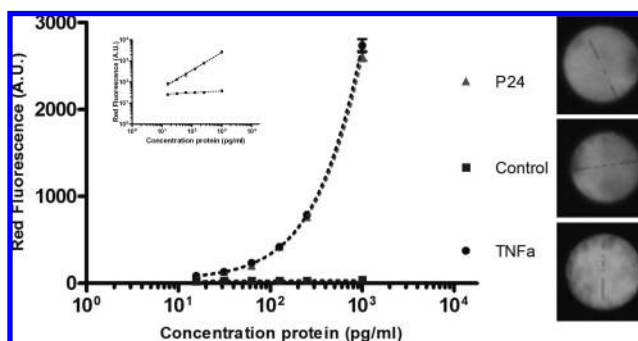


Figure 7. Quantitative duplex analysis of P24 (\blacktriangle , code 9) and TNF- α (\bullet , code 23) spiked in plasma/buffer matrix (25/75; v/v). Negative control microspheres without capture antibody were included in the test (\blacksquare , code 14). Each data point is the average value of the red (surface) fluorescence of 6–13 microspheres (CVs between 4 and 22%). A nonlinear four-parameter logistic plots accurately fit the data (R^2 value = 0.95 and standard recovery was between 92 and 106% for all standards). The LOD equalled 15 and 18 pg/mL for, respectively, P24 and TNF- α . Inset: representation on a log–log plot.

nonlinear four-parameter logistic plot was proven to accurately fit the values. The LOD equals 34 pg/mL.

TNF- α and P24 Multiplex Measurements in Plasma/Buffer Mixtures by LbL Coated Microcarriers. As described in the introduction, the purpose the LbL coated encoded microcarriers is to use them in multiplexing. We subsequently evaluated the potential of LbL coated microcarriers to measure simultaneously TNF- α and P24 in a plasma/buffer mixture (25/75; v/v). Therefore, three types of encoded LbL coated microcarriers were simultaneously dispersed in the TNF- α , P24 spiked plasma/buffer mixtures: to one type of microcarrier (with a “TNF- α dot code”; see inset in Figure 7), TNF- α antibodies were coupled, the second type of microcarriers (with a “P24 dot code”; see inset in Figure 7) was loaded with P24 antibodies, while the third type of microcarrier (with a “control dot code”) did not carry antibodies.

Figure 7 shows the outcome of the simultaneous measurement of TNF- α and P24 in spiked plasma/buffer (25/75; v/v) samples. As observed in the monoplex assays (Figure 5 and 6), a quantitative relationship is seen between the red fluorescence at the surface of the P24 and TNF- α microcarriers and the P24 and

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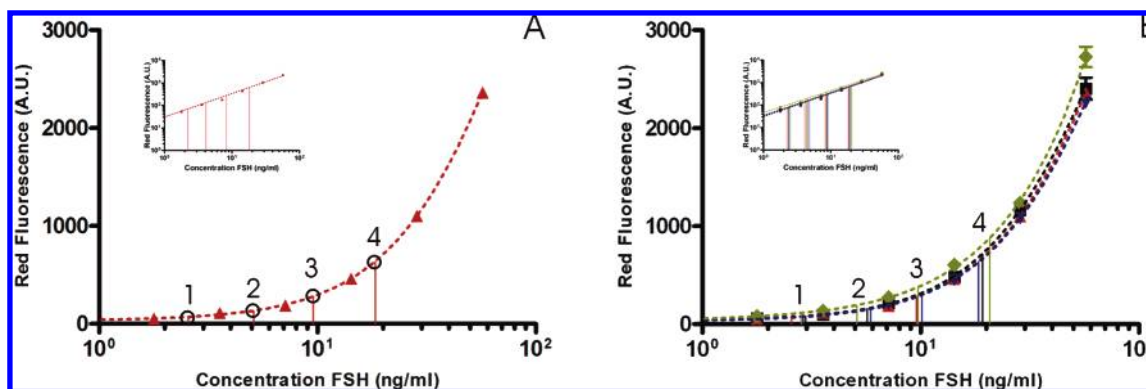


Figure 8. (A) Quantitative analysis of FSH spiked in ELISA buffer (▲) and determination of FSH concentration in 4 unknown samples (○) (extrapolation process: 4 vertical lines). (B) The same assay was repeated 4 times (▲, ■, ▼, ◆). Each data point is the average value of the red (surface) fluorescence of 15–34 microspheres (CVs between 8 and 23%). Four-parameter logistic plots accurately fit the data (R^2 between 0.9999 and 1 and standards recovery was between 92 and 120% for each standard). The LOD equaled between 0.9 and 1.5 ng/mL. Inset: representation on a log–log plot.

