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RESEARCH PAPER

Large-scale protein/antibody patterning with limiting unspecific adsorption

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Abstract A simple synthetic route based on nanosphere lithography has been developed in order to design a large-scale nanoarray for specific control of protein anchoring. This technique based on two-dimensional (2D) colloidal crystals composed of polystyrene spheres allows the easy and inexpensive fabrication of large arrays (up to several centimeters) by reducing the cost. A silicon wafer coated with a thin adhesion layer of chromium (15 nm) and a layer of gold (50 nm) is used as a substrate. PS spheres are deposited on the gold surface using the floating-transferring technique. The PS spheres were then functionalized with PEG-biotin and the defects by self-assembly monolayer (SAM) PEG to prevent unspecific adsorption. Using epifluorescence microscopy, we show that after immersion of sample on target protein (avidin and anti-avidin) solution, the latter are specifically located on polystyrene spheres. Thus, these results are meaningful for exploration of devices based on a large-scale nanoarray of PS spheres and can be used for detection of target proteins or simply to pattern a surface with specific proteins.

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Introduction

In the area of biosensing and bioengineering, one of the challenges is to produce large-scale patterning for specific anchoring of a target protein. This challenge requires an ability to manipulate nano-objects, to order them on large surface areas and to control the location of protein attachment. One of the strategies for the deposition of a large-scale array is the nanosphere lithography using two-dimensional (2D) colloidal crystals that is composed of microscale polystyrene spheres (PS spheres). This technique as introduced by Deckman and Dunsmuir (1982) allows the synthesis of large arrays (several millimeters) (Sakamoto et al. 2008; Bechelany et al. 2009). This method is fascinating because it is rather easy in comparison to the traditional lithographical methods. Using this process, well-ordered and controlled micro/nanostructures could be designed on a large-area substrate using a mono or several layers of PS spheres.

Polystyrene is one of the most extensively used types of plastic, and the number of its applications is growing over the past 20 years. It is an aromatic polymer obtained by polymerization of styrene monomers (Wünsch 2000). It is also biocompatible and is not expected to adversely affect interactions of nanoparticles with biological systems. Polystyrene nanoparticles have been used to fashion large-scale nanoarray for various applications, such

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as biosensors (Velev and Kaler 1999), photonics (Rogach et al. 2000), and self-assembling of nanostructures (Boal et al. 2000). Specifically surface-modified polystyrene nanoparticles are homogeneous, exhibit a low polydispersity index, and form stable colloids in biological fluids (Florence 2004). Because polystyrene nanoparticles can be easily synthesized in a wide range of sizes with distinct surface functionalization, they are perfectly suited as model particles to study the effects of the particle surface characteristics on various biological parameters. The protein micro/nanopatterning using PS spheres has been previously reported. In most of case, the strategy consists to remove the sphere in order to adsorb the proteins (Wang et al. 2005; Mallon et al. 2010; Taylor et al. 2012). There are several limitations for this strategy because the adsorption is an unspecific process which is not suitable to develop specific biosensor. This process does not allow controlling the protein orientation and denaturation.

Thus, the development of large-scale nanoarray requires an effort to control the protein location, integrity, and selectivity; this includes preventing the unspecific adsorption and the grafting of specific probe on the surface. The adsorption process is regulated by the properties of the protein as well as those of the surface of the material, the environmental conditions, and the kinetics of the process (Balme et al. 2006, 2016). In the case of biosensor, unspecific protein adsorption may limit the effectiveness of the surface that provides the active component of the biosensors. This induces a low signal from the sensor and often causes a high background (noise) signal. Thus, the control of the adsorption process is even more critical for applications that use nanostructured substrates with large relative surface areas, such as nanoparticles, microchannels, and thin porous membranes, due to their large surface.

