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Review

The emerging role of nanomaterials in immunological sensing — a brief review

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ABSTRACT

Nanomaterials are beginning to play an important role in the next generation of immunological assays and biosensors, with potential impacts both in research and clinical practice. In this brief review, we highlight two areas in which nanomaterials are already making new and important contributions in the past 5–10 years: firstly, in the improvement of assay and biosensor sensitivity for detection of low abundance proteins of immunological significance, and secondly, in the real-time and continuous monitoring of protein secretion from arrays of individual cells. We finish by challenging the immunology/sensing communities to work together to develop nanomaterials that can provide real-time, continuous, and sensitive molecular readouts *in vivo,* a lofty goal that will require significant collaborative effort.

1. Introduction

Detection of immune-related biomarkers from body fluids is a cornerstone of biomedical research and clinical diagnostics. The development of monoclonal antibodies in the 1970′s and the related development of immunoassay techniques in plates, on cells, and on paper strips facilitated research breakthroughs, and also generated a whole range of devices for clinical diagnostics in both developed and developing areas. Some of the key challenges remaining for immunological sensing include extremely low immune analyte concentrations (pM or lower), and the lack of techniques for robust multiplexing, real-time and/or continuous monitoring of cell-secreted analytes (mainly proteins or peptides), as well as the capture or analysis of extremely rare cells. Microfluidics has made significant in-roads in simplifying and miniaturising a range of immunoassays to begin to address these needs (for detailed reviews see Junkin and Tay, 2014; Yetisen et al., 2013; Chin et al., 2012). This is especially the case in the area of cell capture and analysis, with new devices available for important applications including CD4+ cell counting in the context of HIV management (Glynn et al., 2013), and circulating tumor cell (CTC) isolation, enumeration and single-cell analysis (Hyun and Jung, 2014; Dong et al., 2013). In this brief review, we focus on two key examples of where nanomaterials have been incorporated into immunological sensors and assays, specifically resulting in (a) an improvement of detection limits in traditional immunoassays and (b) the continuous monitoring of cytokine secretion from individual cells. We finally challenge the field to combine these advances and develop new approaches for continuous, *in vivo* monitoring of immunological biomarkers.

From an analytical point of view, we can identify two major classes of biomarkers which are of most interest in immunology, namely the immune cells and the soluble factors that they secrete. The latter are usually peptides and proteins, such as cytokines, cytokine receptors, and antibodies. Traditionally, immune cells have been characterised based on specific combinations of cell-surface and intracellular proteins, which can be interrogated on a cell-by-cell basis using high-speed and multi-parametric flow cytometry (and, more recently, using CyTOF – a combination of cytometry and mass spectrometry that replaces fluorescence labelling of cells with unique rare metal labels for improved detection limits and multiplexing (Bjornson et al., 2013)). Similarly, multiple immune-related soluble factors are often detected by ELISA-type or flow-based approaches (ranging from traditional microplate ELISAs to suspension bead arrays and microfluidic alternatives). Often the experimental detection limits are restricted to moderately abundant markers, and hence the biological fluids which contain them. Thus, whilst sera is often tested, the study of other fluids, such as breath exudate or saliva are still challenging using conventional techniques. Similarly, whilst blood is often tested to sample immune cells in humans, relevant information at the site of the immune reaction (for example the tumour microenvironment) is not easily accessed (Zhou et al., 2014). Hence, evaluations on excised tissues suffer from the

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recovery of low numbers of immune cells for analysis, or the restriction to techniques such as confocal microscopy or immunohistochemistry, which enable assessment of a limited number of cell-associated markers, compared to flow-based techniques. New approaches that could bridge these technical gaps could provide new information for both discovery biology and clinical diagnostics.

Cytokines include the interleukins, lymphokines and cell signalling molecules that allow communication between immune cells, creating dynamic and cross-regulatory networks *in vivo*. While typically collected via blood sampling, this approach precludes both continuous and/or location-specific monitoring. For example, cytokines produced by T helper 2 (Th2) cells will modulate production of cytokines produced by T helper 1 (Th1) cells and vice-versa, and these processes are of great importance to the control of lung allergic airways inflammation (Hardy et al., 2013). Key cytokines such as IL6, and immune cells such as regulatory T cells, substantially fluctuate in levels over the day as well as upon daily sequential sampling, confounding their utility as potential diagnostic and prognostic markers of disease (Madondo et al., 2016). However, such fluctuations in and of themselves, if they could be accurately and continuously measured, could generate new types of diagnostic or prognostic data. As a speculative example, failure to rapidly correct a spike in an inflammatory cytokine back to a homeostatic level could indicate loss of immune regulatory control, which would point to a susceptible state to develop an autoimmune inflammatory disease. Moreover, cytokines produced by immune cells such as monocytes or by cancer cells such as IL10, create immune-suppressive niches in the body, which do not reflect overall peripheral levels. For example, in ovarian cancer, ascites fluid which accumulates in the peritoneum shows substantial enrichment in cytokines such as IL6, TNF, IL10 and TGF-β, which together promote an optimal environment for the maintenance and growth of these tumours (Govindaraj et al., 2013). Monitoring local changes in cytokines in such tumour environments may therefore have diagnostic and prognostic utility.

