A novel method of integration of a silver-polydimethylsiloxane (PDMS) nanocomposite in a microfluidic channel for the realization of a lab-on-a-chip is reported in this work. By using a silver nitrate aqueous precursor solution, silver nanostructures are formed on, and under the surface of PDMS, by in situ reduction. The silver aggregates formed by reduction have a wide absorbance plasmon band in the UV–Visible range. Separated silver nano-islands, having narrow absorbance bands are formed by using the post-reduction annealing process. The plasmonic property of the silver nanostructures is used for the detection of bovine growth hormone. Subsequently, the nanocomposite was integrated into a specially fabricated micro-wall in the channel which is the actual sensing area in the microfluidic environment. The experiments confirmed that the nanocomposite can be integrated into the microfluidic device in order to enhance the detection sensitivity.

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The localized surface plasmon resonance (LSPR) property of metallic nanoparticles has been widely used as a label-free technique for the detection of binding events in real time.\textsuperscript{1-6} The sensing mechanism of LSPR-based methods is based on the change in the position and/or the intensity of the LSPR band, upon the change of the refractive index of the surrounding medium. Silver and gold nanoparticles exhibit strong absorption bands in the visible and near infrared regions, and they show a strong dependence on the refractive index of the surrounding medium, thereby being highly useful for the plasmonic detection of biomolecules.

One of the main steps of LSPR-based sensing is the immobilization of nanoparticles on a substrate.\textsuperscript{7} Subsequently, bio-molecules are attached to the nanoparticles through various linkers and detected by monitoring the changes in the LSPR band, by using an optical setup consisting of a UV–Visible source and a spectrometer. Several techniques such as nanosphere lithography (NSL),\textsuperscript{8,9} vapor deposition,\textsuperscript{10} thermal evaporation\textsuperscript{11} and electrochemical deposition\textsuperscript{12} are reported for the immobilization of nanoparticles on various substrates. Using NSL, ordered nanostructures can be deposited with the help of self-assembled polystrene microspheres and vacuum deposition. A widely reported method of fabrication of nano-island structures is the deposition of thin film of gold on the substrate by vacuum evaporation and annealing to yield a nano-island morphology. In this case, the melting temperature of the thin gold film is highly reduced from that of bulk and hence the gold layer melts at lower temperatures, typically around 500 °C, thereby forming nano-island structures.\textsuperscript{13} Fabrication of gold aggregates or three dimensionally assembled gold nanostructures by convective self-assembly, by evaporating meniscus of aqueous suspension was reported.\textsuperscript{14} In the convective assembly, colloidal particles are driven and deposited onto the substrate immersed in the solution.

In order to adopt the fabrication methods discussed above for biosensing applications in a lab-on-a-chip, gold nanostructures must possess qualities such as good adhesion to the substrates, good optical properties such as narrow absorption peak, feasibility of modifying the structural and optical properties for different applications and the feasibility of integrating them in a microfluidic environment. The direct thermal evaporation and convective assembly are the two commonly used techniques for the fabrication of gold nanostructures for LSPR-based assays. However, the adhesion of the deposited layers is quite poor.\textsuperscript{15} Since the biosassays involve several surface functionalization steps, the nanoparticles must be strongly adsorbed onto the substrates, otherwise, the subsequent measurements would result in errors due to the detaching of the film. In this context, it is highly desirable to develop alternative ways for the LSPR-based sensing. The nanocomposites based on PDMS offer good solution to adhesion issues, as the particles are embedded in the surface of the polymer because the reduction\textsuperscript{16} reaction takes place at the interface of the aqueous solution of the corresponding metal salts and the polymer. Since the nanoparticles are embedded into the surface layer, the adhesion of the particles to the substrate is much stronger than in the case of other deposition processes.

