Sensors and Actuators B 190 (2014) 922–927

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

# Label-free protein detection using a microfluidic Coulter-counter device

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ARTICLE INFO

*Article history:*
Received 8 May 2013
Received in revised form 19 August 2013
Accepted 9 September 2013
Available online 18 September 2013

*Keywords:*
Label-free detection
Protein
Coulter-counter
Microfluidics
Impedance
Bead-based assay

ABSTRACT

A new method for measuring specific protein concentrations in solutions has been developed. The technique makes use of the Coulter effect for detecting and sizing of micro-scaled objects suspended in a buffer fluid. The method is completely label-free as it is only based on the electrical readout when a suspension of microscopic beads flows over a set of electrodes in a microfluidic device. Since no electrode functionalization is needed the same device can be used in a number of different assays. Using goat-anti-rat IgG functionalized polystyrene beads we have shown proof of principle detecting rat IgG in solution. When the analyte (rat IgG) is present oligomers of beads are formed. The electrical readout of the oligomers is different compared to a zero control sample with no rat IgG. Detection of the protein has been performed in a concentration as small as 14 ng/mL. The dynamic range of the system has been demonstrated to be relatively large, ranging from 1 μg/mL to 14 ng/mL. The microfluidic system is made from polymer and glass and very little volume of sample (<10 μL) is needed for analysis.


## 1. Introduction

Proteins are biological macromolecules and are an essential part of all living organisms. They play a crucial roll carrying out the duties specified by the information encoded in genes. Often diseases are associated with a change in concentration of a specific protein. This makes quantification of specific protein levels an important tool in the diagnostics of many human diseases [1].

Assays measuring protein concentration capture the desired protein from a sample and convert this event into a measurable signal. As capture mechanism the immunoassays utilize the specific binding affinity of proteins to antibodies. The dominant immunoassay detection technology is ELISA [2] (Enzyme Linked Immunosorbent Assay) and assays based on chemo-luminescence (e.g. Luminex). Although current commercial assays are highly sensitive and robust they often rely on optical detection, which requires the use of fluorescent labels and delicate optical instrumentation. These factors can limit the widespread availability of such tests with many assays only being available in dedicated diagnostics laboratories and other such centralized facilities. Therefore, for some cases a more cost-effective scheme may be desirable.

Label-free impedance-based methods have already been demonstrated for the detection of protein: micro-electrodes [3,4] and nanowire biosensors [5] are two examples. These methods use electrical rather than optical detection and as such gain from a significant reduction in instrument complexity. However, the electrical immunosensors reported take the format of having a detection antibody attached to a planar surface. This condition makes both the sensor more complicated to fabricate and at the same time limits the applicability of the device towards only one protein type.

A method for detecting protein based on the electrical sensing of functionalized beads in suspension is presented here. The principle of detection is based on the Coulter effect by which micro-particles suspended in a fluid are detected and sized by means of their disruption of an electrical current when transiting through a small orifice in between two reservoirs [6]. Since its invention in 1953, Coulter counters have been used for decades to count and size particles and cells in suspension. During the last few years, there has been a growing interest in developing micro-scaled particle detectors based in this electrical effect due to their potential application to cell detection and analysis [7–10] which could be implemented as low-cost alternative to flow cytometry instruments [11]. In this work, we have used this same principle of particle detection but with a different approach. Namely to study the effect triggered

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http://dx.doi.org/10.1016/j.snb.2013.09.038

PDMS-PDMS replica process

by the concentration of a given antigen in solution over a set of antibody-coated micro-sized polymer beads.

