Gold Nanoparticle Growth Kinetics on Silica/Graphene Surfaces for Multiplex Biological Immunoassays

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Abstract

Kinetics of seed-mediated chemical growth of gold nanoparticles on substrates coated with a thin layer of a number of graphene materials were studied with ultimate goal of employing the developed graphene-nanoparticle composites in light-scattering imaging biosensor applications. The type of the surface coating and chemical conditions influence the surface number density and size of the grown nanoparticles. Importantly, when used as biosensor base, the substrates coated with protein reduced graphene oxide have shown beneficial biological compatibility towards immobilized antibodies resulting in about 20-fold improved antigen capture immunoassay performed on the multiplex photonic biosensor platform.

Keywords: Nanoparticle; Seed-mediated growth; Graphene; Immunoassay; Protein
**Introduction**

Microarray based high-throughput analytical tools and techniques become inevitable in the fields of genomics, proteomics and glycomics. Evolving personalized diagnostics and medicine also relies rapid proteomic profiling by microarray format biosensors (Ahmed et al. 2013). Point-of-care devices required for personalized diagnostics are expected to develop through miniaturization and multiplexing. Although mass spectrometry methods remain ultimate in data quality the associated cost and issues with complexity of clinical samples, when many biomolecules are expected to be detected, limit the spread of the technique. Microarray based analytical tools are thought to complement and supplement the mass spectrometry (Brennan et al. 2010; Hoheisel et al. 2013). Optical properties of nanoparticles recently attracted increasing attention in applications to biosensors used in these fields (Sun et al. 2013).

The change in optical properties in association with graphene surfaces has attracted attention from many and nanoparticle/graphene composite materials have been explored for biosensor applications (Hong et al. 2010; Huang et al. 2013; Mao et al. 2010; Song et al. 2011). The sensor performance enhancements are attributed not only to unique physical properties of graphene (fast electron transfer and large surface area) as a substrate/support for nanoparticles but also synergistic effects are observed (Gupta et al. 2013; Li et al. 2009; Saini et al. 2012; Wu et al. 2009). Although the progress in nanoparticle/graphene composite manufacturing is evident (Bei et al. 2011; Jasuja and Berry 2009; Kamat 2009; Salgado et al. 2012; Xi et al. 2012; Zhang et al. 2012), the subject is still largely restricted to qualitative studies and underdeveloped: each particular application must be optimized in terms of nanoparticle-graphene assembly structure as well as microscopic properties of the nanoparticles, their material composition, shape and size (Watcharotone et al. 2007; Xi et al. 2012; Xiao et al. 2011).

There are two extremes in the approach of nanoparticle/graphene composite synthesis: create a gold nanoparticle/graphene composite by deposition of separately prepared nanoparticles on graphene material; and direct spontaneous formation of the particles by reduction of gold ions in the presence of graphene material. The former offers great control over the particle formation process but involves more synthetic steps, including particle-graphene tethering, while the latter has synthetic challenges to control the shape and size distributions of the resulting nanoparticles (Kong et al. 2009; Xi et al. 2012). A combination of these two methods is a seed-mediated growth of small particles attached to the graphene surface, which improves control over the particle formation
process. The majority of studies on nanoparticle/graphene composites perform the synthesis stem in bulk solution (Jasuja and Berry 2009; Lee et al. 2011; Liu et al. 2012; Muszynski et al. 2008) with only a few in situ growth observations at the solid-liquid (Kim et al. 2010).

The seeded growth process also offers an interesting option for the sensor manufacturing process: the control over the spatial distribution of the nanoparticles on the sensor surface, essential to the manufacture of biosensors in a microarray format. In this paper, the results of a kinetic study of the seed-mediated growth of gold nanoparticles on several graphene material coated substrates are presented and the application of the resultant materials is assessed for label-free protein immunoassay arrays.