Based on the approaches and examples outlined above, non-invasive monitoring of an immune cell (or cells) over substantial amounts of time, as well as the cytokines that they produce under specific conditions could be extremely useful in discovery biology and disease management. These experiments require new ways of transducing this information, within the dynamic range of the proteins relevant to the immune phenomena being studied, and which often include low-abundance proteins. These are examples of important questions that cannot currently be addressed with available technology, however the field of nanomaterials has opened up new opportunities.

2. Metallic films and nanoparticles display unique optical properties leading to signal enhancement

A key advance in immuno-sensing facilitated by nanomaterials is the improvement of detection limits for low abundance proteins, including cytokines. Firstly, as labels, metallic nanoparticles have significantly higher molar absorption coefficients (ε ($M^{-1}cm^{-1}$), used to calculate concentration from absorbance via Beer's Law $-A = \varepsilon CL$, where C is concentration (M) and L is path length (cm)) in comparison to the products of enzymatic substrates commonly employed in ELISA and related techniques (*e.g.* $> 10^8$–$10^9\,M^{-1}cm^{-1}$ for gold nanoparticles vs. $5.9 \times 10^4\,M^{-1}cm^{-1}$ for the product of the common ELISA substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) (Satija et al., 2016)). Furthermore, in the case of nano-scale materials, it is well known that they display very different behaviour from their bulk material counterparts due to enhanced surface area and opto-electronic properties. While an in-depth description of these phenomena is beyond the scope of this article, we briefly describe the key effects below, and direct readers to recent reviews on this topic (Satija et al., 2016; Taylor and Zijlstra, 2017; Singh et al., 2017). For metallic nanoparticles in particular, the oscillations of conduction-band electrons that move through the material are constrained at the particle surface in a manner that does not occur in bulk materials; this leads to "plasmonic effects" at the surface, which are particularly sensitive to the particle size, shape, aggregation state, and local chemical environment. These changes can be observed via detectable shifts in peak wavelengths in absorption spectra ("localised surface plasmon resonance" or LSPR), but may also result in significant enhancement of absorption and/or emission of fluorescent dyes attached to the particle surface ("metal-enhanced fluorescence" or MEF (Geddes and Lakowicz, 2002)). LSPR is easily observed in gold or silver nanoparticles (Satija et al., 2016), with peaks in the visible-NIR wavelength range.

3. Using nanoparticles to improve ELISA sensitivity and multiplexing potential

Over the past 10 years or so, nanoparticles that demonstrate LSPR have been used to replace enzyme substrates for improved protein detection limits in ELISAs, resulting in "plasmonic ELISA" (pELISA). For example, several plasmonic assays have been developed for detection of prostate specific antigen (PSA), with detection limits ranging from fM-aM, in comparison to pM for several commercial plate ELISAs (Yang and Gao, 2015; de la Rica and Stevens, 2012; Rodriguez-Lorenzo et al., 2012; Liu et al., 2014; Liang et al., 2015). In each approach, the immunoassay procedure follows the traditional methods (capture of antigen to an antibody-coated surface followed by detection with an enzyme-labelled secondary antibody), however in the final step, instead of adding colourimetric or fluorigenic substrates, reagents are added to aggregate, grow or modify nanoparticle shape/size in an enzyme-dependent manner (Fig. 1**ai**). The four specific approaches (Fig. 1**aii-v**) include enzyme-dependent: (ii) aggregation of single nanoparticles into larger clusters; (iii) chemical etching which leads to a change in nanoparticle shape; (iv) coating nanoparticles with a shell of different composition to change local refractive index, or (v) nanoparticle growth from soluble components. In each case, the wavelength of the major absorption peak is shifted significantly during the reaction, in a manner dependent on the analyte concentration. Method (iii) is conceptually the most challenging, yet the others are prone to non-specific aggregation in response to small changes in environmental conditions, potentially leading to false negatives or positives. Optimization under field trial conditions will be required for the broad applicability of this approach. Furthermore, the plasmonic modification to the general ELISA strategy does not simplify the laborious and time-consuming nature of the assay, therefore perhaps these assays could be integrated into emerging microfluidic platforms to address this issue.

Inorganic nanoparticles are not the only emerging materials for enhancing detection limits in ELISA technology. Polymeric materials have also been used in innovative ways to detect lower concentrations of rare analytes. For example, Lai, et al., developed stimuli-responsive antibody-polymer conjugates (Fig. 1**b**) which, after binding antigen, could be specifically aggregated and de-aggregated for separation purposes based on a simple temperature change (Roy et al., 2013). The block co-polymers employed comprised a mixture of hydrophilic and hydrophobic blocks, such that above a critical temperature (LCST – lower critical solution temperature) in aqueous solutions, the polymer switched from a hydrophilic material to that of a hydrophobic material, resulting in aggregation. Combining this capture/isolation mechanism with traditional enzyme-labelled detection antibodies, the authors then developed a microfluidic assay whereby upon heating, the complex (capture antibody-polymer conjugate, antigen, detection antibody) non-specifically bound to the walls of the chamber, allowing for substantial washing prior to signal production (Hoffman et al., 2015). Moreover, this approach allowed for successive rounds of low-volume antigen addition steps, leading to detection limits of 1.8–2.2×10^6 molecules, from 0.08–7.5 μL sample. This is comparable to several commercially available systems which take longer to completed and require 20–100 μL sample volume. More recently, this group added magnetic nanoparticles to their assay toolbox (Nehilla et al., 2016),