TNF- α concentration in the samples. The test is specific: there is no cross-reactivity between the different types of antibodies; otherwise one would never observe a quantitative relationship. The specificity was also confirmed under monoplex conditions; microcarriers bearing TNF- α capture antibodies showed neglectable red fluorescence after being dispersed in P24 spiked plasma samples, while microcarriers bearing P24 capture antibodies showed neglectable red fluorescence after being dispersed in TNF- α spiked plasma (results not shown). It seems that, for a given P24 and TNF- α concentration in the sample, the red fluorescence of the microcarriers in the duplex assay (Figure 7) exceeds the one of the microcarriers in the monoplex assay (Figures 5 and 6). This is probably due to less interference with plasma proteins as a 25/75 (v/v) plasma/buffer mixture was used in Figure 7 while a 50/50 (v/v) plasma/buffer mixture was used in Figures 5 and 6.

FSH Measurements in Clinical Serum Samples by LbL Coated Microcarriers. In the experiments above, the potential of LbL coated microcarriers was evaluated in buffer/serum/plasma samples, which were spiked with, respectively, P24 and TNF- α . We subsequently tested whether the LbL coated microcarriers can specifically and accurately measure a protein target (FSH) in (“real, unknown”) serum samples.

Typically, when quantitatively assaying an analyte in serum samples by ELISA, an internal calibration curve is first made by the use of sera spiked with (known) concentrations of the analyte of interest. The same procedure is applied in bead-based assays.¹⁸ The concentration of the analyte in the “unknown” sample is then extrapolated from the calibration curve. Figure 8A shows the FSH calibration curve and the extrapolation of the signal as measured in the four unknown samples (1–4 in Figure 8A). The whole assay was repeated four times to test its reproducibility. Figure 8B shows the four calibration curves and the four measurements on the four unknown samples. Clearly, the calibration curves do not differ significantly. The interassay variability for each of the unknown samples was very low (CV below 8%; see Table 1), which is comparable with the variability observed in other bead-based

Table 1. FSH Concentration in Four Different Mouse Serum Samples Measured with the LbL Coated Microcarriers (by the Extrapolation Process Visualized in Figure 8B)^a

sample	A	B	C	D	average (ng/mL)	SD	CV, %
1	3.0	2.6	2.9	2.8	2.8	0.2	7.1
2	5.7	5.1	5.9	5.1	5.4	0.4	7.4
3	10.1	9.6	10.1	9.7	9.9	0.3	3.0
4	19.1	18.4	18.3	20.8	19.2	1.1	5.7

^a Each sample was analyzed four times (A–D). SD is the standard deviation on the average concentration; CV (in %) is the coefficient of variation (i.e., the standard deviation divided by the mean).

assays.^{18,43,44} All together, Figure 8B indicates that the LbL coated microspheres can measure reproducibly the FSH concentrations in sera. Remark that only 25 μ L of serum was needed to obtain those reproducible results.

Influence of the Number of LbL Coated Microcarriers on the Sensitivity of the Analysis. It has been reported that the number of microcarriers used in a (bead-based) assay may profoundly affect the sensitivity and the dynamic range of the assay. This is explained by the fact that the proteins (antigens) present in the sample will be distributed over a larger surface in case a higher number of microcarriers is used, which lowers the average (red) surface fluorescence per microcarrier.

Figure 9 shows the red fluorescence at the surface of the LbL coated microcarriers dispersed in TNF- α solutions (in buffer); the number of LbL microcarriers in the assay was varied (between 100 and 4000). Clearly, for a given TNF- α concentration, the lower the number of beads used in the assay, the higher the red fluorescence at the surface of the LbL coated microcarriers. Thus, the number of LbL microcarriers used strongly determines both the sensitivity and the dynamic range of the assay. For example, with 1000 memobeads in the assay, a significant difference between 32 and 125 pg/mL cannot be detected anymore, while this remains possible when only 100 memobeads are used in the assay.