A simple microfluidic device made in Polydimethyl-Siloxane (PDMS) and covered by a glass slide patterned with gold microelectrodes has been produced. Two different sets of polystyrene microbeads coated with two different antibodies, specific to the same target protein, were used. The target protein is allowed to react with the functionalized beads. This process triggers the formation of bead clusters driven by the antibody-antigen interaction. The formed clusters are then introduced into our microdevice and conducted to a microchannel with the integrated electrodes while the output voltage is monitored.

detection limit

With this simple method, the presence of rat IgG diluted in PBS at a concentration down to 14 ng/mL has been detected. The main advantage of this device is that it does not need any labelling of the sample as it is the case for more traditional methods based on fluorescence. Also, compared to existents label-free impedance methods [3–5], no electrode functionalization strategy is needed thus making it simpler and reusable. Another clear advantage is the use of a micro-sized device, which enable us to work with very low volume samples below 10 μL thus greatly reducing the cost of sample and reagents used in the analysis.

## 2. Materials and methods

### 2.1. Chip design and fabrication

The microfluidic structure is designed with a gradually reduced width and height in order to properly focus the clusters towards the detection area and, at the same time, avoiding high shear stresses that would tend to break clusters apart. A schematic diagram of the structure of the microfluidic device is presented in Fig. 1. It is made of a poly (dimethyl-siloxane) (PDMS) slab containing the microfluidic network (blue) and a glass slide with deposited gold microelectrodes (orange). The detection scheme is conceived with electrodes perpendicular to the direction of flow to allow fast dynamic measurements of flowing particles. The inlets/outlets A, B and C in Fig. 1 have a width of 2 mm and a height of 0.5 mm while the small detection channel in the middle is only 100 μm wide and 30 μm high.

The micro-fluidic channel network on the surface of the PDMS block is made by a double casting method, described first by Gitlin et al. [12]. Briefly, we first milled the channel structure in poly (methyl-methacrylate) (PMMA) (*Nordplast*, *Denmark*) by using a milling machine (*Folken Industries*, *USA*). A layer of PDMS (*Slygard 184*, *Dow Corning*) is casted on top of it by pouring and subsequently baking it for 2 h at 60 °C. This layer of PDMS is then used as a mould for the actual chip in a PDMS-PDMS replica process. The master PDMS piece is treated 20 min in an HCl solution (pH = 3) with 0.1% w/w of hydroxypropylmethylcellulose (HPMC) (*Sigma Aldrich*) to make it hydrophilic and thus anti-sticking towards PDMS. Using the treated PDMS mould, we can now cast a new layer of PDMS and, after the curing process we can separate the two layers due to the surface treatment.

液体驱动模式

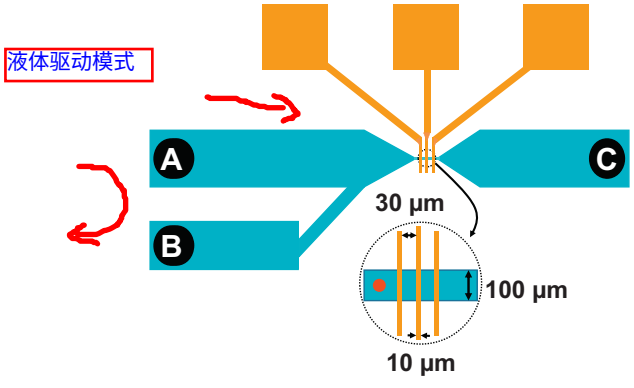


**Fig. 1.** Schematic of the design of the protein detection chamber. The sample is injected through inlet A to fill the volume between inlet A and outlet B. Outlet B is then closed, and a positive pressure is applied at inlet A pushing the sample through the narrow channel where the measurements take place. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The electrodes are microfabricated by standard photolithography on a pyrex wafer followed by gold evaporation and lift-off [13]. Briefly, AZ5214 photoresist is patterned followed by 10/100 nm Ti/Au thermal evaporation deposition. Subsequently, the photoresist is removed by lift-off to produce the desired electrodes structure. At the measurement channel, the electrodes are 10 μm wide and 30 μm separated. The electrode width of 10 μm was selected to be large enough to minimize the double layer impedance effects (which are inversely proportional to the electrode surface area). The inter-electrode distance (30 μm) was chosen to make the detection volume big enough to detect clusters of beads.