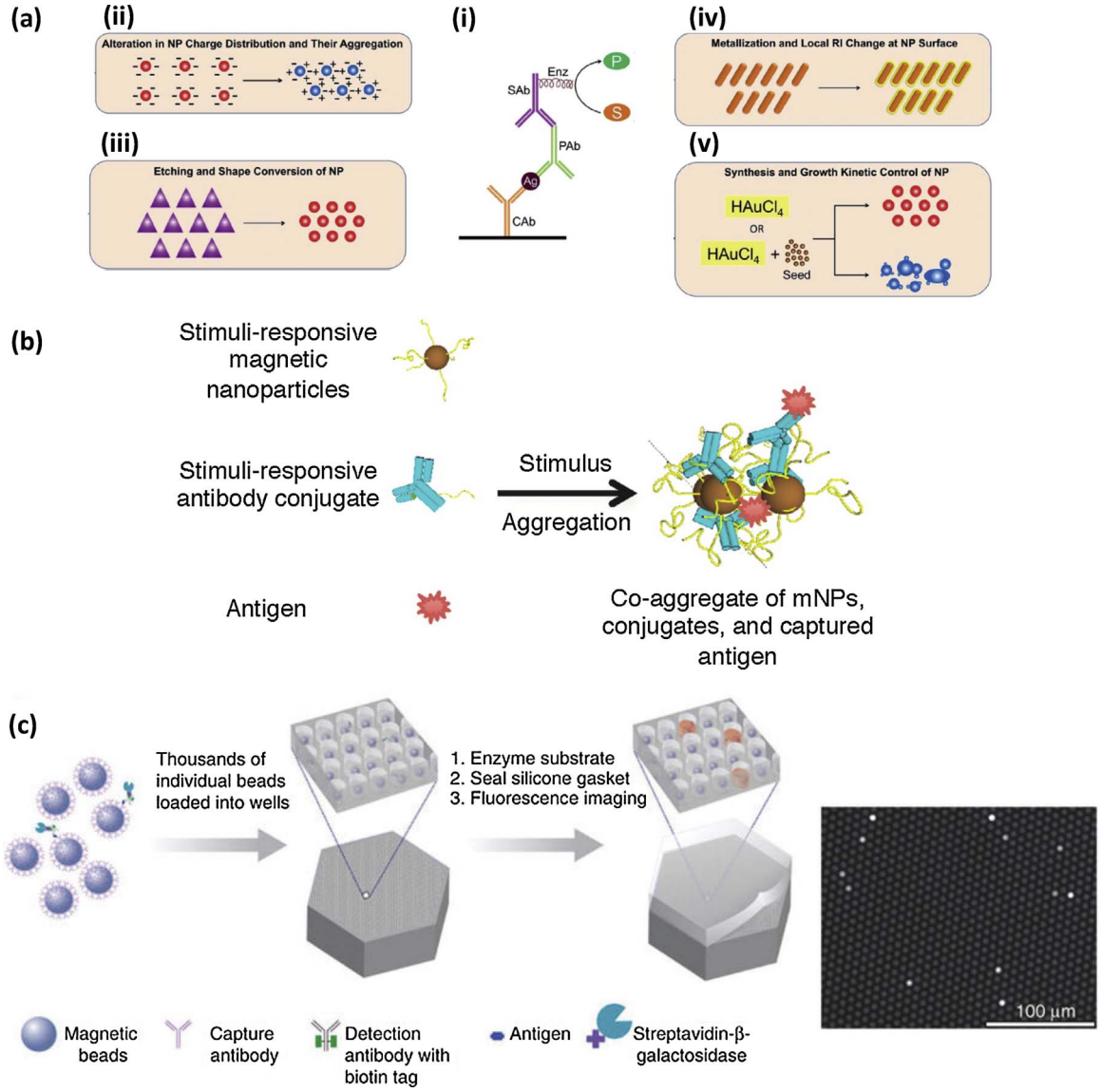

Fig. 1. Plasmonic ELISA (pELISA) involves replacement of the traditional enzyme substrate with plasmonic nanoparticles. (a) i) standard ELISA procedure wherein capture antibodies are coated onto a surface which capture antigens from biological samples, which in turn bind to specific detection antibodies. Enzymatic reporting via LSPR can take place via the following mechanisms: (ii) controlled aggregation of nanoparticles associated with a colour change; (iii) shape changes of nanoparticles via enzyme-dependent etching; (iv) metallization of nanoparticles thereby changing refractive index; (v) nanoparticle nucleation/growth from seeds leading to a colour change. Reproduced (adapted) from Ref. Satija et al. (2016) with permission of The Royal Society of Chemistry (https://doi.org/10.1039/C6RA16750K). (b) Stimuli-responsive nanomaterials in bioassay development; protein antigen binds to polymer-coated antibody in solution in the presence of polymer-coated magnetic nanoparticles. A subsequent change in temperature leads to specific aggregation of antigen/antibody complexes, which can then be separated magnetically for further processing. Reprinted (adapted) with permission from Analytical Chemistry, 2016, 88 (21), pp 10404–10410. Copyright 2016 American Chemical Society. (c) An example of "digital ELISA" based on the Quanterix Simoa technology, in which fluorescently-encoded particles coated with unique antibodies are mixed with antigen mixtures before being captured in single-particle nanowells in an array. Statistically, the majority of particles are associated with only a single target molecule, hence counting "positive" wells based on enzymatically-labelled secondary antibodies provides an estimate of protein concentration. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology (2010, 28, 595–599), copyright 2010.

which allows rapid and temperature-specific aggregation and re-dispersion of antibody-antigen complexes, with higher efficiency and faster kinetics than commercial magnetic microbeads.