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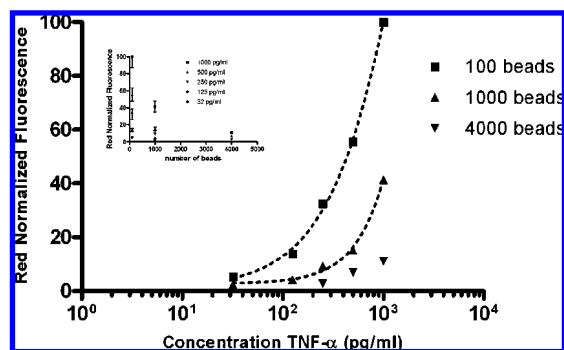


Figure 9. Influence of the number of LbL beads used in the assay on the red fluorescence at the surface of LbL coated memobeads incubated with different dilutions of TNF- α . Main graph: red fluorescence intensity as a function of the spiked concentrations of TNF- α ; 4000 (∇), 1000 (Δ), and 100 (\blacksquare) memobeads were used. In the case of 4000 memobeads, there are too few data points to fit a four-parameter logistic equation. Inset: fluorescence as a function of the number of memobeads for different TNF- α concentrations.

Potential Use of LbL Coated Microcarriers for Protein Analysis in “Whole” Blood. As outlined in the introduction, the encoded LbL coated microcarriers under investigation in this study can be easily decoded by means of a fluorescence microscope (equipped with a semiconfocal module). Therefore, for decoding, it is sufficient to put the microcarriers in a glass-bottomed recipient under a microscope; for this type of encoded microcarriers, microcapillaries are not needed to align the microcarriers to pass in front of the detector. Such microcapillaries (as in a flow cytometry apparatus) are used, for example, in the decoding of spectrometrically encoded microcarriers.¹⁴ As assaying in “whole” blood, instead of in serum or plasma, would be a clear advantage for different reasons, we subsequently studied whether the code in the LbL coated microcarriers and the red fluorescence at their surface could be detected with this easy readout system while the microcarriers remain in blood.

Figure 10 shows the outcome of a multiplex assay in whole blood spiked with TNF- α and IL-2 without separating (washing) the LbL coated microcarriers from the blood sample. The encoded microcarriers, the biotinylated detection antibodies, and the AlexaFluor 647 streptavidin were simultaneously incubated for 3 h in the blood samples spiked with TNF- α and IL-2. Note that in Figures 3–9, detection antibodies and AlexaFluor 647 conjugated streptavidin were added step by step while in Figure 10 an “all-in-one” procedure was used as the microcarriers, detection antibodies, and fluorescent streptavidin were simultaneously added to the blood sample. We observed the following. (a) The digital code of the microcarriers could be still accurately decoded while the microcarriers were in blood (bottom row in Figure 10); (b) It was still possible to measure the red fluorescence at the surface of the microcarriers (middle row in Figure 10). Thus, the blood cells and blood plasma did not hinder the decoding and the quantification process.

DISCUSSION

Bead-based assays become more and more attractive for the analysis of proteins because, compared to ELISAs, they are faster and less expensive and have a broader dynamic range, while they

have the same specificity and reproducibility.^{18,41,45} Also, much less sample is needed.^{19,46} This study shows that microcarriers layer-by-layer coated with polyelectrolytes, as shown in Figure 1, allow quantitative measurements of TNF- α , down to 10 pg/mL in buffer, 16 pg/mL in serum, and 23 pg/mL in a plasma/buffer mixture, which comes close to the LOD reported for commercially available humane cytokine bead-based assays that can detect concentrations down to a few picograms of cytokine per milliliter.^{16,47} One could wonder what the advantage of the layer-by-layer coating there is. The LbL technology is a flexible approach to modify surfaces; e.g., in this study, the LbL coating is bifunctional: the LbL coating incorporates magnetic nanoparticles (needed to properly orient the carriers for decoding) without the formation of aggregates while it also provides the surface of the carriers with a large number of carboxyl groups homogeneously spread over their surface, which improves the intratube variation. We proved that the LbL coated microcarriers can reproducibly measure other proteins like P24 and FSH in complex samples as well. The lower limit of detecting P24 in the plasma samples was 34 pg/mL which is certainly an acceptable value when compared with the commercially available fourth-generation P24 ELISA assays, which measure down to 10 pg/mL.⁴⁸ We observed interassay variation coefficients below 8%, which is comparable with other bead-based assays.^{18,43,44} Note that there remains room to further improve the sensitivity of the LbL coated beads, e.g., by making use of other antibody kits (it is well-known that both the LOD and the dynamic range of a multiplex bead assay is highly dependent on the quality of the capture and detection antibody).¹⁵ For diagnostic purposes, besides the sensitivity and the stability of the LbL coated microcarriers in serum and plasma is also important. However, the LbL coated microcarriers did not show nonspecific interactions with the serum/plasma proteins; no degradation of the LbL coating has been observed.

