Molecular Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Review

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

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ARTICLE INFO

Keywords:
Nanomaterials
Sensitivity
Cell array
Plasmonic
Cytokine

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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https://doi.org/10.1016/j.molimm.2017.12.017
Received 3 October 2017; Received in revised form 13 December 2017; Accepted 19 December 2017

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),