Beyond solution-phase assay formats, another emerging application of nanomaterials in ELISA is related to "digital" assays in which arrays of single molecule reactions on nano-microparticle surfaces can be interrogated simultaneously. Single-molecule array ("Simoa") technology, developed by Walt's group and commercialised by Quanterix (Cohen and Walt, 2017), is perhaps the most advanced example, in which an array of nanowells constructed on a fibre optic cable are filled with individual nanoparticles for analysis (Fig. 1**c**). The nanoparticle size is chosen such that each well only fits one nanoparticle, and capture/detection reactions are performed on the particles in such a way as to statistically limit the number of events per particle to the single molecule level. In this way, one can simply count the number of "positive" wells based on secondary labels in order to determine the number of captured molecules in the experiment. The secondary antibodies are labelled with streptavidin-β-galactosidase, which rapidly converts resorufin β-D-galactopyranoside to the fluorescent molecule resofurin for analysis via fluorescence microscopy. In a recent publication, Wu et al., demonstrated that Simoa technology could detect 10 different human cytokines down to 0.1–1 fM when titrated in 25% calf serum, and consistently detected the presence of each cytokine in healthy human blood samples, covering concentrations ranging from 10 to 70 fM (Wu et al., 2015). Such methods are potentially calibration-free in the same ways that gravimetric (mass counting) or coulometric

(electron counting) approaches do not require calibration. Indeed, Gooding and Gauss argue that due to the rapid and significant advances in the field of single molecule detection strategies in the past 20 years, these measurements can now be considered somewhat "routine", and the challenge now is to monitor many, many events at once in order to develop enough information to answer biologically-relevant questions (Gooding and Gaus, 2016).

4. Cell arrays for detecting cytokine secretion

Beyond the quantification of immune-related proteins in a biological sample, there have also been advances in technology to monitor protein secretion in real-time from individual cells. Key nano/microfluidic platforms in this area include SCBC (single-cell barcode chips), introduced by the Heath lab (Bailey et al., 2007) and commercialised by Isoplexis, and micro-engraving, introduced and developed by the Love lab (Zhou et al., 2014; Torres et al., 2013; Yamanaka et al., 2012; Varadarajan et al., 2012). Both approaches involve (a) isolating single cells by size restriction in nanowells, followed by (b) capturing/detecting secreted molecules in close proximity to the cell over time, and (c) performing these actions in an array format to gather large datasets on many individual cells (Fig. 2**a/b**). In the SCBC, once cells are loaded in the array, it is covered with a microscope slide patterned with arrays of antibodies, such that the secreted proteins from each single cell are captured by a full antibody panel. Once the incubation is complete, the slide is removed and exposed to a panel of fluorescently labelled secondary antibodies, and then slides are imaged and single-cell protein secretion analysed. As an example of the impact of the method, Lu et al. demonstrated that when using a 45-plex antibody array (including 3 controls), they could analyse cytokine secretion profiles for LPS-stimulated macrophages in comparison to unstimulated controls, identifying significant heterogeneity in function and pathogen activation (Lu et al., 2015). The high level of multiplexing was achieved by combining spatial encoding (15 individual bars) with fluorescent encoding (3 dyes). The process is similar in microengraving, except that additional imaging cell cytometry is employed at the same time as conducting microarray analysis of single cell-secreted proteins captured by the antibody-coated slides (Heath et al., 2016). As an example, Love et al. recently used the approach to investigate the quality and breadth of antibody production following vaccination in non-human primates. They were able to determine the distribution and frequency of vaccine-specific antibody-secreting cells and memory B cells in the context of pneumococcal immunisation, determining the antibody isotype produced by each cell type and the fraction of antigen-specific cells (Jia et al., 2017). The key advantage of these approaches over traditional methods is that less cells are required overall for analysis, and the cells are analysed at single-cell resolution rather than generating a single datapoint from an ensemble of cells (*e.g.* ELISpot, *etc.*).

Beyond the nano-well array technology, a number of unique approaches have arisen employing a range of nanomaterials for detecting cytokine secretion from immune cells (Fig. 2**C–E**). Wang, et al., incorporated nanoplasmonic resonators (layers of noble metal interspersed with insulating films) into arrays of nano-wells, leading to metal-enhanced fluorescence in each well. This approach led to pM-nM detection limits of IL-2 dynamic secretion profiles at sub-cellular 2D resolution (Wang et al., 2011). Pui, et al., developed an electrochemical detection system that involved capture and detection of cytokines onto antibody-functionalised silver "nanowires" (AgNWs), requiring only 20 nL analyte in 40 μL samples (Pui et al., 2011). At the nano-scale, the specific binding of cytokines onto the functionalised AgNWs led to minute perturbations in the local charge environment, which could be detected by changes in the conductance of the AgNW. This approach led to fM detection limits for TNF- α and IL-6, although significant dilution of samples (cell-culture or serum) was required for selective binding, suggesting that non-specific binding could be an issue. Raphael, et al., also used gold nanostructures to take advantage of nanoplasmonic effects (Raphael et al., 2013). They functionalised microscope slides with arrays of gold nano-pillars coated in capture antibodies, and by combining fluorescence microscopy (monitoring the cells) with LSPR analysis of cytokine-specific capture, they were able to map the "waves" of cytokine secretion in 2D and over time. This approach was updated by Oh, et al., to combine nanoplasmonics with multiplexed cytokine arrays, where they demonstrated pg/mL sensitivity for monitoring secretion of 4 different cytokines over time (Oh et al., 2016). Most recently, Li et al., have shown that other geometries of nanoplasmonic shapes can also be used to enhance protein detection, using nanohole arrays coated with capture antibodies to monitor secretion of VEGF from HeLa cells (Li et al., 2017a). Confinement of light in the sub-wavelength diameter holes leads to "extraordinary optical transmission", in which minor variations in local refractive index (*e.g.* those caused by binding of VEGF to adjacent capture antibodies) have large effects on signal intensity.

