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Sensitive and rapid protein assay via magnetic levitation

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ABSTRACT

Magnetic levitation (MagLev) is a newly emerging methodology for biosensing that provides a density-based analysis, which is highly sensitive and versatile. In this study, a magnetic levitation based sensor platform was used for protein detection; and sensor platform optimization was performed for both sensitivity and resolution. Bovine Serum Albumin (BSA) was used as a model protein and detection of BSA was carried out by antibody functionalized polystyrene microspheres (PSMs). Various sizes of PSMs were examined and their performances were compared by statistical analyses in terms of limit of detection (LOD), sensitivity, and resolution. Quantification of the protein was done based on the magnetic levitation height differences of antibody functionalized PSMs. For optimization of the methodology, varied PSMs were utilized, and standardization of PSM diameter, concentration of the antibody to be functionalized, and PSM dilution rates were carried out. In conclusion, 20 μm PSMs diluted to 0.005% W/V and functionalized with anti-BSA antibody at a concentration of 28 μg/ml were determined to provide the best resolution for BSA detection. A dynamic range of 100 nM to 1 mM was observed with an LOD value of 4.1 ng/ml. This sensing platform promises a novel approach with a diverse application field and it provides rapid, consistent, and reproducible results with high resolution and sensitivity.

1. Introduction

Protein detection and quantification are integral parts of every study that includes proteins. However functions, molecular structures, and physicochemical properties of proteins show a vast difference, which over complicates the selection of an optimum analysis methodology for each specific protein. That being said, several methods were developed exploiting these differences of proteins (Emilia Manole et al., 2018). These methods show several disadvantages, such as specificity. On the other hand, various reagent-based methods overcame the issues of specificity but they also required a form of labeling; such as colorimetric or fluorescent labelling of either the protein or the reagent (Krohn, 2002; Lequin, 2005). Magnetic levitation (MagLev) based sensing methodology, which provides a density-based measurement, is relatively new in the biosensor field. The fundamental of the technique is the usage of magnetic force to overcome gravitational force; and imitate a region without gravitational effects (Bloxham et al., 2015; Laithwaite, 1975; Thompson, 2000). This phenomenon is generally reached by utilizing magnets, and the simplest form is Helmholtz configuration. A paramagnetic medium is used to suspend objects or samples within the created magnetic field; this provides an uncomplicated and highly sensitive method for resolving extremely small differences in the density of suspended objects. As an object is suspended at a fixed point in the medium, based on its density; if or when its density increases its levitation height decreases and vice versa (Ashkarran and Mahmoudi, 2020; Durmus et al., 2015; Nemiroski et al., 2016; Turker and Arslan-Yildiz, 2018).

Recently few MagLev based sensor platforms were reported for analysis of biological and non-biological substances. A pioneering sensor platform has been designed to monitor the density changes during chemical reactions performed on a solid support. This platform succeeded to determine density differences as little as 0.01 g/cm^3, moreover with further development of the sensor system the accuracy of the platform was improved to 0.002 g/cm^3 (Mirica et al., 2008, 2009). One of the recent biosensor application of these systems was used to successfully separate a mixture of *E. coli* and *S. cerevisiae* cells based on their densities. In conclusion, a label-free and easy to use device was developed, and is able to sort cells with ~100% efficiency at a rate of 10^7 cells/h (Zhu et al., 2012). Similar sensor platforms were developed to procure applications on larger volumes and to simplify the whole process (Amin et al., 2016; Bwambok et al., 2013; Bloxham et al., 2015b; Ge and Whitesides, 2018; Nemiroski et al., 2016; Yenilmez et al., 2016; Zhang et al., 2018). Measurement of cell densities with the idea of possible usage of it in cancer diagnosis was carried out in MagDense

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magnetic levitation sensor platform and various fluorescent polyethylene beads were used to correlate bead densities with levitation heights. It was shown that it is possible to utilize the MagLev sensor platform to monitor the density change in the range of 0.01 g/mL in cellular studies (Baday et al., 2019). Lastly, one of our very own studies proposed a Point-of-Care (PoC) detection method based on MagLev technology; demonstrating *Hepatitis C* (HCV) detection. This study has proven that the MagLev based sensor platform is sensitive enough for protein assays, and it offered advantages such as no need for an external mechanism or device, adaptability, and simplicity (Ozefe and Arslan Yildiz, 2020). In that study it's shown that protein detection was possible with 200 μm diameter carboxylated PSMs, the sensor platform provided qualitative results with fine resolution. Although the platform showed satisfactory sensitivity, the main shortcoming of the platform was the relatively large size of PSMs, which limited its ability to quantitatively detect proteins. However, exhibited sensor platform performance was promising and was open for improvement; with this motivation, optimization and enhancement studies were accomplished in this study.