There are different strategies to prevent unspecific adsorption of proteins, such as coating by bovine serum albumin (BSA), grafting of phospholipids, and use of non-ionic detergents like Tween 20 or of polymeric materials like polyethylene glycol (PEG), as well as combinations of these elements (Washburn et al. 2009; Bog et al. 2015). PEG is one of the most studied materials used in order to obtain antibioadherent coatings, especially in the pharmaceutical, cosmetic, and biomedical fields (Hamming and Messersmith 2008; Obermeier et al. 2011; Soteropulos et al. 2012). The interest of this polymer is due to its unique physical and chemical properties. Indeed, it is highly hydrophilic and has a significant chain flexibility (Uchida et al. 2005). It has also excellent solubility in aqueous and organic and has a low toxicity to living cells (Zalipsky and Milton Harris 1997). Preventing unspecific adsorption via its strong hydration layer and its steric stabilization effect is one of the most widespread applications of PEG (Zalipsky and Milton Harris 1997; Chapman et al. 2000). This coating by PEG is used to control the interaction of biological molecules with the surface of biosensors. This functionalization is commonly done by thiol-PEG self-assembled monolayers (SAM) on gold surface (Ostuni et al. 2001; Ma et al. 2004; Gudipati et al. 2005).

The SAMs produced on various substrates have been extensively investigated for the surface functionalization with active molecules or for protecting them from adsorption of species present in the environment (Ulman 1996; Aswal et al. 2006). The SAM formation is induced by the strong chemisorption between the substrate and the head group of the selected organic molecules. This allows making stable ultrathin organic films with controlled thickness (Bain and Whitesides 1989; Dubois and Nuzzo 1992). These advantages make the SAMs particularly suited to the development of applications in the field of biosensors (Love et al. 2005).This makes them an ideal model in many areas such as bioanalytical, organometallic, physical organic, bioorganic, and electrochemistry (Dubois and Nuzzo 1992; Bishop and Nuzzo 1996; Ulman 1996). Usually, SAMs formed on gold material or surface are attached using thiol-modified molecules. The self-assembled monolayers of alkanethiols on gold are probably one of the best surfaces currently available to obtain the functionalization and the patterning of biosensors with advantages such as flexibility and stability. The gold-thiol monolayers are stable when exposed to air, to water, or to ethanolic solutions for several months (Bain et al. 1989). They are also quite easy to produce, quickly assembled, and well ordered.

This work aims to produce a large surface of nanoarray functionalized with proteins for antibody detection. Herein, we report the synthesis of multifunctional surface with polystyrene spheres and selective functionalization ability. We aim to attach specifically the protein directly to the PS spheres and to limit its unspecific adsorption. In our work, we used a silicon wafer coated with a thin adhesion layer of chromium (15 nm) and a layer of noble metal (50 nm). PS spheres are deposited on the gold surface using the floating-transferring technique. We design a multifunctional surface with array of polystyrene spheres. We used PEG

SAMs to block the unspecific adsorption of protein on the surface of these polystyrene spheres. Another interesting point is relative to the stability of PS sphere layer regarding the multistep functionalization which could create defects on the surface.

Materials and method

Materials

Avidin from egg white (A9275), poly(ethylene glycol) 2-aminoethyl ether biotin (PEG) M_n = 5300 g mol^{-1} (757772), *N*-hydroxysuccinimide (NHS) Mw = 115.09 g mol^{-1} (130672), ethylenediamine Mw = 60.10 g mol^{-1} (E26266), Anti-Avidin, antibody produced in rabbit (A5170), ethanol (absolute alcohol, without additive (02860)), phosphate-buffered saline (P4417), and microparticles based on polystyrene (size 1 μm, 10 wt% aqueous solution (89904)) were purchased from Sigma Aldrich. Thiol PEG, mPEG-SH Mw = 2000 g mol^{-1} (PG1-TH-2k), Cy5-PEG-NHS Mw = 5000 g mol^{-1} (PG2-NSS5-5k) and succinimidyl PEG, and mPEG-NHS Mw = 2000 g mol^{-1} (PG1-SC-2k) were purchased from Nanocs. *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) Mw = 191.70 g mol^{-1} (03449) was purchased from Fluka Analytical. Alexa Fluor 488 TFP ester (A37570) and Alexa Fluor 647 NHS (A37573) were purchased from ThermoFisher Scientific.