It is important to note that the assays and biosensors developed for high-sensitivity and/or continuous monitoring applications need to be robust and reliable. This is particularly important then attempting to detect immune-related proteins which may be in the fM concentration range in humans (Wu et al., 2015). For routine use, nanomaterial-based biosensors should be physically robust, unaffected by sample matrix composition, with internal controls or calibrants to account for signal drift (especially for continuous monitoring). The physical stability of the system is important so that surfaces are not stripped away, and bioreceptors (*e.g.* antibodies or enzymes) are not degraded, during operation or regeneration cycles. In the case of plasmonic ELISAs, signal generation is governed by complex colloidal phenomena which are sensitive to changes in the local environment, so these techniques will need to be rigorously field tested (Satija et al., 2016). For all biosensors, it is important that the sample matrix (*e.g.* blood, saliva, tissue lysate, *etc.*) does not affect the sensing chemistry to the point that the system no longer shows a linear response across the relevant physiological range of the target analyte. Finally, for those techniques that require it, calibration sites or ratiometric comparators are vital to confirm that analyte concentrations are faithfully tracked over a number of sensing cycles, without being dramatically affected by sample and environmental noise.

5. From in vitro to in vivo − new applications of nanoparticles in immuno-sensing

While detecting extremely low concentrations of multiple cytokines in extracted blood samples, or dynamically secreted from cultured cells, has been facilitated through advances in nanomaterials and micro/nanofluidic technologies, monitoring key immune biomarkers *in vivo* remains an elusive goal. While taking blood samples and measuring cytokine concentrations from one moment in time is extraordinarily useful (indeed it is the basis of most clinical diagnostic assays!), the ability to monitor temporal changes in concentration during an immune process or disease time-course would be far more revealing. Frequent blood sampling can provide course-grain dynamic profiles, however immunological processes take place in specific locations outside of circulating blood (except perhaps for haematological processes), where opportunities for frequent sampling is limited. Furthermore, from a medical point of view, blood samples are only taken once symptoms are apparent, hence early detection of life-threatening events (*e.g.* sepsis, cancer) is extremely difficult. The logical solution is to develop minimally-invasive biosensors that can be deployed to specific locations in the body to report back calibrated changes in the levels of various cytokines and immune biomarkers in a continuous manner. We suggest that nanoparticle sensors ("nanosensors") could pose a unique solution, however equally there are a number of challenges which need to be overcome.

While nanoparticle-based imaging labels are being translated into the clinic (Smith and Gambhir, 2017), there are relatively few reports