Recently, the integration of nanoparticles in microfluidic devices\textsuperscript{17} has attracted significant attention because it facilitates the fabrication of highly sensitive sensing platforms in a compact form. Microfluidic chip-based bioassays have many benefits, including consumption of low sample volumes, fast response and the feasibility of developing high throughput detection systems in portable formats.\textsuperscript{18} Silicon, glass and polymeric materials such as PDMS are the commonly used structural materials for microfluidic chips. PDMS is an excellent material used for the fabrication of microfluidic and optical devices. PDMS has several advantages. It is easy to prepare for the fabrication of high-aspect ratio 3D microfluidic structures by soft lithography, it is easy to bond with glass or PDMS itself to provide a hermetic sealing of the microfluidic channels, it is biocompatible and highly transparent in visible lights and, most importantly, the material and fabrication cost are very low, compared to conventional microfabrication processes. In addition, because of the low glass transition temperature, excellent flexibility, high thermal and oxidative stability, PDMS is a very attractive polymer for the fabrication of nanocomposites. Gold (or silver)-PDMS nanocomposites are important functional materials with interesting potential applications such as sensors.\textsuperscript{19,20} Previously, the composite materials were produced by immersing the PDMS substrate having a rich curing agent content, in the aqueous solution of corresponding metal salts.\textsuperscript{16} However, the absorbance spectrum of nanocomposite\textsuperscript{16} showed a wide resonance band, not adequate for sensing purposes.

The application of silver-PDMS for the detection of antigen-antibody interaction of bovine growth hormone, called bovine somatotropin (bST) is reported in this work. The silver-PDMS nanocomposite is produced by immersing the PDMS substrate in the silver nitrate aqueous solution, followed by annealing, to tune the morphology and optical properties. The nanocomposite produced in
this way, is used for the detection of bovine somatotropin (bST) through antigen-antibody interaction.

Bovine somatotropin is a polypeptide growth hormone, naturally produced by the anterior pituitary gland in mammals. The effect of bST on the production of milk was identified in 1937. Later, by the 80’s, with the emergence of the recombinant DNA technologies, large quantities of hormones were produced and used for increasing the milk production in USA and some of the Asian countries. The use of bST is controversial because, its traces may be found in milk and meat and hence it may potentially harm the health of animal and humans. Hence, it is highly desirable to develop an efficient analytical method to provide to the consumers meaningful information about the traces of hormones in food. With the use of recent developments in internet of things (IoTs), fifth generation (5 G) communication, artificial intelligence (AI) and machine learning (ML) technologies, the nanocomposite-based sensing materials can be used to develop smart lab-on-a-chip devices for rapid screening hormones in food.

Traditionally, the concentration of bST was estimated by using ELISA (enzyme-linked immunosorbent assay) or radioimmunoassay (RIA). ELISA provides the information of antibody in a relative form that is based on a combination of affinity and concentration. Liquid Chromatography-Mass Spectrometry (LC-MS), combined with electrospray ionization is a recently developed method which can distinguish the natural and recombinant forms of somatotropin. However, it is a complex method, requiring expensive instruments. In this context, developing a low-cost microfluidic bio-sensing platform with the capabilities of detecting accurately and rapidly the growth hormones is very important.

In this work, a sensing element is integrated in a microfluidic environment, across an optical path. The sensing element (silver-PDMS nanocomposite) is synthesized in a microfluidic sensing cell. A schematic illustration of the proposed Lab-on-a-chip is shown in Fig. 1. The device is designed to be fabricated in PDMS. The PDMS chip formed by soft lithography consists of a sensing cell, a micro-sensing wall as the sensing element and two optical fibers directly integrated in the cuvette of the spectrophotometer and measurements are taken. The drawbacks of this approach are that it needs large amount of reagents and expensive instruments. Hence the integration of the nano-sensing platforms into a microfluidic environment is a useful approach for developing a Lab-on-a-chip based on plasmonic technique.