Here we demonstrated density based detection and quantification of proteins via MagLev biosensor platform. First we've fabricated the platform to be used, and then size, dilution, and saturation based standardization studies of polystyrene microspheres (PSMs) were carried out. Bovine Serum Albumin protein was chosen to be used as a model protein due to its ease of access and widely studied nature (Wischke, 2006; Estey et al., 2006; Fielding et al., 2005; Mahdavinia et al., 2018). The developed platform allowed to detect and quantify BSA and provided a novel, simple, rapid, and sensitive protein quantification method based on density variance.

2. Materials and methods

2.1. Magnetic levitation sensor platform

Fabrication of the Mag-Lev sensor platform has begun with the fabrication of structural frame out of Polymethyl methacrylate (PMMA) sheets of 2 mm thickness via Laser ablation. Saddles for four mirrors, two magnets and one capillary were carved into PMMA layers. Two permanent N52-grade Neodymium (NdFeB) magnets were arranged in an anti-Helmholtz configuration (same poles oriented towards each other). Four mirrors were positioned such that they would provide an image of the lateral section of capillaries. A borosilicate capillary tube was used to introduce the samples into the sensor platform; the images were then captured by Zeiss Axio Observer microscope. A detailed schematic of parts of the device and the assembled form can be seen in our previous works (Ozefe and Arslan Yildiz, 2020; Turker and Arslan-Yildiz, 2018).

2.2. PSM size standardization

Varied diameters of PSMs (5 μm, 20 μm, and 200 μm diameter) were used to calculate standard deviation of sample free PSMs (s_0), standard deviation corrected for LOD calculations (so'), estimated LOD, and p-value of normality test (Magnusson and Örnemark, 2014). PSMs with carboxyl group surface modification were commercially obtained from Lab261. PSMs with 5 and 20 μm diameter were delivered in suspension (1% solid) and 200 μm diameters were delivered in pure solid form; each PSM had 1.06 g/cm^3 density.

2.3. PSM and antibody concentration optimization

Varied concentrations of PSM suspensions (0.02%, 0.01%, 0.005%, and 0.0025%W/V) were used to investigate its affect on resolution; samples with each dilution rate were introduced to the capillary channel and microscopy images were taken after 5–20 min. Aggregation and interactions between capillary channel borders and PSMs were analyzed.

For antibody functionalization on PSM surfaces, the EDC/NHS protocol described elsewhere (Ozefe and Arslan Yildiz, 2020) was applied Antibody solutions with concentrations of 0.875, 1.75, 3.5, 7, 14, 28, and 42 μg/ml were added to PSM suspensions and were softly agitated. Centrifugation was used to remove unattached antibodies. The supernatant was removed and then sediment was resuspended with 50 mM paramagnetic agent Gadavist (Gx) solution with 0.01% Tween 20 which contained antibody functionalized PSMs.

2.4. BSA protein detection with magnetic levitation sensor platform

To analyze BSA protein 25 μl of anti-BSA antibody functionalized PSM suspensions were mixed with BSA protein solutions equivolumetrically. BSA concentrations of 1000, 100, 10, 1, 0.1 and 0.01 μM were prepared in paramagnetic agent solution (50 mM Gx with 0.01% Tween20) and 30 μl of the sample mixtures were introduced into the capillary channel for microscopic analysis. Due to the interactions between anti-BSA antibody and BSA, proteins were captured by PSMs and microscopy images were taken after 5–20 min (based on PSM size) after the introduction of samples to the capillary channel. A summary of the analysis protocol that's been followed can be seen in Fig. 1.

2.5. Image and data analyses

Measurements of magnetic levitation heights were carried out by MATLAB 2018b software. For this purpose, firstly, the acquired microscopy images were converted to black and white. Afterwards Sobel method was applied to identify PSMs and then images were converted to DICOM format. Structural element addition method was used to clarify the borders of each PSM and constituted shapes were filled. Weighted centroid of each shape was determined. Removal of artifacts and noise is carried out by clearing of lines and shapes with unlikely structures and sizes. Lastly, the distance between each PSM centroid and bottom of the capillary was measured.