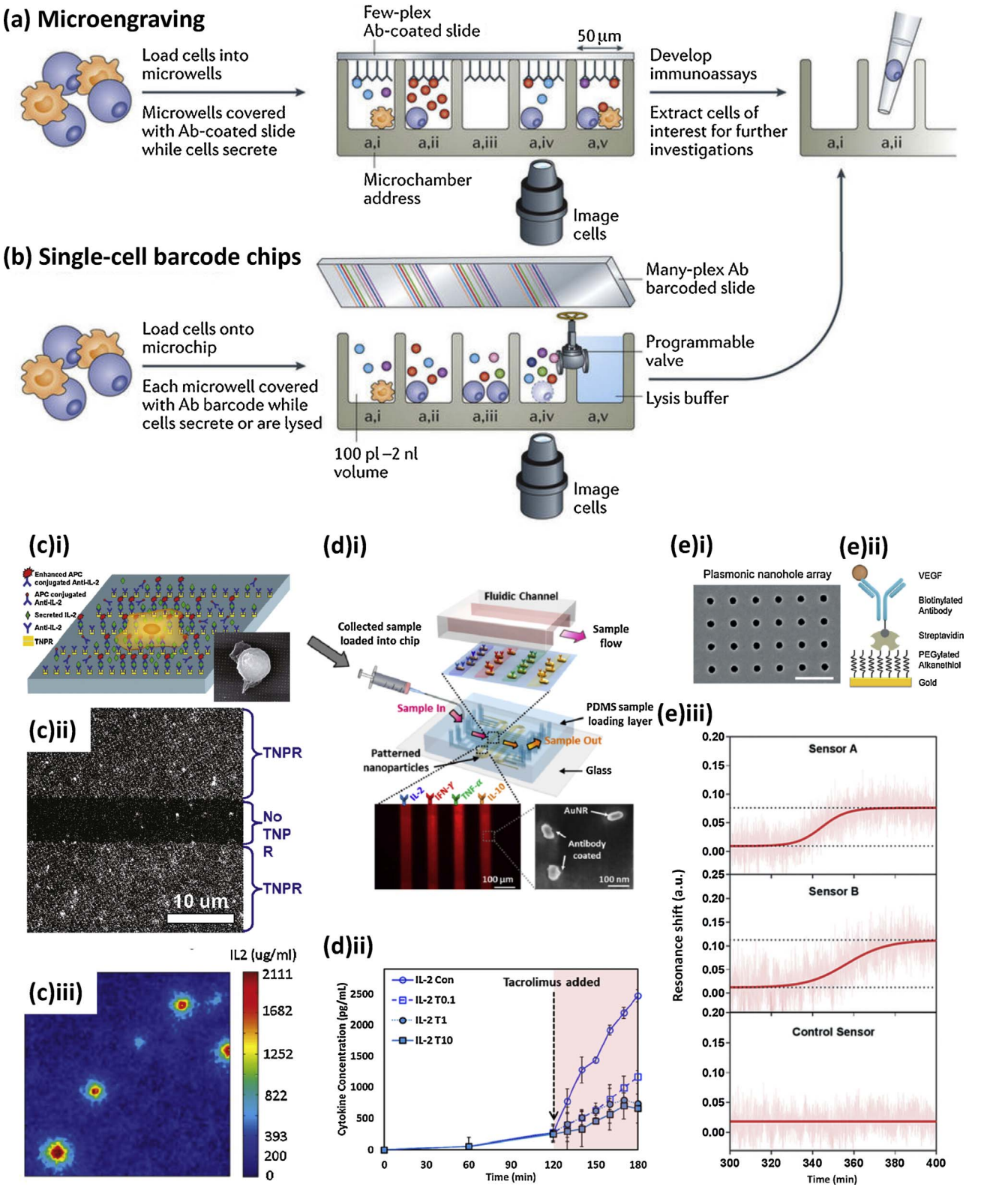

Fig. 2. Cell arrays with integrated nanoplasmonic structures for monitoring cytokine secretion from individual cells. (a) Schematic showing the workflow for microengraving and (b) single-cell barcode chips. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (2016, 15, 204–216), copyright 2016. (c)i) TNPR arrays for IL2 detection secreted from cells, with high spatial resolution. (ii) Fluorescence micrograph showing the enhancement of signal due to the TNPR. (iii) Quantitative and high spatial resolution of IL-2 signal from single T cells. Reprinted (adapted) with permission from Ref. Liang et al. (2015) (*Nano Letters,* 2011, 11, 3431–3434). Copyright, 2011, American Chemical Society. (d) i) Fluidic channel design for detection of four cytokines secreted from stimulated cells, using capture antibody-coated nanoplasmonic sensors on the floor of the channel. (ii) Quantitative and time-resolved secretion of IL-2 from stimulated cells in the fluidic chip. Reprinted (adapted) from Ref. Nehilla et al. (2016) (*ACS Sensors*, 2016, 1, 941–948) with permission from the American Chemical Society (further permissions related to the material excerpted should be directed to the ACS; https://doi.org/10.1021/acssensors.6b00240). (e)i) Alternative plasmonic structures, sub-wavelength "nanoholes"; light confinement in the holes at nanoscale makes the holes extremely sensitive to local changes in refractive index, such as those caused by (ii) antigen capture onto capture antibodies located adjacent to the holes. (iii) Individual sensors allow time-resolved quantitation of VEGF secretion from HeLa cells. Reproduced from Ref. Cohen and Walt (2017) with permission of The Royal Society of Chemistry (https://doi.org/10.1039//C7LC00277G).