We showed that the number of LbL coated microspheres in the protein assay may affect profoundly the sensitivity. The same finding was observed by Kohara et al.,⁴⁹ who used microcarriers for DNA assaying, while other authors did not experience significant differences in sensitivity when changing the number of microcarriers.³⁸ The higher sensitivity when lowering the number of microcarriers is due to the fact that a higher number of antigens become coupled per microcarrier, which results in a stronger red fluorescence of the microcarrier. Some authors argue, however, that a sufficiently high number of microcarriers should be used to precisely determine the concentration of the antigen, especially in low antigen concentrations, and to shorten the confidence interval.⁵⁰ In our study, the red surface fluorescence of only some tens of microcarriers was measured per antigen concentration. Though, the interbead variation for beads incubated

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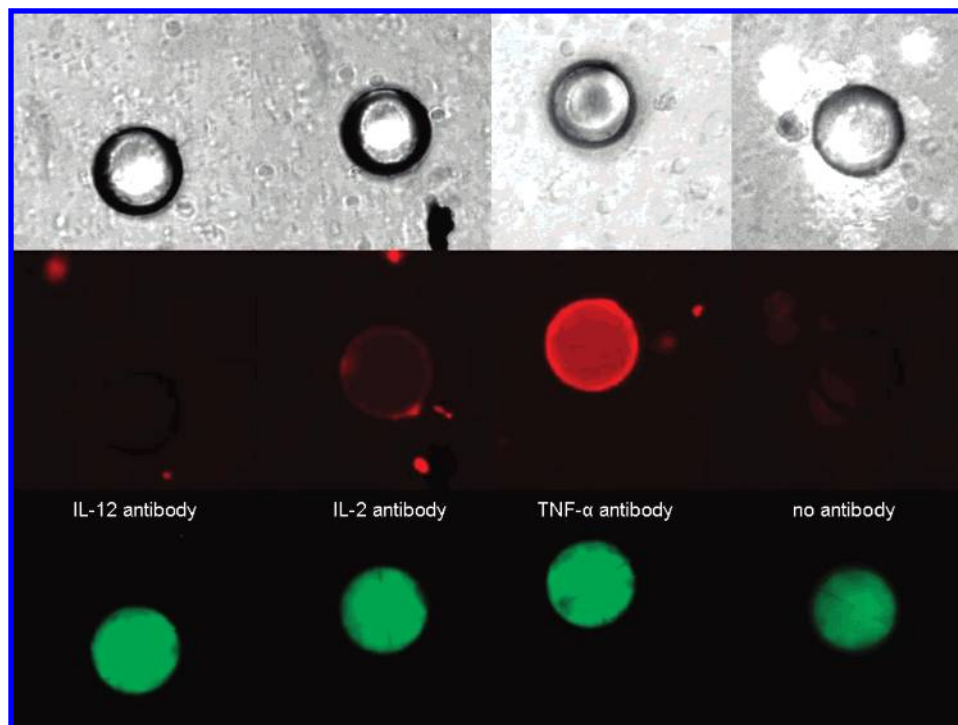


Figure 10. Multiplexing in “whole” blood using LbL coated (encoded) microcarriers. The analysis of the microcarriers occurred while the microcarriers remained in the blood (thus without prior wash steps). Differently encoded LbL coated microspheres were coupled to IL-12 capture antibodies, IL-2 capture antibodies, or TNF- α capture antibodies. A mixture of encoded TNF- α , IL-2, IL-12, and blanco microcarriers (i.e., microcarriers without capture antibody) was incubated during 3 h in a whole blood sample spiked with TNF- α and IL-2 (together with the biotinylated detection antibodies and the AF647 conjugated streptavidin). Top row: transmission image showing the microcarriers suspended between blood cells. Middle row: red fluorescence image of the microcarriers; only the surface of the TNF- α and IL-2 microcarriers turns red fluorescent as the samples were only spiked with TNF- α and IL-2. Bottom row: green fluorescence confocal image of the microcarriers, which reveals the code.