**Materials and Methods**

**Chemicals and Materials**

Gold (III) chloride trihydrate (HAuCl$_4$·3H$_2$O, 99.9%), silver nitrate (AgNO$_3$, 99%), cetyltrimethyl-ammonium bromide (CTAB, 95%), sodium citrate (C$_6$H$_5$Na$_3$O$_7$·2H$_2$O, 99.9%), BSA (98%), fibrinogen (60%), were obtained from Sigma-Aldrich; sodium borohydride (NaBH$_4$, 98%) was from Lancaster; (L+) ascorbic acid (C$_6$H$_8$O$_6$, 99%) was from Acros. Dithiobis-succinimidyl propionate (DTSP, 97%) was from Fluka. Glass slides coated with aminoalkylsilane, (Silane-Prep Slides) were from Sigma-Aldrich. Human transferrin, TRA (4 mg/mL) was obtained from Invitrogen; bovine serum albumin, BSA (98%), goat polyclonal antibody to CRP, aCRP (10 mg/mL IgG fraction) were from Abcam; sheep polyclonal antibodies to TRA, aTRA (34.3mg/mL, IgG fraction) were supplied by AbD Serotec UK. The protein and antibody solutions were prepared and used in standard phosphate buffed saline, PBS, containing 0.005 wt% Tween 20 surfactant, supplied by Sigma-Aldrich. Throughout the experiments 18 MΩ cm de-ionized water was used as solvent when required, and 100 mM phosphoric acid solution was used as regeneration buffer.

**Substrate Preparation**

Three reduced graphene oxide (rGO) samples were prepared by reducing graphene oxide (GO) supplied by Sigma-Aldrich using BSA and FG proteins according to procedure described by Liu et al (Liu et al. 2010), and using MB as suggested by Cai et al (Cai et al. 2011). All three prepared samples and original GO were purified of the residual water-soluble chemicals by repeated centrifugation, discarding the supernatant and re-suspending the sample in pure water. The changes
associated with reduction of GO are clearly recognized by sample colour change: the original brown-yellow GO turns into black rGO-BSA/FG or blue-black rGO-MB suspension. The samples remained stable water suspensions after several weeks of preparation. Both protein reduced graphene oxide coated substrates showed very similar behaviour in the performed experiments and further data and analysis are presented only for BSA-rGO samples.

The aminated silane glass slides (ASG) were immersion-coated with GO, rGO-BSA, rGO-FG, and rGO-MB aqueous solutions. The room-temperature dried slides were further incubated at 50°C for 30 minutes to facilitate satisfactory surface adhesion of the graphene flakes, which otherwise showed tendency to lift off the substrate surface in aqueous environment.

**Seeded Array Patterning**

The seed gold nanoparticles were prepared by a rapid sodium borohydride reduction of auric chloride in the presence of a citrate surface ligand (Murphy et al. 2005): 0.6 mL of ice-cold 0.1M NaBH₄ solution was quickly added while stirring to the 20 mL solution containing 2.5×10⁻⁴ M HAuCl₄ and 2.5×10⁻⁴ M sodium citrate. The resulting gold nanoparticles are spherical in shape with a diameter typically 4-5 nm (determined by TEM). The resulting gold colloid was left to mature for 2-3 hours before the printing step. The final concentration of the seed colloid is estimated as 55±5 nM from the mass balance. Three concentrations of the seed nanoparticle colloid were inkjet printed in 12 × 8 rectangular arrays on bare and GO/rGO coated glass substrates; a part of the developed array is shown in the Fig. 3 insert. The printed arrays were incubated over two days allowing all seed particles to settle on the substrate surface. The concentration of the printed seed particles determines the subsequent surface number density of the grown particles.

**Growth of God Nanoparticles on GO/rGO substrates**

The growth of the printed gold seed nanoparticles forming the photonic surface was performed directly in the flow cell of the imaging instrument at 23±0.5°C. The growth solution was flowed over the surface at 0.1 mL/min volume or 2 mm/min linear rate. The flow conditions in the cell correspond to a laminar flow regime. The standard composition of the growth solution, or ×1 developer, is based on the gold rod nanoparticle synthesis protocol (Murphy et al. 2005) optimised to manufacture the sensor array for the light scattering platform (Olkhov et al. 2009; Olkhov and Shaw 2008): 0.05 M CTAB, 2.5×10⁻⁴ M HAuCl₄, 2×10⁻⁶ M AgNO₃, and 4.5×10⁻⁴ M ascorbic acid. In contrast to the bulk synthesis, that leads to the growth of the gold nanorods, the growth of the seed
particles on the substrate surface results in nearly-spherical truncated polyhedral particles. If silver nitrate is omitted from the growth solution, a small fraction of seeds develops into rod and flat polygonal particles (Olkhov and Shaw 2008). Several variations of the developer were used to study the effect of the HAuCl₄ concentration on the growth process. The concentration of the CTAB surfactant was kept constant at 0.05M.