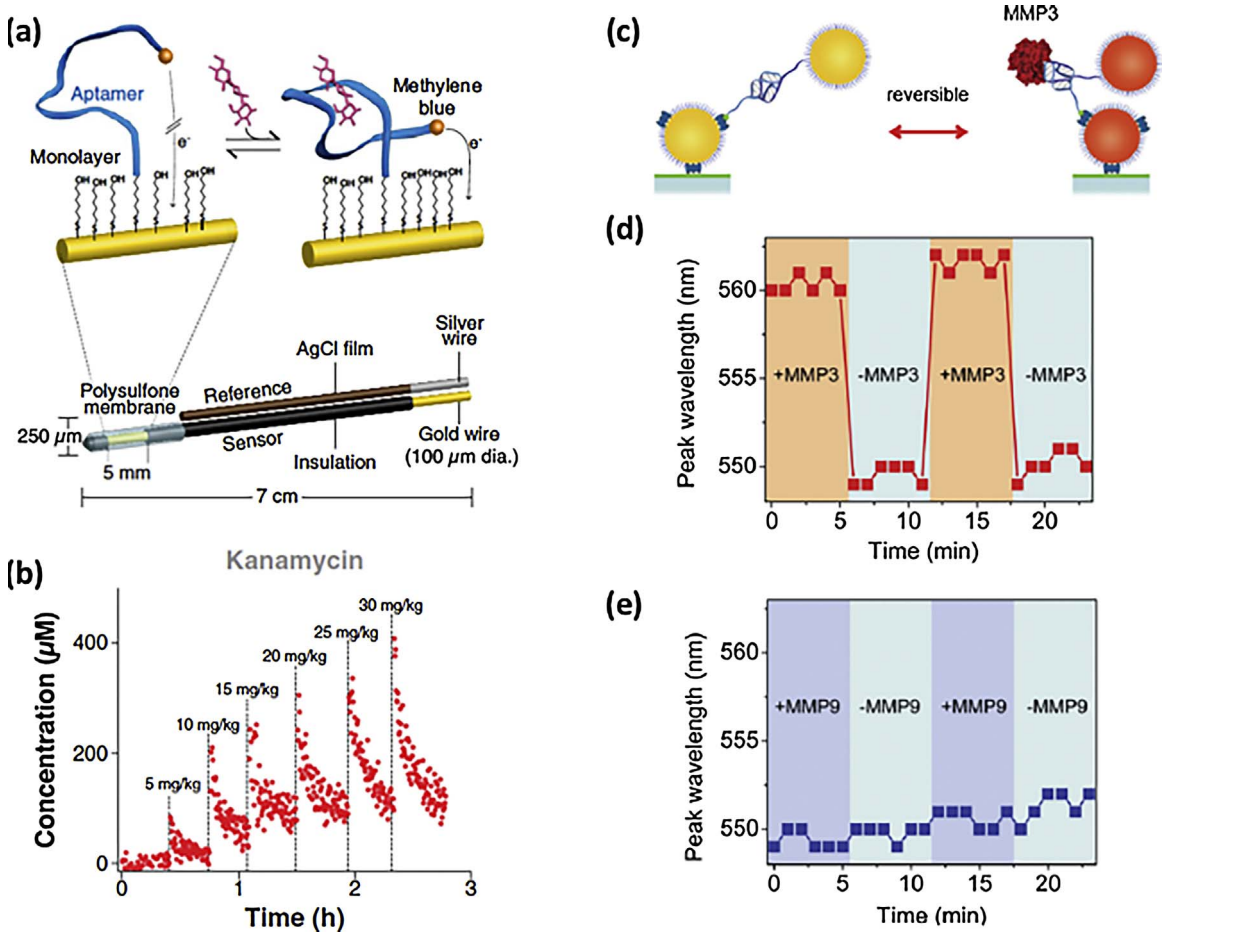

Fig. 3. Examples of aptamer-based bioreceptors used for continuous monitoring applications. (a) Electrochemical aptamer biosensor (E-AB), in which the proximity of methylene blue (MB; electron donor) to the electrode determines current flow. Target binding/unbinding brings MB closer to or away from the surface, and a polysulfone membrane minimises non-specific interactions. (b) One example of the E-AB is the continuous monitoring of aminoglycosides, in this case, kanamycin. Adapted from Ref. Arroyo-Currás et al. (2017), Copyright 2017 National Academy of Sciences. (c) "Plasmon Rulers' are another example of continuous aptamer-based biosensor, in which an aptamer is tethered between two plasmonic nanoparticles, and target binding changes the distance between particles, yielding detectable shifts in LSPR. (d)-(e) The plasmon rulers could detect 9 nM MMP3 with response time in minutes, yet did not respond to MMP 9. Adapted with permission from *Nano Letters*, 2015, 15 (7), pp 4564–4570. Copyright 2015 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

describing nanomaterials that are capable of continuously monitoring analyte concentration *in vivo*. The design characteristics of biosensors for *in vivo* application are well known for small molecule sensing, and are a good starting point for immunosensing. For continuous monitoring applications, sensors must be selective, sensitive, and bind reversibly to the target with a dynamic range that encompasses the expected physiological range. Beyond these design traits that are common to most biosensors, *in vivo* sensors also need to be easily implantable and removed/degraded, they need to be stable over the required sensing time, they need to have minimal "off-site diffusion" (in other words, they need to remain in the implant location), and their signals need to be readily transmitted and detected outside of the body. The most successful examples occur in the area of metabolite and small-molecule drug sensing, where optical (Balaconis et al., 2015; Cash et al., 2015; Awqatty et al., 2014; Ruckh and Clark, 2014) and electrochemical (Arroyo-Currás et al., 2017; Kang et al., 2017; Li et al., 2017b) strategies have been designed for continuous monitoring of all analytes in the standard blood panel, following the commercial successes and challenges of short-term (~2 weeks) electrochemical glucose sensors (Soto et al., 2017). However, extending these systems to protein biosensing *in vivo* poses unique problems which will need new solutions.

To date, most biosensors and bioassays have relied on high affinity antibodies (equilibrium dissociation constant, $K_D < 10^{-8}$ M) as the bioreceptor of choice. For example, ELISA assays (including those discussed earlier) involve immobilizing antibodies on surfaces for rapid target capture (high association rate constants, k_a) with minimal target release (low dissociation rate constants, k_d), to allow secondary detection reagents to selectively bind at another site on the target protein for signal amplification and detection purposes. While such methods are increasingly becoming more sensitive, and applicable to measuring the rate of accumulation of cytokines (as discussed earlier), this approach is not an avenue for continuous sensing. This is because continuous sensing requires the time-resolved monitoring of rates of accumulation *and depletion* of the target analyte on a similar time scale. This requires reversible bioreceptors with binding characteristics in the range of $K_D < 10^{-8}$ M and $k_d > 10^{-3}\,s^{-1}$ (Jang et al., 2016). Recently Paek et al. showed that anti-myoglobin antibodies and antibody fragments can fit these requirements, showing response times of ~1–10 min (defined here as the time taken to reach 90% of the steady state analyte concentration following a step-wise change). These antibodies were selected in blood samples collected from the early phase of the immune response in mice, before affinity maturation had taken place (Jang et al., 2016; Kim et al., 2012). However, this is a labour-intensive process not particularly amenable to rapid development of novel bioreceptors.