Protein labeling

Avidin was labeled with Alexa Fluor 647 NHS. Typically, 100 μL of protein solution (1 mg Avidin and 100 μl PBS pH = 8.3) was added to dry fluorophore Alexa-647 and allowed to react for 0.5 h at 20 °C. Anti-Avidin was labeled using Alexa Fluor 488 TFP ester. One hundred microliters of protein solution (5 μL Anti-Avidin and 100 μl PBS pH = 8.3) was added to dry fluorophore Alexa-488 and allowed to react for 0.5 h at 20 °C. Then, the unreacted dyes and proteins were separated by centrifugation (16,000*g*, 1 min) using filter (Biospin P6) according to the supplier of the kit.

Polystyrene sphere deposition on the Si/Cr/Au surface

The nanoarrays of polystyrene spheres were designed using the following procedure. A deposition of 15 nm of Cr and 50 nm of Au were performed on p-type Si (100) wafers (2 × 2 cm^2 pieces of boron doped (8–25 Ω cm)). The substrates were treated by O_2 plasma using a pressure of 0.6 mbar and a power supply of 0.15 A in order to eliminate all the organic matters from the surface. The polystyrene spheres (PS spheres) were then deposited using the floating-transferring technique.

Typically, the monolayers of spheres were prepared using monodisperse polystyrene particles with the diameter of 1 μm. Forty microliters of the polystyrene solution (10 wt%) was diluted by an equal amount of ethanol. The solution was applied onto the Si substrate and displayed over the Si surface. The Si substrate was maintained stationary for few seconds in order to allow a good dispersion of the PS spheres. The Si wafer was then slowly immersed into a glass vessel filled with deionized water (80 mL). A non-ordered monolayer of PS spheres was formed on the water surface. A droplet of 10% sodium dodecyl sulfate (SDS) solution was added to the water in order to modify the surface tension. This step targets to consolidate the PS spheres. A large monolayer of PS spheres with highly ordered areas was achieved. This monolayer of PS spheres was transferred to our target substrate (Si/Cr (15 nm)/Au (50 nm)). After drying, the Si substrate covered with the PS was heated at 100 °C for 10 min in order to sinter the PS spheres and to improve their adhesion into the Si substrate. After sintering, the diameter of the PS spheres was reduced with etching by O_2 plasma for different times: 1, 5, 10, and 15 min. In this experiment, the utilized pressure of O_2 plasma is 0.6 mbar and the power supply is 0.15 A.

Functionalization of nanoarrays

The PEGylating of gold surface by SAMS The nanoarray was placed into 1 mL of solution of thiol PEG (mPEG-SH) (4 mg) in unmodified ethanol for 2 days. Then, the samples were rinsed with ethanol.

Polystyrene sphere biotinylation After PEGylation by SAMS, the nanoarrays were placed in a solution of 0.5 mg mL^{-1} PEG-biotin-NH_2, 2 mg mL^{-1} EDC, and 1 mg mL^{-1} NHS for 24 h. After this period, the sample is rinsed with ethanol to remove the absorbed PEG.

mPEG-NHS and Cy5-PEG-NHS grafting on polystyrene sphere In order to link mPEG-NHS or Cy5-PEG-NHS on the polystyrene surface, we have firstly grafted

ethylenediamine on COOH groups as follows: (i) the nanoarrays were immersed in 1 mL ethanol solution which contained 2 mg mL^{-1} EDC and 1 mg mL^{-1} NHS for 2 h. (ii) Then, the sample is rinsed with ethanol and it is placed in solution of mPEG-NHS or Cy5-PEG-NHS (0.2 mg mL^{-1}) for 1 h. (iii) Finally, the samples were rinsed with ethanol.

Protein attachment assays For protein attachment assay, we used Avidin, Avidin labeled with Alexa Fluor 647 NHS (noted Avidin*), and Anti-Avidin labeled with Alexa Fluor 488 TFP ester (noted Anti-Avidin*). The nanoarrays were immersed for 30 min in 1 mL of protein (10 μg mL^{-1}) in PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C). Then, the samples were rinsed with PBS.

Microscopy

The morphology of the samples was observed with scanning electron microscopy (SEM, Hitachi S-4800). Fluorescence images were performed using an epifluorescence microscope DM6000. The Alexa Fluor 647 and 488 were detected using CY5 and FTIC cube respectively.