### 2.2. Sample preparation

The principle of cluster formation is schematized in Fig. 2. Bead oligomers (typically 2–10 beads per cluster) can be formed by mixing equal volumes of two different undiluted functionalized polystyrene micro-beads: (1) 7.4 μm, RtPXA-60-5 (*Spherotech*) coated with goat-anti-rat IgG (H&L), and (2) 6.7 μm, RtPFc-60-5 (*Spherotech*) coated with goat-anti-rat IgG (Fc) and adding diluted rat IgG serum (*R9759-5ML*, *Sigma*). To prevent unspecific bead interactions and unspecific protein binding rat serum is diluted in PBS-t, i.e. phosphate buffered saline solution containing 0.1% v/v of the surfactant Tween 20 (*Sigma*).

Samples are prepared by mixing 200 μL of different dilutions of IgG protein (approximately 100 μg/mL–20 ng/mL) with 50 μL bead solution 1 and 50 μL of bead solution 2. Thus, bead concentration is fixed to $10^6$ beads/mL for all the experiments and the IgG protein concentration varies. A negative control was also prepared consisting on mixing the same volumes of the two bead solutions and PBS-t. After mixing the beads and proteins were incubated at room temperature on a tilting table for 20 min. During this time, the beads

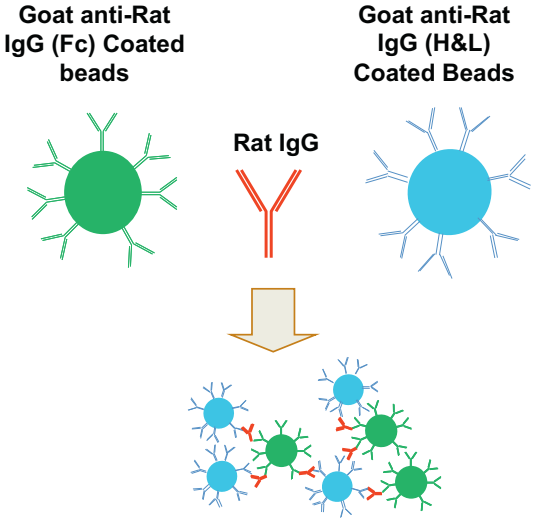


**Fig. 2.** Diagram of the bead oligomers formation. Mixing two sets of anti-rat coated polystyrene microbeads together with rat IgG (target protein) triggers the formation of bead clusters driven by the antibody-antigen affinity.

are able to react with the sample and form oligomers. Finally, 10 μL of the sample is injected into the microfluidic device for analysis.

### 2.3. Experimental set-up

In order to interface the chips with external fluidic and electrical connections, a custom-made holder was fabricated by micro-milling (*Folken Industries*, *USA*) in a 5 mm PMMA sheet (*Nordplast*, *Denmark*) and cut out in the dimensions of a microscope glass slide (75 mm × 25 mm). The microfluidic channel network and the glass electrode slide are aligned by eye and placed inside the holder. The holder is made of two different plates that sandwiches the chip and squeeze it with screws to make sealed connections. The top PMMA plate includes holes for inlets/outlets that fit with the corresponding connection ports in the PDMS, as well as electrical spring connectors (*RS*, *Denmark*) which fit with the electrode external pads. Teflon tubing (*BOLA*, *Germany*) is inserted to reach the chip reservoirs and make tight sealing within the PDMS bulk structure.

The chamber is first filled completely with PBS and subsequently the sample is injected through inlet A (Fig. 1) so that it is allowed to fill the volume between inlet A and outlet B. Due to the high differential fluidic resistance between the branches, only very little sample will go through the electrodes towards outlet C. Outlet B is then closed and inlet A is connected to an elevated water reservoir through Teflon tubing while outlet C is left open at atmospheric pressure [14]. This pushes the sample through the narrow channel where the electrodes are situated and the measurements take place. The typical time duration of the transitions is 0.05 s–0.1 s depending on the size of the particles and the height between the device and the reservoir.