within the same well was acceptable (CV between 4 and 30%) and comparable to bead-based assays in which a higher number of beads was used.^{41,51}

We observed that the LbL coated microcarriers are stable for at least 20 days after the assay. This means that the red fluorescence of the surface of the microcarriers has not to be read out immediately after the assay, as long as the microcarriers are kept in the dark. We found before that the code in the microcarriers is very light stable when the memobeads are stored in daylight (this period is even longer when the beads are stored in the dark; unpublished results). Hence, the stability of the code is not the limiting factor. It has been reported that spectrometrically encoded beads (which are loaded with a mixture of fluorophores, which encodes the beads) lack stability: in this study, the decoding of such beads becomes problematic after 1-month storage at 4 °C after antibody coupling, although no other papers were found in the literature that confirm this observation.⁵¹ Furthermore, such beads should also be handled in the dark as bleaching of the fluorophores by prolonged exposure to light results in false codes.³⁸

The detection of antigens in “whole blood” instead of in serum and plasma is highly desired in the field of diagnostics. Indeed, assaying in whole blood would shorten the processing time and lower the costs, two main issues in diagnostic and research settings. Additionally, in whole blood, the assay occurs in a more

physiological environment as, during the preparation of serum and plasma, the antigen may be degraded or absorbed and even antigen cellular production is possible.¹⁵ This study (Figure 10) showed that the red fluorescence at the surface of the LbL coated microcarriers can be measured even when the beads remain in whole blood. Importantly, while in blood, the digital code in the beads can be perfectly read out; Although blood is significantly more viscous than water and blood cells are extensively present, the orientation of the LbL coated microcarriers in blood by applying a magnetic field (which is necessary for the decoding of the microcarriers) remains possible. We conclude that the digitally encoded LbL coated memobeads are good candidate materials to be used in near-patient testing, which aims rapid and simultaneous diagnosis of many antigens in whole blood of a patient while he is in the doctor’s office.

Importantly, the encoding of microcarriers by means of photobleaching provides them with a digitally accurate code. As the decoding of such microcarriers occurs with 100% certainty, it means that, theoretically, one microcarrier per antigen type in the assay would be sufficient. Other bead encoding technologies do not permit the use of a very small number of microcarriers as errors may occur in the decoding process, which necessitates a statistical analysis of the decoding result of a larger number of microcarriers to obtain the correct code. It means that, typically, at least 100 microcarriers per antigen type are used in the assay. Considering the fact that a low number of digitally encoded microcarriers per antigen in the assay is sufficient and considering

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the increase in sensitivity when performing assays with a very small number of microcarriers (see above), one may conclude that the LbL coated microcarriers described in this paper are well suited for assaying biological samples in microfluidic chips,⁵² which are loaded with a limited number of the encoded beads. Assaying in microfluidic chips may have a number of advantages. It is, for instance, well-known that (bio)chemical reactions occur much faster in a microfluidic environment.^{53–55} Those chips, however, often lack multiplexing capabilities. The use of the digitally encoded LbL coated microcarriers in microfluidic based devices could give new opportunities for point-of-care micro total analysis systems.

CONCLUSION

This study shows that LbL coatings, loaded with capture antibodies, at the surface of digitally encoded microcarriers allow the quantitative and sensitive detection of proteins, such as TNF- α , P24, and FSH, not only in buffer but also in complex media

like serum and plasma. When incubated in serum or plasma, the LbL coatings remain stable at the surface of the microcarriers while nonspecific binding of serum/plasma molecules to the capture antibody loaded LbLs was not observed. Importantly, we observed that (a) the digital code of the microcarriers could be still accurately decoded and (b) the red fluorescence at their surface could be quantified even when the microcarriers remained in “whole blood”. These properties make the LbL coated digitally encoded microcarriers investigated in this study ideally suited for the simultaneous (multiplexing) assaying of proteins in “whole” blood instead of in serum or plasma. We showed that using a lower number of LbL coated microcarriers in the protein assay even profoundly improves the sensitivity of the assay, an interesting feature when one wants to make use of the microcarriers for assaying in microchips that only allow using a rather low number of microcarriers. Based on the observations in this study, we suggest that the LbL coated digitally encoded microcarriers may be ideally suited for protein multiplexing in whole blood by microchips.

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