Results and discussion

The present work aims to produce large-scale nanoarrays which permit to control the protein grafting and limit the unspecific adsorption. The large-scale nanoarrays can be produced by nanosphere lithography using the floating-transferring technique of polystyrene sphere. These spheres can serve as anchor site for protein attachment. However, the main limitation comes from the unspecific adsorption between the spheres. In order to tackle this limitation, one way is the functionalization of these free spaces with a high density of PEG molecules. This can be achieved by thiol PEG SAM strategies on gold surface since it is a well-known strategy to limit the non-specific adsorption of protein. The overall process for nanoarrays design is presented in the schematic diagram (Fig. 1).

The first step consists to deposit a thin layer of gold (50 nm) on Si water. In order to improve the stability of this layer, 15 nm of Cr is deposited on the Si substrate before the Au deposition. The Si/Cr/Au substrates were treated by O_2 plasma to remove all organic matter from the surface. After the pretreatment, an ordered monolayer of polystyrene spheres (PS spheres) was prepared and deposited on the substrate by floating-transferring technique (Rybczynski et al. 2003; Mikhael et al. 2011). The sample was prepared as described in the experimental section and dried in air at room temperature. The obtained spheres were self-assembled into a close-packed two-dimensional ordered area via attractive capillary forces (Danov et al. 2001). After drying, the Si substrate covered with PS was heated in an oven at 100 °C for 10 min to sinter the PS and to improve their adhesion to the Si/Cr/Au substrate. The diameter of spheres was decreased with etching by O_2 plasma. This step permits also as well to oxidize the polystyrene surface to create a COOH group on their surface. The latter step is essential for further functionalization of PS spheres. The SEM image (Fig. 2) of PS spheres shows the unformal decrease of the diameter with the plasma treatment. In addition, it confirms that the transfer permits to obtain the monolayer of PS spheres. The dependence mean diameter of PS spheres as the function of different times of etching by O_2 plasma is also reported in Fig. 2. The characterization of protein and antibody on the nanoarrays is performed by epifluorescence microscopy. The minimum size required to detect the PS spheres is around 900 nm. Thus, we have fixed the etching time to 5 min. This time permits to decrease the PS spheres diameter from 956 to 933 nm.

After the plasma etching, the sample is composed by zone. The first one is the gold metallic surface which is uncovered by polystyrene spheres. The second one is the surface of PS spheres which exhibits the COOH group. These two parts of the sample could be functionalized differently because we expect to graft proteins and antibodies specifically on PS spheres. This imposes to prevent the absorption between the PS spheres. To do it, the gold surface between PS spheres was functionalized by SAMS of PEG-thiol.

After this step, we are able to have two different functionalizations on spheres and between them on the gold surface. We attached on polystyrene spheres the PEG-Cy5 following two steps. The first one consists to link ethylene diamine on carboxyl groups using EDC/NHS and the second one is the addition of NHS-PEG-Cy5. Figure 3a shows a fluorescence microscopy image of the prepared sample. We can observe that fluorescence came only from the spheres and there is no fluorescence between them. The diameter of spheres