A variance component analysis was used to determine the influence of experimental factors (*e.g.* PSM size, dilution rate, antibody concentration), analysis of variance (ANOVA) was applied. In brief, one-way ANOVA (OriginPro 2016) was performed using levitation height as the dependent variable and all other variables (*e.g.* PSM size, dilution rate, antibody concentration) as covariates. Kolmogorov-Smirnov normality test was applied to test whether the data were normally distributed ($p > .05$). Pairwise comparisons were carried out by contrast analyses where relevant.

3. Results and discussion

Effect of PSM diameter on sensitivity and resolution was analyzed by using varied sizes of PSMs. Comparison of PSMs with diameter of 5, 20, and 200 μm was carried out first, 0.04% (W/V) concentration was used for 200 and 20 μm *diameter* PSMs, and 0.0025% (W/V) concentration for 5 μm sample. No levitation past 600 μm was observed in samples of 200 μm diameter, this upper levitation height was between 700 and 800 μm for smaller PSMs. It indicated that a higher resolution and sensitivity can be achieved by using smaller PSMs since their levitation heights provided a wider range of levitation. Although 200 μm PSMs were observable with naked eye and it was possible to take their images even with a smartphone, their inferior sensitivity and resolution proved smaller sized PSMs to be better suited for a protein assay. Microscopy images of smaller sized PSMs in comparison with 200 μm diameter PSMs can be seen in Fig. 2.

Levitation of all sizes was carried out in 50 mM Gx solution and succeeded. However, the time period needed for levitation increased with decreasing PSM diameter where levitation of PSMs with 200 μm took about 3 mins; PSMs with 5 μm needed around 20mins to stabilize at a certain levitation height.

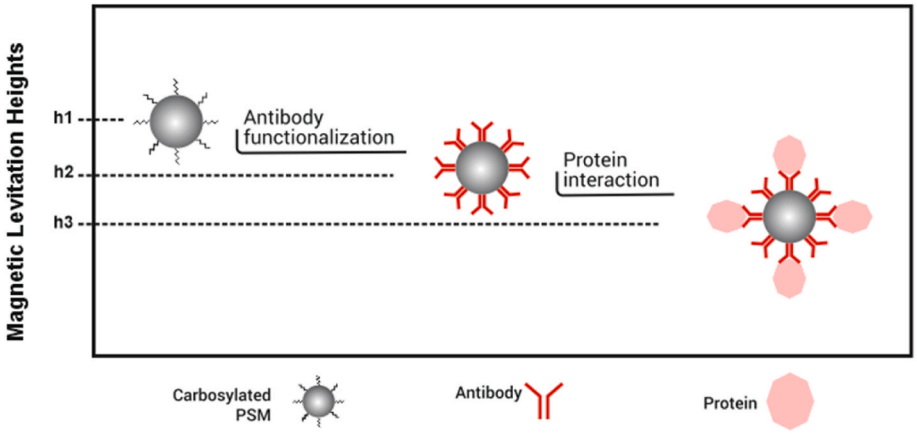

Fig. 1. Schematic of the MagLev analysis methodology: From left to right each image represents one step of the application. Where PSMs and surface activated PSMs show a similar magnetic levitation height; functionalization decreases this value and interaction with proteins decreases it even further, moreover interaction of functionalized PSMs with protein solution that have higher concentrations decrease the levitation height of PSMs more drastically compared to lower concentration solutions.

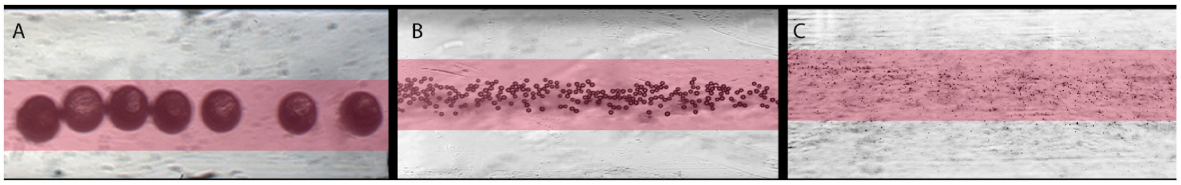

Fig. 2. Comparison of PSMs with different diameters A) 200 μm, B) 20 μm, C) 5 μm diameter.

Table 1 shows Kolmogorov-Smirnov Normality test results with corrected standard deviation (S′0) and estimated LOD (in μm) values for each PSM diameter.

200 μm PSMs didn't provide a large enough sample size compared to smaller PSMs; 20–30 PSMs per sample due to their larger size and lower number per unit sample volume. All other samples that are used for calculations shown in Table 1 were randomly derived from a larger pool. On the other hand smaller PSMs showed a hundred-fold higher sample sizes. That's why it'd be more appropriate to compare 20 μm and 5 μm in a calculation which included larger sample sizes, which is given in Table 2.