In this work, label-free sensing of bST, by using the localized surface plasmon resonance (LSPR) of silver in the silver-PDMS nanocomposite is presented. The silver-PDMS nanocomposite is synthesized by a simple and low-cost process. The LSPR spectrum of the silver-PDMS nanocomposite, produced by immersing the PDMS substrates in silver nitrate solution, is found to have a broad plasmon band, which is not ideal for bio-sensing with high sensitivity. Hence, the morphology and the LSPR band of Ag are tuned by annealing at various temperatures and times. The annealed silver-PDMS nanocomposite is demonstrated for the detection of antigen-antibody interaction in the case of the growth hormone bST. Further, the synthesis of Ag-PDMS is carried out in a microfluidic device for the realization of a lab-on-a-chip. A micro-sensing wall is fabricated across the optical path realized by integrating optical fibers directly into a microfluidic device. Subsequently, the micro-sensing wall was integrated with silver-PDMS nanocomposite through the reaction with the silver nitrate aqueous solution in the device. This sensor chip was demonstrated for the detection of antigen-antibody interaction of bovine somatotropin (bST).

Conventionally, the plasmonic bio-sensing experiment is conducted with the help of a spectrophotometer, in which, the samples having the nanoparticles are placed directly in the cuvette of the spectrophotometer and measurements are taken. The drawbacks of this approach are that it needs large amount of reagents and expensive instruments. Hence the integration of the nano-sensing platforms into a microfluidic environment is a useful approach for developing a Lab-on-a-chip based on plasmonic technique.

**Experimental**

*Materials.*—The Sylgard® 184 elastomer kit and curing agent for the PDMS fabrication were purchased from Dow Corning. The silver nitrate was purchased from Sigma Aldrich. Deionized (DI) water obtained from NANOpure ultrapure water system (Barnstead) with 18MO resistivity was used in all the experiments. Natural bovine somatotropin (bST, MW 20 kDa) and its corresponding antibody (Anti-bST raised in a guinea pig) were obtained from...
Preparation of silver-PDMS nanocomposite.—The PDMS base and curing agent are mixed in 4:1 (wt.) ratio. The mixture was placed in a vacuum desiccator and degassed to remove the gas bubbles. PDMS was cast onto a flat polished silicon wafer surface to prepare the substrates. Prior to casting of PDMS, the silicon wafer was silanized by using 0.1 ml of the trichlorosilane in a covered Petri dish on a hot plate at 55 °C for 5 h. The silicon wafer was placed in a Petri dish and PDMS was poured on the wafer to a thickness of ~2 mm and baked at 80 °C for 5 h. Then, the PDMS layer was cut into samples of 0.5 × 4 cm size. The silver nitrate solution of 22 mM concentration was used for the preparation of samples and the PDMS samples were incubated in the silver nitrate solution for 20 h. The dependence of optical properties of the silver-PDMS on the immersion time of PDMS sample in silver nitrate solution was reported in Ref. 31. The optical and structural properties of the samples under different annealing conditions were investigated by using UV-visible spectroscopy, scanning electron microscopy (SEM) and Atomic Force Microscopy (AFM).

Procedure for the bio-sensing on silver-PDMS substrate.—The silver-PDMS substrate was treated with oxygen plasma for 35 s to transform the surface of the sample in a hydrophilic one. Figure 2 shows various steps in the bio-sensing protocol by using silver-PDMS nanocomposite. The silver-PDMS substrate shown in Fig. 2a was functionalized with around 150–200 μl of 1-mercaptoundecanoic acid (in ethanol) and the cross-linker (N, N'-disopropylcarbodiimide and N-hydroxysuccinimide) for one hour in order to attach the peptides covalently to the silver nanoparticles. Figure 2C shows the Ag-PDMS sample after introducing the linker and cross-linker. The sample was washed in PBS and after 10 min, the spectrum of the functionalized substrate is measured. The linker and cross-linker attached the antibody of the bST covalently to the silver nano-islands. Then, the antibody corresponding to the bST was introduced onto the sensing platform and kept in contact with silver NP for at least one hour. The change in the position of LSPR corresponding to the binding of antibody was measured with the UV–Visible spectrophotometer (LAMBDA 650, Perkin Elmer). The concentration of antibody on the functionalized silver-PDMS was kept constant and the concentration of antigen was varied between 5 and 10000 ng ml⁻¹. The anti-bST obtained in powder form was dissolved in PBS (5000 ng ml⁻¹) and stored in refrigerator at 3 °C–5 °C.