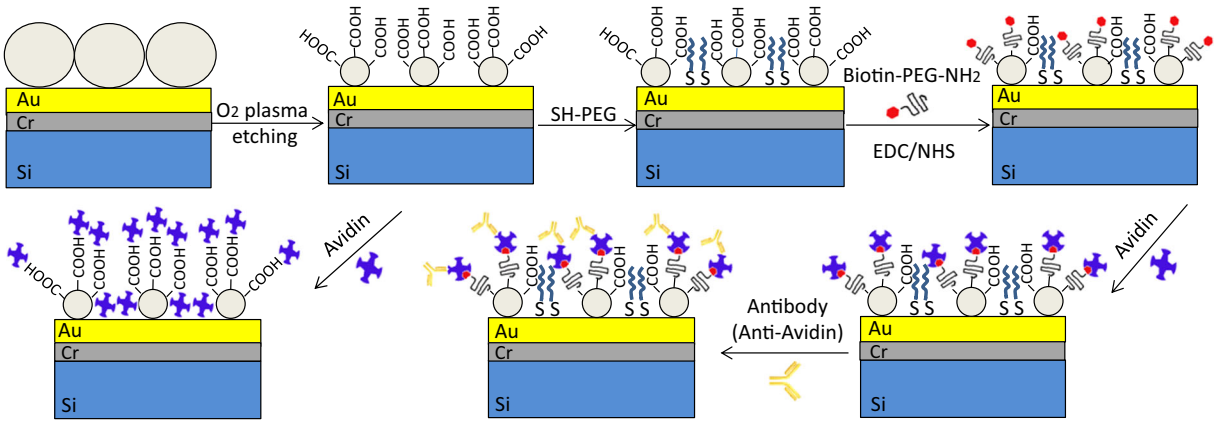

Fig. 1 The schematic diagram of overall process for protein immobilization

around 0.9 μm is in good agreement with the one determined by SEM.

Several additional experiments were performed as blank to confirm that the fluorescence comes from the dyes. PS spheres do not exhibit self-fluorescence under excitation wavelength of 590–650 nm provided by Cy5 cube. In addition, the images performed after each step of functionalization (after reduction by plasma, gold coating and PEG-biotin grafting) do not reveal fluorescence emission under the same condition. A weak autofluorescence of PS sphere was only observed under excitation wavelength lower than 450 nm (using Hoeechst and CFT cubes).According to that, we can conclude that the PEG-Cy5 is attached on *PS spheres*. Between the PS spheres, we observe some defects. However, PS spheres cover the majority of the surface of the sample (here 90.48 × 67.6 μm^2). At this step, our results prove that our strategy allows designing a large scale of monolayer of PS spheres and that our strategy of functionalization is relevant to functionalize PS sphere surface and gold surface with different functions.

We wanted to use similar strategy to attach the protein directly to the spheres, but not between them. To do it, we have done a variety of experiments using the avidin-biotin strategy. The latter is commonly used to design biosensor device. The first step consists to graft PEG-biotin on PS spheres. This was performed using PEG-biotin-NH_2 and EDC/NHS. In the second step, the avidin labeled with Alexa-647 was added and their localization was investigated by epifluorescence spectroscopy (Fig. 4). As

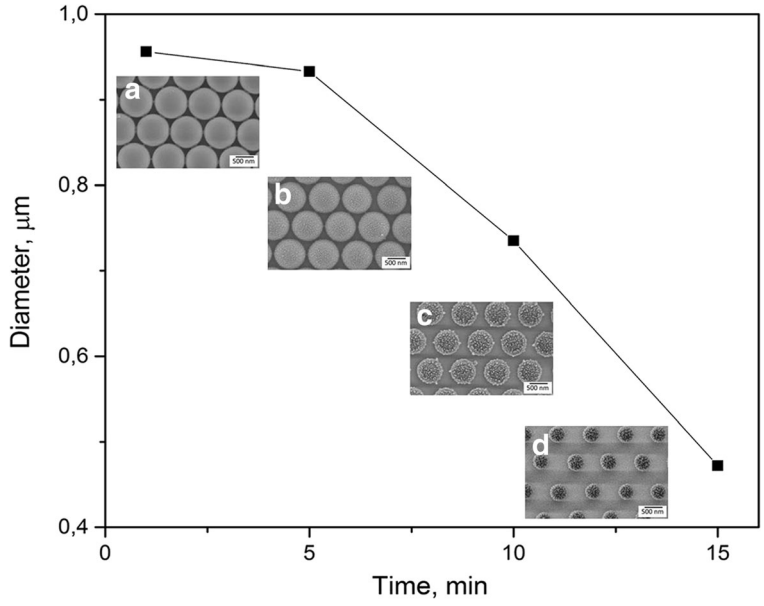

Fig. 2 Diameter of PS spheres as the function of the O_2 plasma etching. The insets show PS spheres with different O_2 plasma etching times: 1 (**a**), 5 (**b**), 10 (**c**), and 15 (**d**) min, respectively, as determined by SEM (scale bars 500 nm)