When larger sample sizes were taken into account, LOD values of both PSMs were decreased while they retained normality and a relatively close standard deviation. Comparisons were also made between suspensions of 5 μm microspheres with various PSM concentrations; the related images are presented in Fig. 3.

Each sample has shown levitation approximately in 20mins, but no consistent levitation was observed. Also there was a perpetual circular motion in the capillary, which hindered clear imaging of samples. These motions can be explained by intra-particle and particle-capillary wall collisions moreover, as previously described by Tjfirooz et al., a reduction in particle size increases the time needed for magnetic levitation to stabilize (Tajfirooz et al., 2021). Magnetic levitation can be performed only along the vertical axis, however with larger number of particles, the levitation region may shift and this off-axis levitation may cause rotation of the PSMs and an unstable levitation due to the this constant motion (Liang et al., 2021; Zhang et al., 2020). Table 3 shows the related statistical analyses results for 5 μm PSMs.

As seen in Table 3 in terms of LOD and standard deviation; concentration is an effective factor. Overall, the rest of the experiments were carried out with 20 μm PMSs both for sake of lower LOD values and also for better resolution in microscopy images. Next step was to determine the optimum concentration of PSMs. The concentration used in Fig. 2B caused slight aggregation of PSMs and interactions between PSMs and capillary channel walls were monitored. Fig. 4 shows different concentrations of 20 μm PSMs in comparison with each other.

At each concentration PMSs reached to a stable levitation height in less than 10 min and showed normal distribution ($p > .05$). Reducing the PSM concentration also prevented aggregations; both 0.005% and 0.0025% (W/V) PSMs showed no aggregation and provided a sufficient number of PSMs that would be enough for further statistical analyses. 0.005% PSM concentration was decided to be used for further steps, since higher number of PSMs per sample was obtained with no aggregation and minimal interaction between PSMs and capillary channel walls was observed. As the next step antibody concentration for surface saturation was examined. For this purpose PSMs were functionalized with different concentrations of antibodies; 0.875, 1.75, 3.5, 7, 14, 28, and 42 μg/ml then magnetic levitation heights were measured. Fig. 5 summarizes the results.

As expected, magnetic levitation heights decreased with increasing antibody concentrations; due to the interaction between the activated surfaces of PSMs and antibodies. This decrease also validated the surface functionalization with antibody. Fig. 5A indicates PSM surfaces get saturated to antibodies around 14–28 μg/ml antibody concentration, where magnetic levitation height stops decreasing with increasing antibody concentration. Non-linear regression analyses were also carried out to support this claim and Fig. 5B shows the regression plot.

Table 1
Kolmogorov-Smirnov Normality test results with corrected standard deviation (S'_0) and estimated LOD (in μm) values of PSMs with various diameters.

PSM size	P-Value	Std. Deviation	S'_0	LOD (μm)	n
5 μm	0.2253	94.312	17.823	53.467	28
20 μm	0.0528	41.978	7.798	23.393	28
200 μm	0.1349	71.514	13,515	40.455	28

Table 2
Kolmogorov-Smirnov Normality test results with corrected standard deviation (S′0) and estimated LOD (in μm) values of PSMs with increased sample sizes for 5 and 20 μm diameter PSMs.

PSM size	P-Value	Std. Deviation	S'_0	LOD (μm)	n
5 μm	0.2253	104.790	6.722	20.167	243
20 μm	0.0569	47.016	2.000	8.997	243