It is important to note that, at the bead concentration used in the experiments (ca. $10^6$ beads/mL) there is 1 bead for each 1 nL ($10^{-6}$ mL) of sample volume. However, the detection volume (the volume considered between the three measurement electrodes) is only 0.27 nL, that is, almost 4 times smaller. In these conditions, the probability of having multiple events is very much reduced and is then neglected in this work.

A typical instrumentation scheme of a lock-in amplifier with a pre-amplifying step [15] was chosen for this system. Three electrodes are used to perform differential impedance measurements (Fig. 3). An AC signal of 1 V at 1 MHz is applied to the central electrode and the resultant currents from the lateral electrodes are pre-amplified (*HF2CA Current pre-amplifier*, *Zurich Instruments*), subtracted, demodulated and acquired using a lock-in amplifier (*HF2IS Impedance Spectroscope*, *Zurich Instruments*). The pre-amplification stage in this system is performed for each channel ($I_1$ and $I_2$) separately in mode single (not differential) using a shunt-resistor based amplifier with 2 channels (*HF2CA* in Fig. 3). The input impedance was set to 1 kΩ and the output gain to 1 for both channels of the current amplifier. The used lock-in amplifier (HF2IS in Fig. 3) worked with the following specifications: typical input noise: 5 nV/Hz$^{1/2}$; input offset amplitude (max): 20 mV; typical Common Mode Rejection Ratio (CMRR): 75 dB. The measurements were done using a bandwidth of 1 kHz and the acquisition with a sample rate of 14400 samples/s. Custom-made software has been developed using *MATLAB R2010a* to perform automatic data analysis and display the results.

The principle of particle detection can be explained in the following way. As shown in Fig. 3, particles flow from left to right in the microchannel over the electrodes. When a particle (or cluster of particles) transits through the detection region it creates a disequilibrium in the generated currents that is amplified and converted into a differential voltage. Indeed, while the detection volume is free of particles, the currents coming out from both branches (currents $I_1$ and $I_2$) have approximately the same values and the differential output approaches zero. When the particle is flowing between the first and second electrode, the impedance corresponding to this volume is increased and current $I_1$ is reduced so a positive peak is obtained at the output. Similarly, when the particle flows between electrodes 2 and 3, the differential output change its sign and a negative peak arises. A voltage offset is always present in the signal due to the fabrication process imperfections and small tolerances in the instrumentation. However, this voltage offset is automatically removed from the signal to facilitate analysis.

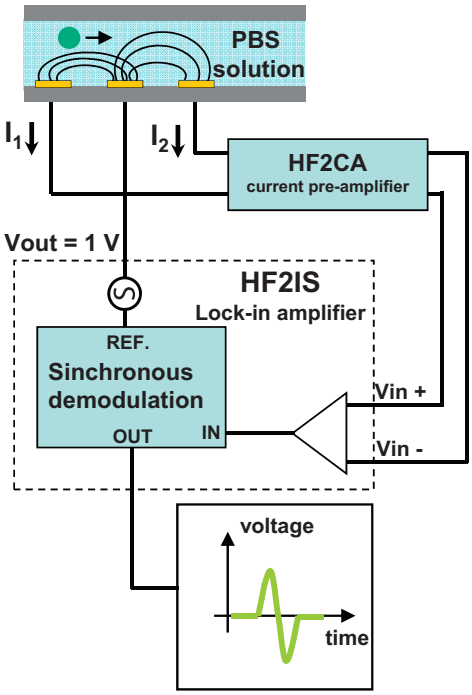


**Fig. 3.** The principle of differential particle detection based on impedance. Particles flow from left to right in the microchannel and over the electrodes giving rise to a characteristic differential signal.