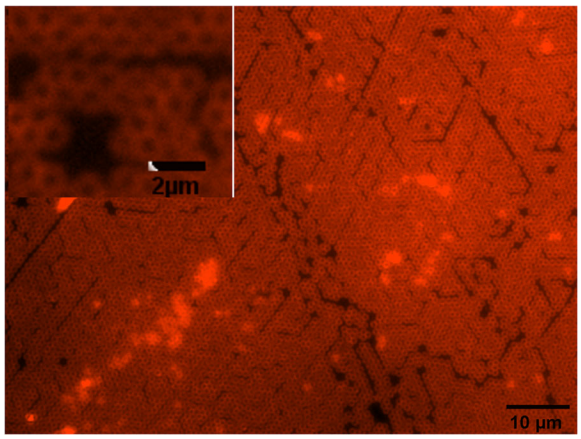

Fig. 3 Fluorescent images of Cy5-PEG-NHS-modified polystyrene spheres (the inset indicates a zoom)

expected, the fluorescence comes specifically from the PS spheres. The lake of fluorescence between the spheres proves that there is no protein. Thus, the functionalization of gold surface is efficient to prevent the unspecific adsorption of protein. In order to prove that the avidin is on PS spheres due to the presence of biotin, a similar functionalization with mPEG on PS spheres was performed. After immersion, under labeled avidin, the epifluorescence reveals a weak fluorescence. Since the PS spheres and the functionalization do not reveal fluorescence emission using the CY5 cube, we can admit that the signal is due to avidin likely adsorbed on sphere. Actually, this is not surprising because PEG layer has to be dense to prevent the protein adsorption. In the case of PS spheres, the PEGs are grafted on a carboxylate group. Thus, the density of these groups is likely too weak to induce a dense layer of PEG. In this case, proteins could be adsorbed on the defects and/or between the PEG chains.

In order to prove that the large-scale nanoarrays are suitable for antibody detection, different samples were functionalized with PEG-biotin and unlabeled avidin. After this first step, no fluorescence can be detected under excitation wavelength of 460–500 nm as well as 590–650 nm. Then, they were immersed in solution containing Anti-Avidin* labeled with Alexa Fluor 488. As expected, the epifluorescence image shows that anti-avidin is specifically located on PS spheres (Fig. 5). The functionalization with SAMs of PEG prevents the antibody as well as the avidin adsorption. We can observe that some microspheres have different intensity of green fluorescence. This could be explained by the following: (i) the number of dyes on antibodies is not homogeneous or (ii) the number of antibodies attached on microspheres is different.

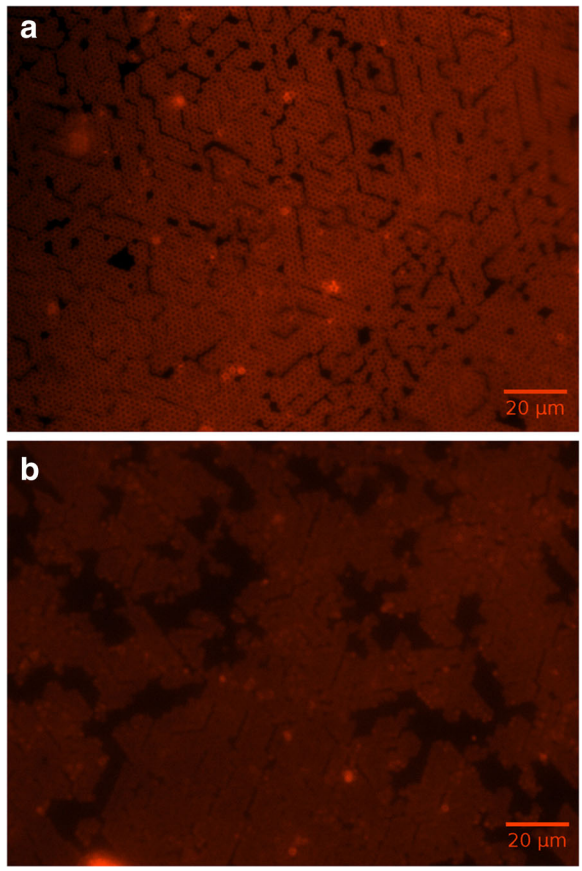

Fig. 4 Fluorescent images of PS spheres functionalized with biotin-PEG-NH_2 (**a**) and mPEB (**b**) after addition of avidin (labeled with Alexa Fluor 647)