A range of protein-based antibody and non-antibody scaffolds have been discovered via "directed evolution" library approaches (Williams and Sooter, 2015). In the case of antibodies, phage and yeast libraries

have been used for selection of a range of high affinity antibodies and related antibody fragments, however most effort in this area has focussed on the selection of therapeutic binders rather than reversible antibodies for biosensing. A range of antibody "mimetics" have also emerged, with reduced molecular weights, μM-pM affinities, and relatively high denaturing temperatures (indicative of high stability), which could yield advantages over native structures, especially in terms of physical stability (Yu et al., 2017). Beyond antibodies, the Plaxco group, among others, have engineered single-stranded DNA molecules ("aptamers") for reversible binding to small molecules and proteins, achieving fast response times using either optical or electrochemical detection. The advantage here is that while the *in vitro* selection of aptamers is complex, once the sequence is known they can be chemically synthesised in automated processes, and sensor characteristics rationally tuned (Ricci et al., 2016) in comparison to protein-based bioreceptors. Recently, Plaxco and colleagues have demonstrated continuous, real-time *in vivo* monitoring of doxorubicin and several antibiotics in live animals (Arroyo-Currás et al., 2017; Ferguson et al., 2013), demonstrating the stability of aptamer biosensing *in vivo* and the potential for individualised therapeutic drug monitoring and dosing (Fig. 3**A/B**). In another example of the interest in aptamer technology, Lee et al. demonstrated the use of a "plasmon ruler", in which an aptamer targeted against MMP3 protein was tethered between two gold nanoparticles, one of which was attached to a surface through biotin/streptavidin interactions (Lee et al., 2015). In the absence of target the inter-particle distance was relatively large, while in the presence of target the particles were brought closer together, resulting in a significant red-shift in the peak absorbance. While the affinity constants were not reported, the response time in the presence/absence of 9 nM MMP3 was ~1 min, with negligible response for the non-specific target MMP9 (Fig. 3**C–E**). At this stage the plasmon rulers have not been tested in real biological solutions, and this will be required to determine potential utility. However, these examples show that aptamers can be designed with rapid reversible binding for not only small molecules, but also macromolecular proteins. The key challenge in designing or evolving bioreceptors for rapid cytokine monitoring is that their concentrations in fluids and tissues may be significantly lower than those used in the studies mentioned, and biosensor response times increase with decreasing analyte concentration. However, if we consider the dynamics of cytokine production in the skin (Sjogren et al., 2012; Sjogren and Anderson, 2009) as an example of a reasonable sensor location (9–16 h to return to baseline following sterile trauma), response times for fM concentrations in the range of 1–15 min could still generate useful data.

In addition to the need for unique bioreceptors, *in vivo* sensors must also be biocompatible (implantable and removable/degradable), non-toxic, and transmit a calibrated signal from the desired sensing location. Most examples to date focus on the design of sensors that are implanted into the subcutaneous tissue, mainly to make signal transduction (usually optical or electrical) possible. While this may be a useful site for monitoring relatively small, circulating, molecules that can perfuse the tissue from local vessels, it is tantalising to consider the possibility of targeting a sensor to an internal tissue for monitoring local immune activity in the context of disease monitoring or discovery biology. Recently there has been a research trend towards miniaturising sensors such that they can act on the length scale of cells and tissues. This is most clearly identified in the work directed at the design of neural sensing components, which are being miniaturised form the large planar electrodes that are still in current use (Kang et al., 2016; Kang et al., 2014). Interestingly, there is a growing body of knowledge and evidence around the targeted delivery of nanoparticle-based drugs to specific internal targets (including lymphatics (Trevaskis et al., 2015)), and we suspect that the field of *in vivo* sensing will benefit a great deal from what is already known in this field. Nanoparticles can be coated in a range of surface chemistries and targeting molecules with the goal of controlling biodistribution profile and targeting drugs to specific locations (Nel et al., 2009). Current challenges include increasing the targeting success at the tissue level, as a recent highly publicised review showed that over the past 10 years, targeting rates are still hovering around 1% of the injected dose (Wilhelm et al., 2016). Furthermore, new ways of transducing biosensor signals from deep tissues will be required, as optical imaging has limited depth penetration, and most electrochemical sensors require wiring and power sources for operation. Biomedical imaging techniques including near-infrared (NIR), magnetic resonance imaging (MRI), *etc.*, are likely to be targeted for these applications. Early examples are emerging in the area of small molecule sensing with MRI (Sowers et al., 2014), and NIR-imaging of organosilica contrast agents for sentinel lymph node mapping is showing promise in clinical trials (Phillips et al., 2014).

In conclusion, nanomaterials have facilitated significant increases in detection limits for traditional and emerging immunoassays, and have also allowed the direct and continuous monitoring of secreted protein accumulation from individual immune cells. We expect that the technology will continue to improve beyond the proof-of-concept stage, into clinical trials and products. Looking forward, we present a new challenge to the immunology and sensor science communities. Nanomaterial-based sensors that achieve the following objectives could yield significant further advances:

- Continuous monitoring of immune-related protein in a dynamic biological environment
- Targeting of specific tissues for monitoring immunologically relevant microenvironments – e.g. tumors, lymphoid organs, etc.
- Transducing of analytical signals from deep tissues with sensitivity and detection limits appropriate for targets of immunological interest.

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