The imaging platform is based on the total internal reflection sensor illumination arrangement described in details elsewhere (Olkhov et al. 2009). Before the nanoparticle development step, the sensor surface does not scatter light with sufficient intensity for the array to be visible. The chemical growth of the seed particles significantly increases their light scattering efficiency and is recorded as a brightness change in the areas where nanoparticles were printed. The time-dependent responses from the 16 array spots printed with the same seed concentrations are averaged together, producing corresponding kinetic growth curves of the gold seed nanoparticles in the chosen developer.

**Biosensor Immunoassay**

Several sensor arrays were used in immunoassay experiments to examine the effect of the graphene-coated substrate on the assay performance. The gold nanoparticle surface was first activated for protein binding using Lomant’s reagent and then the slides were returned to the inkjet printer and functionalized with two antibodies, aTRA and aCRP, and A/G, TRA, and BSA proteins, the detailed biosensor manufacturing procedure is described elsewhere (Olkhov et al. 2009). The biosensor arrays were employed in biological immunoassay: after the array was stabilized in a flow of PBS buffer, 200 nM anti-transferrin and 100 nM transferrin solutions were injected sequentially in the flow cell for ca. 10 minutes each with buffer wash step in between the injections. The observed transient responses were averaged over the similarly bio-functionalized array spot sets.

**Results and Discussion**

Sensitivity and performance of an optical biosensor based on the plasmonic properties of the gold nanoparticles depends critically on the shape and size of these particles which can then be optimised for a specific application. Chemical growth of the nanoparticles directly on the sensor substrate surface allows straightforward control of their location in an array format, their size and also their number densities. While the number of the particles is determined by the seed particles concentration in the printed solution, the final size of the grown nanoparticles is a function of parameters such as: temperature, chemical composition of the developer, properties of the interface
Graphene is known to influence redox reactions (Shi et al. 2013) and therefore it is expected that the growth of the gold particles by chemical reduction of the auric chloride could be different on the graphene coated surfaces.

The experimental kinetic growth curves are recorded as change of the light scattering. The observed intensity change can be converted into gold mass change by making some assumptions about the evolution of optical properties with particle size. The grown particles are approximately spherical in shape and the scattering and absorption properties of spherical particles can be calculated using Mie theory, if dielectric properties of the metal particle is known (Scaffardi and Tocho 2006). As a first approximation, the bulk gold permittivity can be used but when the size of the nanoparticles is smaller than the electron mean free path in bulk gold (~50 nm (Link and El-Sayed 1999)), the electron scattering from the particle boundary becomes significant and affects the dielectric function (Averitt et al. 1997). The contribution of the bound electrons to the dielectric function is usually considered size-independent for particles larger than 2 nm, and only the free electrons contribution is taken as particle size dependent (Scaffardi and Tocho 2006). The complex dielectric function for the free-electrons contribution is described by the following equation (Kreibig and Genzel 1985; Link and El-Sayed 1999; Scaffardi and Tocho 2006):

\[
\varepsilon_{\text{free electrons}}(\omega) = 1 - \frac{\omega_p^2}{\omega^2 + i\omega(\gamma_{\text{bulk}} + C_{\text{F}})}
\]

Equation 1

where \(\omega_p\) is the bulk plasma frequency \(13 \times 10^{15}\) Hz, \(\beta_F\) is the electron velocity at the Fermi surface \(14.1 \times 10^{14}\) nm s\(^{-1}\), \(\gamma_{\text{bulk}}\) is the dumping constant for free electrons = \(1.1 \times 10^{14}\) Hz, \(R\) is the radius of the particle, and \(C\) constant associated with details of scattering processes was taken as 0.8 (Scaffardi and Tocho 2006). The complex dielectric function depends on the particle size, with about twofold increase in the imaginary part for 10 nm particles compared with the respective bulk metal value, while the real part changed by <1%, Fig. 1A.
Fig. 1. Dependence of optical properties of gold nanoparticles on their size: (A) real (top) and imaginary (bottom) parts of the refractive index, with dotted lines corresponding to the respective bulk gold values; (B) scattering efficiency in medium with RI of 1.3335 at 660 nm, the wavelength used in kinetic particle growth experiments.