## 3. Results and discussion

### 3.1. Protein detection system

In order to demonstrate the principle of detection, two different samples were prepared as described in Section 2.2 by diluting the protein to a concentration of approximately 0.7 μg/mL. A negative control (no protein present) was also prepared. After sample preparation, 10 μL are placed in a microscope slide for optical inspection. Microscope pictures of the corresponding samples are shown in Fig. 4a and b. A completely different appearance can be easily observed for the beads with and without the presence of the antigen. When the protein is not present (Fig. 4a), most of the particles are singles and only few of them form little clusters due to unspecific binding. However, when the solution contains the protein, the beads start to interact giving rise to cluster formation as is evidenced on Fig. 4b. Cluster formation was also optically monitored over time and it was observed to reach equilibrium after approximately 20 min of mixing. This was used as the period of incubation for all samples.

Right after sample preparation, electrical measurements were performed. Within a certain frequency range (~500 kHz–3 MHz) the electrode interface is expected not to have considerable effects in the dynamic of the system impedance. Under these conditions, the electrical response of the system is dominated by the bulk

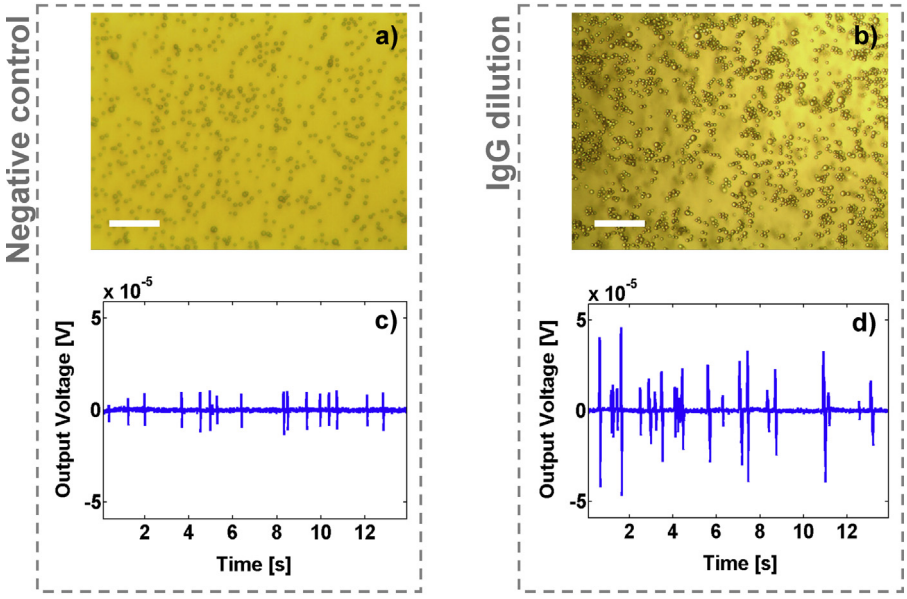


**Fig. 4.** Sample imaging and detection signals. When the protein is not present in the sample (Negative control), little or no cluster formation is observed in the sample (a). However, when the protein is added (IgG dilution), much more clusters are visibly distinguished (b). This condition is also clearly distinguished through the electronic transition peaks obtained for the negative control (c) and the protein dilution sample (d). Scale bars = 100 μm.

resistance of the liquid [16] and the amplitude of the transition peak will increase with the particle volume. As a consequence, the transition of a bead cluster should be distinguishable from that of a single bead based on the amplitude of the electrical transition. This can be clearly observed from the graphs in Fig. 4. A typical experimental signal obtained when single beads are transiting through the detection volume is shown in Fig. 4c. Similarly, Fig. 4d shows the corresponding output when (at least some of) the particles are bead clusters. The amplitudes for clusters transitions are roughly 4–5 times larger than the ones for single beads what correlates with the fact that the electrical readout depends on the particle volume and that the clusters are formed by a few number of beads.