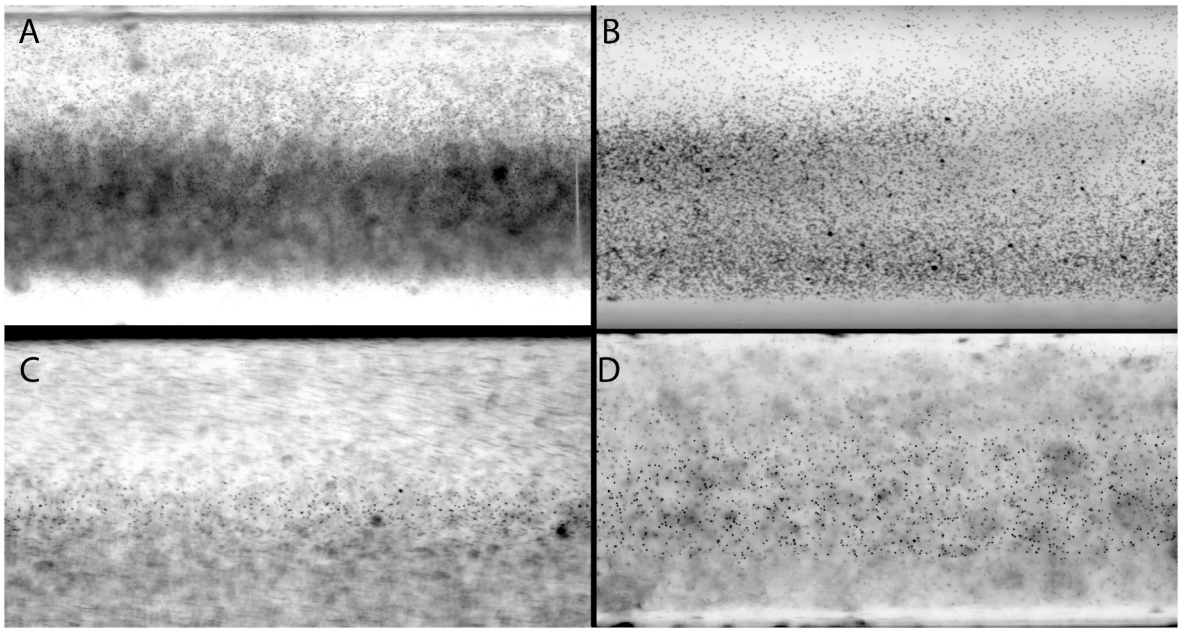

Fig. 3. Images of 5 μm PMSs, with concentrations of A) 0.01% W/V, B) 0.005% W/V, C) 0.0025% W/V, D) 0.00125% W/V.

Table 3
Kolmogorov-Smirnov Normality test results with corrected standard deviation (S′0) and estimated LOD (in μm) values of PSMs with increased different dilution factors for 5 μm diameter PSMs.

Concentration (% W/V)	P-Value	Std. Deviation	S'_0	LOD (μm)
0.01	0.2253	104.790	6.722	20.167
0.005	0.0160	86.216	5.531	16.592
0.0025	0.0314	80.332	5.153	15.460
0.00125	0.0484	73.751	4.731	14.193

Regression analyses also showed that an estimated 1 μm mean levitation change had occurred between the PSMs that were functionalized with 28 μg/ml antibody concentration and 42 μg/ml concentration. Therefore, it would be safe to assume 28 μg/ml antibody concentration is sufficient enough to saturate PSM surfaces with antibodies. Related statistical analyses results are shown in Table 4.

To further support this claim, one-way ANOVA analysis was performed and showed that the model was significant [$F(6,311) = 28.932$, $p < .001$]. Further analyses were carried out as contrast tests. Results of the contrast tests can be seen in Table 4 and it is evident that 28 μg/ml concentration is the saturation point of PSMs. Although there was no significant levitation height difference between 3.5 μg/ml and 7 μg/ml antibody concentrations ($p = .054$) this might be explained by a trend. Also statistically significant further decrease in PSM magnetic levitation height values with increasing antibody concentration suggests that it is a concentration that is below saturation point.

The study was followed by an analysis step where protein capturing and detection were carried out. For this purpose, functionalized PSMs were used as capturing agents to detect BSA in solution; results can be seen in Fig. 6A. Non-functionalized PSMs, and antibody functionalized PSMs, were used as control groups.