Figure 6 depicts the fluorescence image of a sample where both avidin* and anti-avidin* are labeled with Alexa Fluor 647 and 488 respectively. The first one is

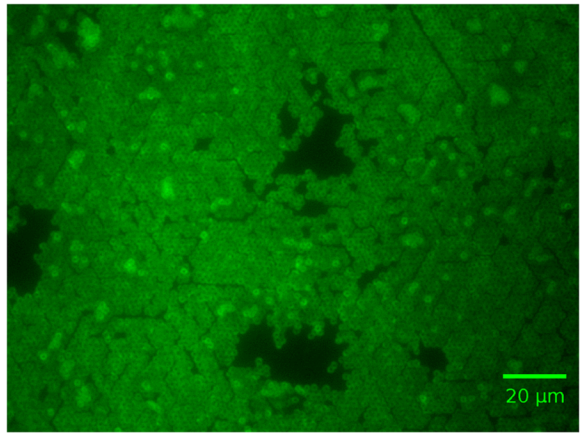

Fig. 5 Fluorescent images of PS spheres functionalized with biotin-PEG-NH_2 and avidin (unlabelled) after Anti-Avidin* (labeled with Alexa Fluor 488)

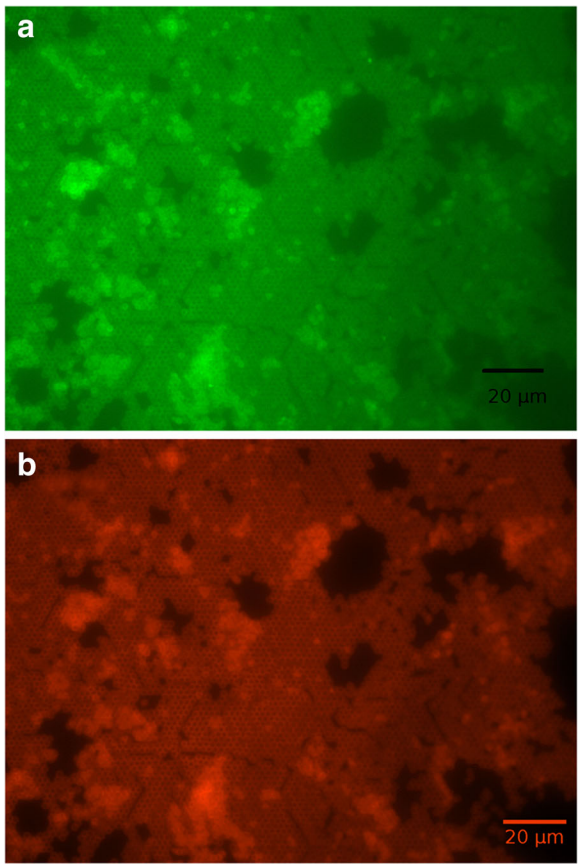

Fig. 6 Fluorescent images of PS spheres functionalized with biotin-PEG-NH_2 and avidin (labeled with Alexa Fluor 647) after Anti-Avidin (labeled with Alexa Fluor 488) addition recorded with FTIC cube (**a**) and Cy5 cube (**b**)

characterized with CY5cube and the second one with FTIC cube. The perfect superposition of both images confirms that both avidin and anti-avidin are located on PS spheres.

Conclusion

In summary, we have presented a novel method based on nanosphere lithography in order to design a large-scale protein patterning. This technique based on two-dimensional (2D) colloidal crystals (polystyrene sphere (PS spheres)) allows the fast fabrication of large arrays (up to several centimeters) by reducing the cost. The nanoarrays are formed by a monolayer of polystyrene sphere deposit by floating-transferring technique on gold surface. On the one hand, the gold surface between spheres was functionalized with SAMs of PEG to prevent the unspecific adsorption. On the other hand, the PS spheres were functionalized with PEG biotin. After addition of avidin and anti-avidin, the proteins are located only on PS spheres confirming that our strategy is suitable to control their location. Our results are meaningful for exploration of devices based on large-scale protein patterning on PS spheres. Regarding the versatility of avidin/biotin system, this strategy can be extended for biosensor development as well as for other applications which require large-surface patterning of proteins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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