Results and Discussions

Tuning of morphology and LSPR absorption band of the silver-PDMS nanocomposite.—Figure 3A (a)) shows the PDMS samples after incubating in the silver nitrate solution for 20 h. The presence of the curing agent, together with the oligomers in the PDMS permits the formation of silver nanoparticle on the surface layer of the PDMS by reducing AgNO₃. The silver-PDMS nano-composite produced in this way was found to have a dark gray color as shown in Fig. 3a. The sample was annealed at various temperatures to tune the morphology and optical properties. The color of the sample turned to yellow as shown in Figs. 3A (b) and (c) when the sample was annealed. The samples annealed above 350 °C were found slightly deformed and the color turned to a dark yellow as shown in Fig. 3c. The structural change of nanocomposite upon annealing was investigated using SEM and AFM.

The morphology of the silver-PDMS nanocomposite after incubation in silver nitrate solution for 20 h is shown in Fig. 3a. A thin layer of gold of thickness around 5 nm was sputtered on the PDMS to clearly image the morphology. The surface of the composite is found to have silver NP aggregates of size ranging from 100 nm to 1 μm. When the substrate was annealed at temperatures in the range of 340 °C to 370 °C for 20 min, the morphology changes as shown in Figure 3 is thought to be induced by melting of the silver aggregates and detaching from the PDMS surface. Indeed, it is known that the melting temperature of metallic nanoparticles is strongly reduced (by hundreds of degrees) due to the smaller particle size. The smaller particles diffused into the surface layer and remained on the surface. When the annealing temperature was 340 °C, the silver aggregates were found not completely removed as shown in Fig. 3b. For the complete removal of the silver aggregates, the annealing temperature was further increased to 370 °C, which resulted in a surface embedded with well-ordered nano-islands, having the diameter of around 30 nm as shown in Figs. 3B(c) and 3B(d).
The absorbance of the nanocomposite was investigated by using the UV–Visible spectrophotometer. The spectrum of the non-annealed sample showed in Fig. 3C(a) shows a wide band extending between 370 to 650 nm, with an absorbance maximum at 440 nm and belonging to the silver aggregates. When the sample was annealed at 340 °C, the band is blue shifted to 410 nm and became narrower as shown in Fig. 3C(b).

The wide band of the silver aggregates results from the near field coupling effects of neighboring particles in the aggregates, that is, when the distance between the particles is short, an electrodynamic interaction mechanism is expected. Upon annealing, aggregates are melting and breaking into smaller particles and the inter-particle distance is increased. As a result, the near field coupling between the particles is decreased, resulting in the blue shift of band to 412 nm. When the annealing temperature was further increased to 370 °C, the band becomes much narrower, and the absorbance intensity is considerably increased as shown in Fig. 3C(c). For the bio-sensing experiments, the sample having sharp resonance peak is useful, however, the annealing at higher temperatures results in the embedding nano-islands into the PDMS, as revealed by the AFM images. Since the particle is inside the PDMS matrix, less surface area of the particle is accessible to bind the bio-molecules, which results in less sensitivity for the sample annealed at higher temperatures.