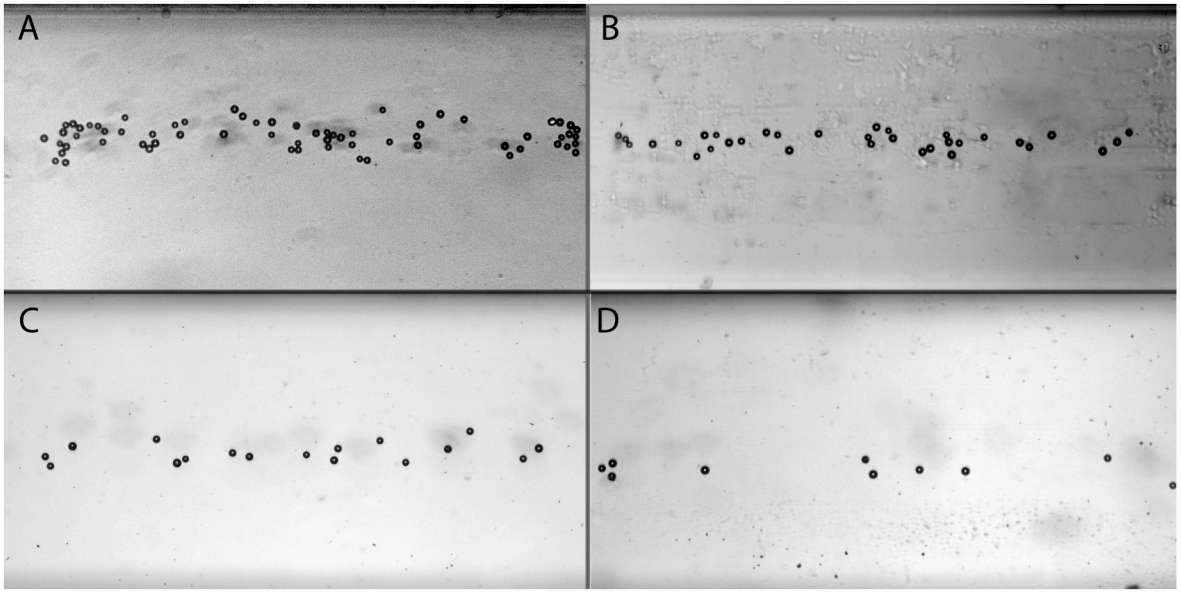

Fig. 4. Images of 20 μm PMSs, with concentrations of A) 0.02% W/V, B) 0.01% W/V, C) 0.005% W/V, and D) 0.0025% W/V.

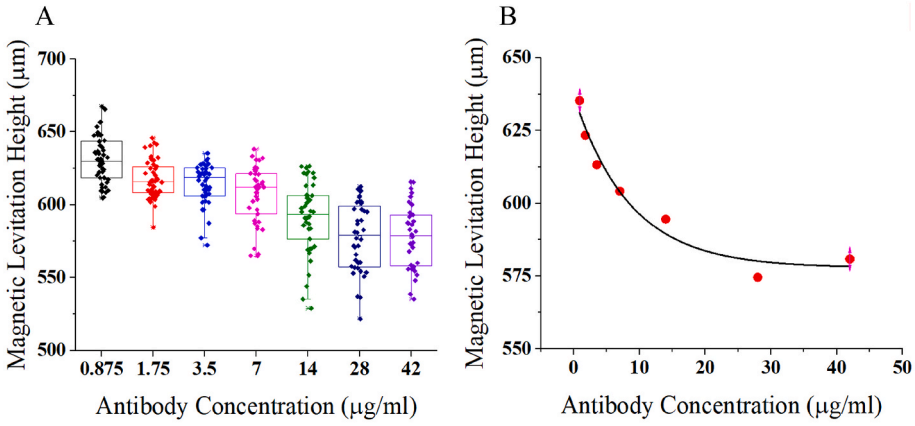

Fig. 5. A) Distribution graphic of antibody functionalized PSMs with increasing antibody concentrations; regarding surface saturation study with various antibody concentrations, B) Nonlinear regression results of antibody saturation study, mean levitation heights of PSMs with $R^2 = 0.97$, [$F_{(3,7)} = 33337.02$, $p < .001$].).

Table 4
Descriptive statistical analyses results of PSMs functionalized with various concentrations of anti-BSA antibody.

Concentration (μg/ml)	N Analysis	Mean	Standard Deviation	SE of Mean	P Value[a]
0.825	57	635.284	22.497	2.980	.004
1.75	52	623.369	20.997	2.912	.027
3.5	42	613.269	13.684	2.220	.054
7	44	604.181	17.631	2.658	.033
14	46	594.567	25.692	3.788	.001
28	41	574.594	22.499	3.51	.189
42	40	580.813	22.481	3.554	n/a

[a] These values are given as contrast tests with sequent concentration i.e. 0.825 with 1.75 μg/ml or 3.5 with 7 μg/ml.

Fig. 6A shows that there is a slight interaction between carboxyl groups of PSMs and BSA protein and also this interaction is insignificant compared to the interaction between antibody functionalized PSMs with the protein. Lastly various concentrations of BSA protein solutions were used to test the sensing capacity of magnetic levitation sensor platform. PSMs functionalized with anti-BSA antibody (28 μg/ml) were used for this purpose and they were mixed with samples of BSA protein solutions. Results can be seen in Fig. 6B. It is clear that interaction between antibody functionalized PSMs and BSA protein caused a decrease in levitation height based on the BSA attachment and it is evident that the developed magnetic levitation sensor platform was able to successfully capture and detect BSA protein down to 100 nM concentration. Statistical analyses related to Fig. 6A, comparing carboxylic group interaction of PSMs with antibody interaction can be seen in Table 5.