After the particle size correction has been applied to the optical properties of the bulk gold (Johnson and Christy 1972), the corrected dielectric function was converted to a size-dependent refractive index, Fig. 1A, which in turn was used in Mie theory calculations (Charamisinau et al. 2005) to give wavelength and particle-size dependent scattering and absorption efficiencies, Fig. 1B. The variation of the gold dielectric function with temperature is small and was neglected (Link and El-Sayed 1999).

Assuming non-interacting particles, the overall observed scattering brightness is proportional to the number density of the particles on the sensor surface. Although a known concentration of the seed particles was printed per array spot, the total number present in the array spot depends on the surface abilities to attract and immobilise particles from the printed colloid with a reasonable affinity.

The substrate arrays were imaged in scanning electron microscope (SEM) at low resolution / large field-of-view settings after each of the growth experiments to determine the particle number density by counting the particles in several images, and the overall gold surface coverage as a bright fraction of the image area. The higher magnification images were used to measure the particle size distribution, Fig. 2 and suppl. Fig. S2. The overall gold surface coverage can also be calculated using the counted number density from low-resolution images and the particles size values from
high-resolution images. The gold surface coverages estimated by these two approaches are in good agreement (the correlation is shown in supplemental material Fig. S3).

The observed particle number densities on different substrates are shown in Fig. 2A. The BSA-rGO substrate supports the largest density of nanoparticles, up to 80 particles per \( \mu m^2 \), reflecting the enhanced affinity of the seed particles for the protein surface (Liu et al. 2010). In a separate experiment the used graphene material suspensions in water were incubated with gold colloid (15 nm diameter citrate reduced gold particles); only the BSA/FG-rGO samples effective captured the gold particles, while GO and MB-rGO were hardly successful (supplemental Fig. S1). The lowest particle number density, about 18 particles per \( \mu m^2 \), was observed on the MB-rGO substrate which carries a strong negative charge from the methyl blue dye and therefore repels similarly charged colloidal gold particles. The array spots on the MB-rGO coated substrate also show small regions of very high particle density (supplemental Fig.S5), which are interpreted as gaps in the graphene layer where bare, positively-charged aminated glass surface is exposed and captures the particles efficiently.

![Fig. 2.](image-url) (A) Surface number densities, grey, of the nanoparticles printed with stock 55nM seed colloid, and their sizes, black, observed in SEM image data after 40 minutes growth in the standard developer. (B) SEM images of the grown nanoparticles on different substrates, scale bar is 1\( \mu m \).
The concentration of the printed seed nanoparticles is the dominant factor in determining the final number of particles on the surface. Although the used concentrations were sequential three-fold dilutions of the stock 55 nM seed colloid, the observed surface number densities were not accurately proportional to the printed seed concentrations (supplemental Fig. S4). On the ASG, GO, and BSA-GO the lowest used dilution of seed colloid, 6 nM, deposited significantly less, ca. 50%, particles on the surface than expected comparing with the middle seed concentration, 18 nM. Similar effect was observed for the 55 nM and 18 nM printed seed concentrations on the MB-rGO sample. The effect limits the utility of the seed colloid concentration as a control for the desired surface density of the nanoparticles.

Fig. 3. Seed mediated growth of gold nanoparticles on the BSA-rGO substrate surface: (A) experimental kinetic traces corresponding to the three colloids which are printed with varying seed solution concentrations, a – 55 nM, b – 18 nM, and c – 6 nM, the insert shows part of the developed sensor image with printed spots; (B) particle radii derived from the kinetic traces in A using the scattering efficiency shown in Fig. 1 and the final particle sizes and number densities measured from SEM images, dash lines are simulations.