We thus have a good principle of detection valid for measuring the presence of a given protein in solution. This system though, cannot be based only on the measurement of one or few transitions since, as indicated by the pictures in Fig. 4a and b, the negative control always contains some clusters and within the clusters in the protein solution there are always few single beads. In addition, there is also an intrinsic dispersion in the electrical readout associated with the fact of the electrodes being coplanar and thus the field inhomogeneous in the channel height [17].

However, the presence of clusters at a given proportion must give a different overall output and this can be certainly measured in a statistical fashion. Within the same experiments shown in Fig. 4, a total of 246 transitions were analyzed for each of the samples (negative control and protein dilution) using MATLAB. For each transition, the maximum peak value (voltage amplitude) is stored. The histograms in Fig. 5 summarize the results for the distribution of amplitudes of the electrical transitions.

Eye-guide curves have been drawn over the histograms so that the two distributions can be distinguished without any doubt. The statistical means of the distributions are 12.6 μV and 21.8 μV respectively for the control and the rat IgG experiments. This means that when the sample that contains protein is injected in the system, it give rise to transitions with amplitudes that are higher in average than in the case where the protein is not present (by a factor of 1.73). This result demonstrates the potential of our developed device to detect rat IgG protein in solution at a concentration of 0.7 μg/mL. This concentration is lower than the total concentration of IgG in most animal and human fluids (in the level of 1–10 mg/mL, [18,19]). In order to perform a clinical experiment we would have to dilute the serum around 1000 times prior to executing the assay.

### 3.2. Protein concentration measurements

In the event of a clinical experiment, it could be of interest to determine the concentration of the given protein in serum. In order for our system to do this, we have to demonstrate that we can differentiate different amounts of protein in solution. To do this, four dilutions were prepared for rat IgG having final concentrations of 70, 1.4, 0.14, 0.014 μg/mL and 0 μg/mL. A total population of approximately 26,000 particle transitions were detected using the developed system and analyzed with the software to extract the maximum value of each transition.

In order to analyze all the obtained data in a comprehensive manner, a figure of merit (FOM) representing the percentage of particle transitions with amplitude over a given threshold for all 5 different dilution experiments is presented in Fig. 6. Given that

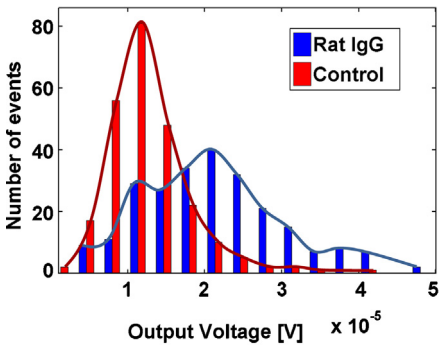


**Fig. 5.** Histograms of the maximum impedance peaks of electrical transitions of rat IgG sample dilution (blue bars) and a negative control (red bars). The means of the distributions are 21.8 μV and 12.6 μV respectively.

**Table 1**
Size distribution of clusters for 4 different protein dilutions and the negative control. The results have been obtained from optical analysis of microscope images.

| Protein dilution (μg/mL) | Number of beads per cluster | | | | | | Mean cluster size (beads) |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6+ | |
| 70 | 127 | 40 | 11 | 5 | 2 | 2 | 1.51 |
| 1.4 | 112 | 57 | 24 | 11 | 5 | 12 | 2.10 |
| 0.14 | 51 | 19 | 9 | 10 | 12 | 15 | 3.86 |
| 0.014 | 49 | 22 | 16 | 5 | 5 | 28 | 4.87 |
| Control | 172 | 49 | 14 | 12 | 6 | 6 | 1.76 |

an increased proportion of clusters in the sample will necessarily influence the amplitude distribution queues, the threshold has been taken to be the mean value plus the standard deviation ($\mu + \sigma$) of the negative control sample distribution. However, it is been also observed that a different choice of the threshold within ±50% of the aforementioned value would also show the same tendency. Error bars in the figure refers to the standard deviation of two different runs within the same experiment. There is a clear dependence showing that the percentage of amplitude transitions above the threshold is larger for a small concentration of the protein (only 14 ng/mL) than for the control and that this value then drops when the protein concentration is increased.