As the table shows there is normal distribution in each sample in itself. ANOVA analyses showed that there is significant difference ($p < .005$) between means of populations which indicates that the reduction in magnetic levitation height is due to BSA protein and anti-BSA antibody interaction moreover the interaction between carboxylic group of non-functionalized PSMs and BSA is much lower than functionalized PSMs and BSA for the same protein concentration. Statistical analyses of functionalized PSMs in samples with various BSA protein concentrations are shown in Table 1S.

Each sample showed normal distribution in itself and ANOVA showed significant difference ($p < .005$) between means of populations and post-hoc tests showed that there was a significant difference between each of samples except for control group and 10 nM sample results. Overall quantification of BSA protein was possible in the range between 100 nM and 0.1 mM. For further investigation non-linear regression and linear regression of linearized values of BSA concentrations were carried out and can be followed in Fig. 6C and D respectively. Mean values of magnetic levitation heights were used for analyses. The results of the sample with the lowest BSA concentration and antibody functionalized PSMs showed that the difference between their mean levitation heights is statistically significant ($p < .05$).

For detection range calculation linearization was utilized, slope of Fig. 6D was used in calculations and logarithmic equation was engaged to calculate the LOD value. An LOD value of 4.1 ng/ml was calculated. The calculated LOD is highly comparable with current gold standard for BSA detection; ELISA. LOD values in the literature are 64 ng/ml for ELISA, 157 ng/ml for BCA assay, and 138 ng/ml for Bradford assay (Khamehchian et al., 2008; Protein Analysis using Microplate-based Quantification Methods, 2021). A state-of-art study reported an LOD value of 5.33 ng/ml with a novel developed quartz crystal microbalance (QCM) assay (Sudjarwo et al., 2021).

The developed methodology provides an extended lower limit of detection compared to counterparts that are commonly utilized and that are newly designed. The sensor platform delivered results in 5 min, without the need of fluorescent and/or colorimetric labelling. Moreover it only needed light microscopy to carry out the assay, it was possible to work with volumes as low as 30 μl which reduced both the sample volume needed for analyses and also required antibody and paramagnetic agent amounts. Parts needed for the platform are easily accessible, moreover only part that is disposable is the capillary channel which further increases the inexpensiveness of platform and ensures measurements with no contamination or impurity.

4. Conclusion

Herein we developed and standardized a novel, simple, rapid, and sensitive method of protein detection and quantification based on density difference. Investigations of the effects of PSM diameter, antibody concentration for functionalization, and PSM concentration on sensor sensitivity and resolution were carried out. In conclusion 20 μm 0.005% (W/V) PSMs and functionalization with anti-BSA antibody at a concentration of 28 μg/ml had appeared to be superior in terms of resolution and sensitivity and were used for the rest of the study for higher reliability and reproducibility. Density based detection and quantification of BSA was carried out with the developed system; a dynamic range of 100 nM to 1 mM was observed with an LOD value of 4.1 ng/ml (62.04 nM). The developed magnetic levitation based biosensor platform has the potential to detect and quantify proteins; it would provide simple, rapid, inexpensive, and sensitive results with significance. In conclusion the developed sensor platform exhibits several advantages such as visual real-time output, sensitive density-based detection and quantification, lower sample size requirement, and adaptability.