A simple description of the particle growth kinetics can be based on an assumption of either reactant diffusion or surface reaction limited mechanism. There is a large excess of the reagents in the bulk of the developer solution flow and their concentrations are treated as constant. If diffusion of the reactants to the particle surface is a rate determining process then the particle size shall increase with a $t^{1/3}$ dependence, whereas a surface reaction controlled growth would lead to a linear particle size increase with time.
The observed growth behaviour is more consistent with the surface reaction limiting step in the growth process. Similar mechanism with a linear growth law was postulated in a study of gold nanoparticle growth in solution (Averitt et al. 1997). All recorded kinetic traces display an initial fast scattering increase phase with further growth that shows approximately linear growth after 15 minutes, Fig. 3A. The kinetic mechanism for the growth of nanoparticles has two extreme processes: reagent diffusion limited and surface redox chemistry limited. There is an indication of growth rates slowing down after 16-17 minutes, which might be associated with an onset of the reagent transport limiting kinetics. Fitting the initial 15 min period of the kinetic trace to a line function, Fig. 3B, results in a slope parameter describing the particle growth rate and an offset corresponding to the initial radius of the seed particles. The offset falls within the range 2.6-3.1 nm, in reasonable agreement with the known size distribution of the used seed particles, 4-5 nm diameter. The linear growth rate has been derived for each surface and is presented in Table 1.

Table 1. Characteristics of the gold nanoparticles grown on the studied substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Surface number density, (particles μm²)</th>
<th>Grown particle radius, (nm)</th>
<th>Growth rate, (nm s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASG</td>
<td>51±3</td>
<td>63±9</td>
<td>0.018±0.004</td>
</tr>
<tr>
<td>GO</td>
<td>56±3</td>
<td>41±5</td>
<td>0.014±0.002</td>
</tr>
<tr>
<td>BSA-rGO</td>
<td>79±4</td>
<td>45±4</td>
<td>0.014±0.002</td>
</tr>
<tr>
<td>MB-rGO</td>
<td>17±1</td>
<td>78±7</td>
<td>0.031±0.002</td>
</tr>
</tbody>
</table>

The particle growth rates obtained for developer compositions with varying auric chloride concentration, Fig. 4, show that the rate is approximately proportional to the concentration of gold ions in the developer within the range 0.1-0.5 mM, hence it can also be used as a growth rate control if required.
The ability to grow different shapes of nanoparticles on graphene substrates is significant advantage for metal-graphene nanocomposites in new applications (Jasuja and Berry 2009; Lee et al. 2011). The control of particle shape is not straightforward and it is known that the bulk solution methods show different results in size and shape compared to nanoparticles grown at the solid-liquid interface (Olkhov and Shaw 2008). The gold nanorods growth method with CTAB surfactant (Murphy et al. 2005), on which the current development is based, was also reported to produce rounder thorny/star-shaped gold nanoparticles (Nehl et al. 2006) in slightly modified conditions (NaOH presence) which disturb CTAB binding. Application of the current development procedure results in nearly spherical particles, with some indication of sharper geometrical features forming at the later stage of the growth (supplemental Fig. S6). If other shapes are desired then different development chemistry shall be used, for example decylypyrene surfactant was reported to be more effective in nanorod formation than CTAB when particles are grown on graphene surface (Kim et al. 2010).

The derived values of the gold nanoparticle radius growth rates within initial 15 minutes of the process are shown in Table 1. The seed particles appear to grow somewhat slower on the surface of graphene oxide and protein decorated reduced graphene oxide, compared with the reference aminated glass surface. By contrast, the growth rate is almost twofold increased on the surface of the
methyl blue reduced graphene oxide. A tentative explanation might be related to the conductivity of the surface layer on which the seed particles are deposited. ASG and GO substrates are not conductive, and the protein decoration of the BSA-rGO layer may also prevent particle contact with the conductive rGO sheet, while small aromatic methyl blue decoration of MB-rGO cannot shield the seed particle from interaction with conductive reduced graphene (Cai et al. 2011), which might serve as a source of electrons and/or reduce electrochemical potential for Au(I) reduction at the vicinity of the seed nanoparticle.

The derived growth rates and SEM data allow the average gold mass deposition rate on the substrate surface to be estimated as ≪6×10⁻⁴ g cm⁻² h⁻¹ for the range of the used samples. This can be compared with the reported gold influx in the relevant study of dendritic gold nanostructures grown on graphene oxide of ca. 0.13 g cm⁻² h⁻¹ at 25 °C in diffusion controlled process (Jasuja and Berry 2009). The two-hundred-fold slower gold influx estimated here is consistent with the postulated reaction-limited rate at the beginning of the growth, when seed particle radius is increased by factor of ten, its area, which determines gold reduction rate, becomes hundred times larger and the growth process is expected to be diffusion controlled. The side effect of the diffusion control at the later stage of the growth is the observed inverse correlation between the final size and the number density of the nanoparticles on the surface.