The AFM images of the silver-PDMS nanocomposite shown in Fig. 4 indicate that the morphological change due to annealing is similar to the one observed in the SEM micrographs (Fig. 3). In addition to the SEM results, the line profile of the composite corresponding to the samples annealed at various temperatures shown in Fig. 4d indicates that annealing at higher temperatures results in the embedding of nano-islands deeper into the composite. The average height of the nano-islands in the non-annealed composite was ∼120 nm, and the height is reduced to ∼70 nm for samples annealed at 340 °C. The height of nano-islands was further reduced to ∼20 nm for the sample annealed at 370 °C. The top view of the samples is shown in Fig. 4d. These results are in good agreement with those on embedding of evaporated gold nano-islands into the softened glass substrates while the melting point of PDMS is −40 °C and the glass transition temperature is very low (−125 °C), cured PDMS has a softening point around 50 °C–60 °C. That means that Ag nano-islands could be embedded into PDMS and migrate deeper and deeper as the annealing temperature increases. The Fig. 5 shows the AFM image taken across the cross-section of the samples annealed and non-annealed. The non-annealed sample has lots of silver clusters accumulated towards the surface of the samples and the sample annealed at 370 °C showed smaller particles diffused across the sample as shown in Fig. 5c.

The samples annealed at 340 °C and 370 °C were further tested for the bio-sensing experiments. During the sensing process, the LSPR spectrum of the silver-PDMS nanocomposite was recorded after each step of bio-sensing. The anti-bST (100 ng ml⁻¹ concentration) was added to the surface functionalized silver-PDMS nanocomposite. Figure 6a shows the absorbance spectrum of the Ag-PDMS sample after adding the anti-bST. The antigen (bST, 100 ng ml⁻¹) was added to the sample and kept for one hour, which resulted in a shift of 8 nm as shown in Fig. 6b. In addition, the band is found to become wider. The experiments were repeated with various concentrations of antigen in order to assess the sensitivity of the platform as shown in Fig. 7.

The shift in LSPR band against the concentrations of antigen shown in Fig. 7 is linear in the range of 50 to 100 ng ml⁻¹ concentration for the samples annealed at 340 °C. In order to investigate the reproducibility of the results, the sensing experiments were conducted on 5 samples for each concentration. An error bar representing the standard deviation is included in Fig. 7. The
detection limit of the silver-PDMS nanocomposites annealed at 340 °C was found to be as low as 20 ng ml$^{-1}$.

The sensing experiments conducted on the sample annealed at 370 °C or above showed a lower sensitivity as seen in Fig. 7. Only one or two nanometer shift is observed for the concentration of 200 to 500 ng ml$^{-1}$ of antigen. The reason for the reduction in sensitivity for the samples annealed at higher temperature is the partial embedding of nano-islands into the substrate. The AFM images presented in Figs. 4 and 5 revealed that the annealing of sample at higher temperature causes the sinking of the islands deeper into the PDMS, which essentially make their surface less accessible for binding of bio-molecules, resulting in a reduced sensitivity.

Fabrication of Lab-on-a-Chip

The lab-on-a-chip is realized by in situ synthesis of silver-PDMS nanocomposite in a microfluidic channel. Different process steps involved in the fabrication of the lab-on-a-chip are shown in Fig. 8. PDMS chip. Soft lithography is used for the fabrication of the PDMS chip. The mold for the soft lithography was fabricated by

Figure 4. AFM image of nanocomposite annealed at 340 °C, (c) annealed at 370 °C and (d) line profile showing the height of the nanoparticle in composite (e) schematic of the nanostructures integrated onto the surface of the PDMS at various annealing temperatures and (f) AFM image of the nanocomposite showing the grouping behavior of nanoislands while annealing at 370 °C.
UV–photolithography. The SU8 negative photoresist (SU8 2035 Microchem) was spun on the silicon wafer at 2000 rpm for 30 s, which resulted in an SU8 layer with a thickness of 60–70 μm. Then, the photoresist was baked at 80 °C for 10 min. Since the diameter of the fiber used to couple the light to the microfluidic channel is of 125 μm (core diameter 9 μm), the depth of the slot for inserting the fiber must be ~125 μm. As the thickness of the photoresist layer decides the depth of the channel, the photoresist was spun again at 2000 rpm for 30 s to yield a thickness of ~125 μm. The resist was baked again at 80 °C for 10 min. The photoresist was exposed to UV light for 30 s through a mask. The UV–exposed photoresist layer was baked at 80 °C for 10 min and developed in SU8 developer for about 15 min. The fabricated mold shown in Fig. 10 was silanized for 8 h before casting the PDMS on it.