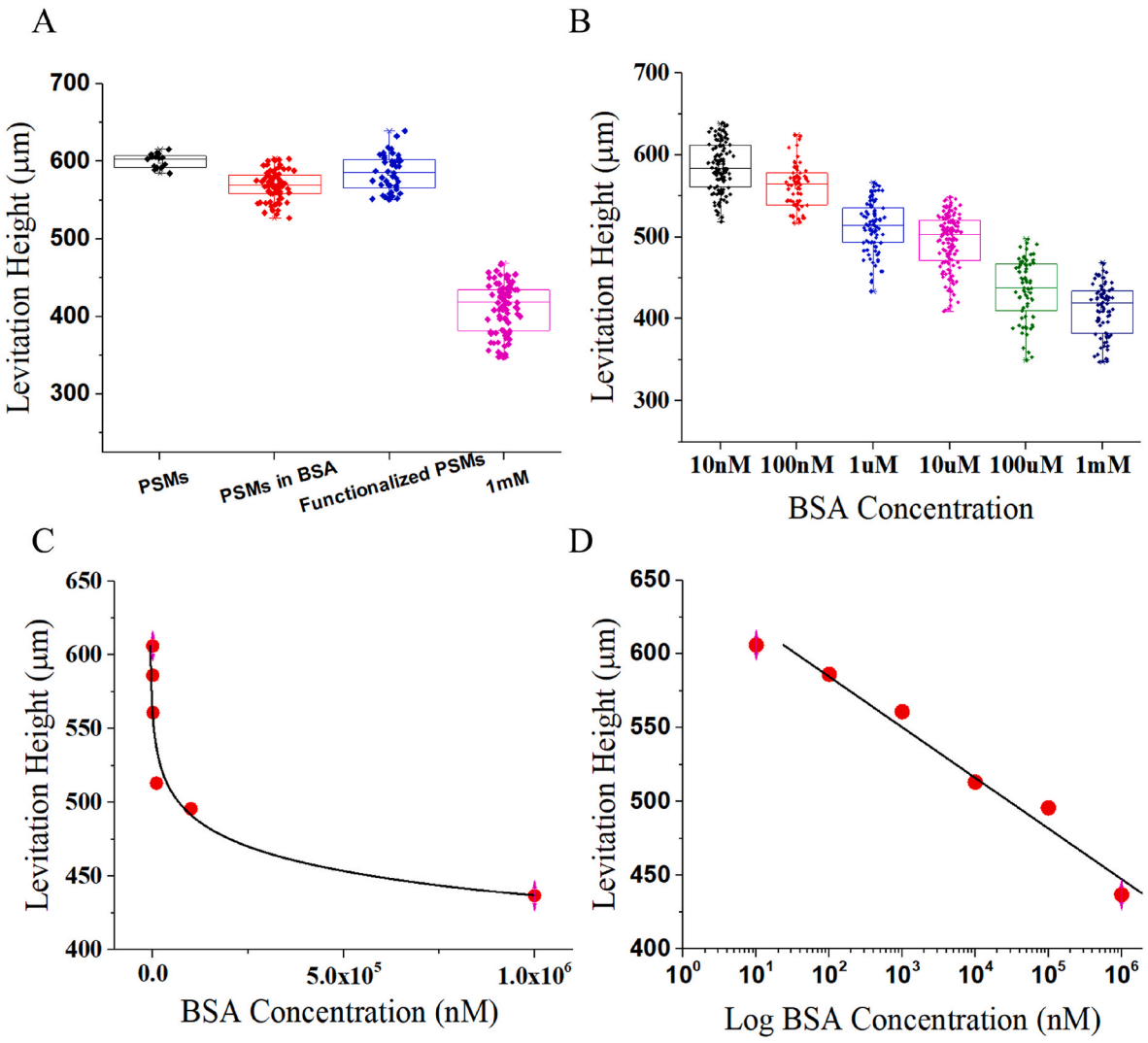

Fig. 6. A) Comparison of antibody functionalized PSMs and non-functionalized PSMs against BSA solution. Left to Right: 1st PSMs with no functionalization, 2nd PSMs with no functionalization in 1 mM BSA solution, 3rd Functionalized PSMs, and 4th functionalized PSMs in 1 mM BSA solution. B) Interaction between suspensions of antibody functionalized PSMs of 20 μm diameter and BSA protein solutions of various concentrations. C) Non-linearized regression of magnetic levitation height and BSA concentrations; $R^2 = 0.99$, [$F_{(3,6)} = 116.54$, $p = .001$], D) Linearized BSA concentration data has been used for regression; $R^2 = 0.97$, [$F_{(1,5)} = 259.99$, $p < .001$].

Table 5
Descriptive statistical analyses and Kolmogorov-Smirnov normality test of BSA protein detection via magnetic levitation sensor platform.

	N Analysis	Mean	Standard Deviation	SE of Mean	P-Value
PSMs in PBS	36	600.366	9.179	2.295	0.707
Non-Functionalized PSMs in 10^6 nM sample	75	568.926	18.755	2.166	0.843
Functionalized PSMs in PBS	45	585.097	23.005	3.430	0.982
Functionalized PSMs in 10^6 nM sample	60	405.762	22.680	2.928	0.213

CRediT authorship contribution statement

A.B. Sözmen: Methodology, Investigation, Visualization, Writing – original draft. **A. Arslan-Yıldız:** Conceptualization, Supervision, Validation, re-writing, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biosx.2022.100137.

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