Special attention should be paid to the fact that the percent of large amplitude transitions decay with the increased protein concentration, even dropping to a value which is under the negative control. That means that large concentrations of protein would give rise to the formation of many single-bead particles. This is due to the competitive behaviour of the antibody antigen interaction. When the amount of protein present in the sample is high enough, it will bind and completely block all the sites on the surface of both types of beads (H&L and Fc) thus inhibiting the formation of clusters and promoting the singles. This effect is very strong for high concentration of the protein even drastically reducing the unspecific binding between beads giving rise to a lower number of clusters than in the case of the negative control.

Optical control experiments have also been performed for the protein dilutions mentioned before. For each case, after the incubation period, 10 μL of sample were placed in a microscope slide, pictures were taken using an optical microscope and the clusters size distribution was studied. The results are summarized in Table 1. It can clearly be appreciated that the size distribution of clusters follows the same tendency than the one shown for the electrical readout in Fig. 6. The mean size of clusters decreases when the protein concentration increases and for sufficiently large protein concentration, the clusters size is even smaller than for the negative control.

The dependence shown in Fig. 6 (and Table 1) demonstrates that the system can be used to detect protein concentration in solution. We have proved this for the specific case of rat IgG diluted in PBS at concentration between 1 μg/mL and 14 ng/mL. However this method is general and could, in principle, be extrapolated to any protein in solution. For any specific case, the sensitivity, specificity and range of application should be studied.

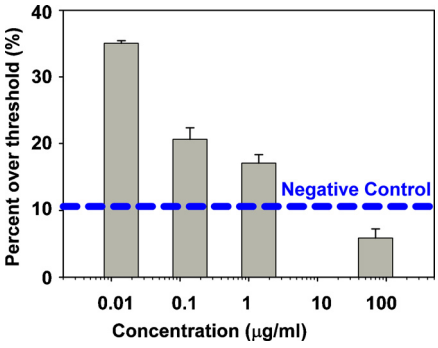


**Fig. 6.** Figure of merit (FOM) representing the percentage (with respect to the total number of transitions in the experiment) of particle transitions with amplitude over a given threshold. It depends highly on the concentration of protein in solution.

## 4. Conclusions

In this paper we have presented a compact microsystem for detecting protein concentration in solution by means of a simple label-free bead-based assay. The system is based on a qualitative measure of the change in the impedance readout of the bead assay compared to a negative control. The system is made from glass and polymer by means of a soft-lithography technique; it does not need any fluorescent labelling or electrode functionalization procedure for its operation. The sensitivity of the system was investigated by diluting the sample concentration down to 14 ng/mL. The system demonstrated a large dynamic range from 1 μg/mL to 14 ng/mL, which is in the order of the physiological concentration of many proteins in serum. The bead based assay system presented here could easily be adapted to other proteins with other physiological relevant dynamic range, as the method has a unique possibility for large dynamic range to be investigated.

## Acknowledgments

The authors would like to thank financial support from the Danish Research Council for Science and Technology through Proof of Concept (POC) funds and from EU 6th Framework Marie Curie Project CELLCHECK (MRTN-CT-2006-035854).

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**Winnie E. Svendsen** completed her master degree with honours in 1993 from the University College Dublin. She received her doctorate in Atomic Physics in 1996 from Copenhagen University, Denmark. Thereafter she was a postdoctoral researcher at the Max Planck Institute for Plasma Physics, Garching, Germany. She returned to Denmark in 1998 and was appointed Associate Professor at Copenhagen University in 1999. Since 2001 she has been at the Technical University of Denmark where she established her own research group Nano Bio Integrated Systems (NaBIS) in 2006.