The PDMS microfluidic chip shown in Fig. 1 was fabricated by soft lithography. The PDMS base and curing agents are mixed as explained in section 2.2 and casted to the mold as shown in the step 1 of Fig. 8. A thin PDMS slab of 2 mm thickness was bonded with the chip as shown in Fig. 8(2). Two holes of diameter of 1 mm were punched on the PDMS layer corresponding to the place of liquid reservoirs in the microfluidic chip to insert the inlet and outlet tubes of the lab-on-a-chip. The thin PDMS slab and the PDMS microfluidic chip were exposed to oxygen plasma for 35 s to seal the device. Then the samples were immediately kept in contact in order to yield and strong leak proof bond. The fluidic tubes were inserted to the holes of the sealing layer of the device as shown in Fig. 8(3). Two single mode pigtailed fibers with SMA connectors were inserted to the slots of the fibers with the help of micro-positioners.

**Integration of silver-PDMS nanocomposite into a LOC environment**—A schematic sketch of the experimental setup for the silver-PDMS nanocomposite integrated LOC is shown in Fig. 9a. Light from a UV–visible source is coupled to the device through the fiber integrated with the device and another fiber integrated in the other side of the micro sensing wall couples the light to a spectrometer as shown in Fig. 9. A closer view of the Lab-on-a-chip fabricated on PDMS is shown in Figs. 9b and 9c. The experimental setup used for the lab-on-a-chip shown in Fig. 9d includes a UV–visible source and a spectrometer (USB 2000 Ocean Optics). Two single mode pigtailed fibers are integrated to the device, and each connected to the light source and the spectrometer as shown in Fig. 9d. For integrating the fiber to the device, two high precision micro-positioners were used. The spectrometer was connected to a computer in order to record the spectra. The UV–visible spectrum of the device is recorded by the spectrometer as a reference signal for the subsequent measurements. Then, the silver nitrate solution was pumped to the device and kept for 20 h and afterwards, the device was cleaned by pumping the DI water through the LOC for 2 min. The UV–visible spectrum recorded from the device is similar to the one of the Ag-PDMS substrate (Fig. 5). The fiber and fluidic tubes
were removed, and the device was kept in an oven and annealed at 340 °C for 30 min. The bonding of the device was found unaffected by the annealing. Then the fibers were inserted to the slot and fluidic tubes were connected and sealed. The absorbance spectrum was recorded again as shown in Fig. 10. The spectrum 10(a) and (b) show the UV–visible absorbance spectrum for the silver-PDMS nanocomposite integrated in the LOC before and after annealing respectively. The spectra recorded before and after annealing is the similar to that of obtained on Ag-PDMS substrate presented in Fig. 5. After annealing, the spectrum was blue shift to around 410 nm and became narrower as shown in Fig. 10.

**Bio-sensing in the lab-on-a-chip.**—The sensing procedure explained in the section 2.4 was repeated in the device by pumping all the reagents. The absorbance spectrum was recorded after pumping the antigen and keeping antibody for one hour as shown in Fig. 11a. The spectrum recorded after adding the antigen shows a shift in wavelength of around 6 nm in the LSPR spectrum as shown in Fig. 11b. The shift of LSPR peak obtained for the experiments on the substrate is closely matching with the shift obtained on the device. Few experiments repeated on the LOC platform confirm that an LOC can be realized with the silver-PDMS nanocomposite by preserving the sensitivity obtained on the Ag-PDMS substrate.
forces the embedding of silver islands deeper into the substrate, which could be the reason for the reduced sensitivity. Subsequently, a silver-PDMS nanocomposite integrated lab-on-a-chip is fabricated, and its performance is tested. The lower consumption of bio-molecules and reagents for the LOC (10 times lesser than that for the macro scale experiment) is the main benefit of carrying out the detection in the lab-on-a-chip.

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