The ultimate aim of the current study consider the potential of the graphene-composite materials in the production of biosensor surfaces in the array format multiplex biosensor. While GO and MB-rGO coated substrates have shown no improvement over the biosensor arrays printed on the standard ASG substrate, the BSA-rGO samples delivered important improvements in a particular type of the immunoassays, where antibodies are printed on the surface of the sensor to detect proteins in solution; a label-free protein screening array.

The biologically specific array sensors were manufactured with immobilized protein A/G, aTRA, TRA, and aCRP. Protein A/G binds antibodies via their non-specific Fc region thus allowing to create one specific-protein sensor channel with the antibody immobilized on the surface during the course of the assay. When the sensor array is first exposed to aTRA solution, Fig. 5, the analyte antibody binding is observed in the TRA and protein A/G channels, the latter in effect corresponds to the formation of the second TRA sensitive channel on the sensor. After a wash with running buffer the antigen TRA protein was flowed over the surface to measure the sensor binding sensitivity. At the end of the assay, the entire sensor surface was re-generated with an acid wash removing adsorbed aTRA and TRA. The ASG sensor can be reused about 10 times with 2-5%
performance loss per assay sequence. The BSA-rGO sensor was more susceptible to the degradation during the regeneration step, losing about half of the detection performance after a single regeneration step.

Fig. 5. TRA and aTRA kinetic immunoassays performed on the sensor arrays manufactured using BSA-rGO (A) and ASG (B) substrates. aTRA antibody solution was injected at the start of the experiment, it binds to the immobilized TRA (a) and is also captured by protein A/G (b). The TRA solution was injected at ca. 28 min., resulting in evident sensor response in aTRA (c) and aTRA-protein A/G (b) channels on BSA-rGO substrate (A) while only aTRA-protein A/G (b) channel show visible response on standard ASG substrate (B). Relative response corresponding to the antibody activity in aTRA-protein A/G channels is about twofold higher for BSA-rGO compared with ASG. The printed aTRA antibody (c) retained its activity only on BSA-rGO substrate. aCRP channel (d) presented as a non-specific reference.

The sensor channels with protein A/G and TRA were responding to the injection of aTRA antibody, the former capturing immunoglobulin at Fc region, and the latter forming specific antibody-antigen complex. Apart from the weaker, about twofold, overall response from BSA-rGO based sensor compared with the standard ASG the sensors performed similarly in the antibody capture assay step. aTRA captured by protein A/G forms a second aTRA sensor channel in addition to the one created during sensor functionalisation with printed biomolecules. The injection of TRA analyte at ca 28 minutes is expected to be detected in both printed aTRA, (c) in Fig. 5AB, and captured aTRA-protein A/G, (b) in Fig. 5AB, channels. The standard ASG sensor array responded only in aTRA-protein A/G (b) with ca. 30% activity from the ratio of the detected TRA to the captured antibody amount, no significant signal, <3%, was detected in aTRA channel (c) indicating that the printed antibody has lost its antigen recognition activity. The sensor based on BSA-rGO substrate showed
about 60% in corresponding aTRA-protein A/G detection efficiency and, more importantly, it also showed similar in value response in the printed aTRA channel, Fig. 5A(c), implying that the BSA-rGO surface is about 20-fold more effective (60% against <3%) in maintaining the activity of the immobilized aTRA antibody.

**Conclusions**

Several substrates coated with thin layer of graphene materials were studied as candidates for spatially controlled surface gold nanoparticle growth with ultimate target of their usage as multiplex biosensors. Both the deposition of the seed nanoparticles and the kinetics of the seed mediated particle growth were influenced by the surface functionalization with graphene samples. The rates of the particle growth indicate that the process is reaction controlled at the employed experimental conditions on all tested surfaces. The choice of the surface coating and the development conditions (concentration of printed seed particles, chemical composition of the growth solution, development time) allow some control over the surface number density and size of the grown particles. Among the tested samples the BSA reduced graphene oxide coated substrate appears to be a promising candidate as a base for antigen capture array biosensors, although further optimisation of the overall sensor sensitivity and stability is desired.
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