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Recent advances in cytokine detection by immunosensing

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Abstract

The detection of cytokines in body fluids, cells, tissues and organisms continues to attract considerable attention due to the importance of these key cell signalling molecules in biology and medicine. In this review, we describe recent advances in cytokine detection in the course of ongoing pursuit of new analytical approaches for these trace analytes with specific focus on immunosensing. We discuss recent elegant designs of sensing interface with improved performance with respect to sensitivity, selectivity, stability, simplicity, and the absence of sample matrix effects. Various immunosensing approaches based on multifunctional nanomaterials open novel opportunities for ultrasensitive detection of cytokines in body fluids *in vitro* and *in vivo*. Methodologies such as suspension arrays also known as bead assays together

with optical fibre-based sensors, on their own or in combination with microfluidic devices will continue to have an important role to address the grand challenge of real-time *in vivo* multiplex cytokine detection.

Keywords: Cytokines; Immunosensing; Sensitivity; Real-time detection; Microfluidics; Review

1. Introduction

Cytokines, low molecular weight (~ 6-70 kDa) soluble proteins secreted from the immune and non-immune cells are core indicators of the functional status of the body, and strongly associated with the immune system including the modulation of immune reactions such as sensitization.(Stenken and Poschenrieder 2015) Cytokines also play critical roles in chemically-induced tissue damage repair, in cancer development and progression, in the control of cell replication and apoptosis and in many other aspects of physiology. Consequently, monitoring cell functions and cell-to-cell communication by using their cytokine secretions has enormous value in biology and medicine.(O'Shea et al. 2011) The effects of cytokines are very potent as they engage various downstream amplification processes. As a result, only a few cytokine molecules may be sufficient to induce a significant cellular response.(Xue et al. 2015)

Cytokines are classified into lymphokines (cytokines made by lymphocytes), monokines (cytokines made by monocytes), chemokines (cytokines with chemotactic activities), and interleukins (cytokines made by one leukocyte and acting on other leukocytes).(Nicola 1994) Based on effects of cytokines in the context of an inflammatory disease, they can also be divided into inflammatory or anti-inflammatory,(Wojdasiewicz et al. 2014) and produced both with and without stimuli such as lipopolysaccharide.(Zhao et al. 2011) Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). Cytokines can also act addictively, synergistically or antagonistically, and induce one another.(Whicher and Evans 1990)

Cytokine detection and measurement is important as elevated concentrations of cytokines may indicate the activation of cytokine signaling pathways associated with inflammation or disease progression. Consequently, these proteins are widely used as biomarkers to characterize the immune function, understand and predict disease, and monitor effects of treatment.(Catalfamo et al. 2012) Measurement sensitivity is always an issue for cytokines because they are released into the extracellular milieu resulting in pM concentration range.(Schenk et al. 2001) In addition to low concentrations, it is difficult to measure physiological concentrations of cytokines accurately and reproducibly due to some challenges (Figure 1) such as significant interference from heterophilic antibodies,(Bolstad et al. 2013) the rheumatoid factors,(Bartels et al. 2011) and specific or non-specific cytokine binding proteins,(Whicher and Evans 1990) and an extremely dynamic, transient cytokine secretion process.(Kulbe et al. 2012)
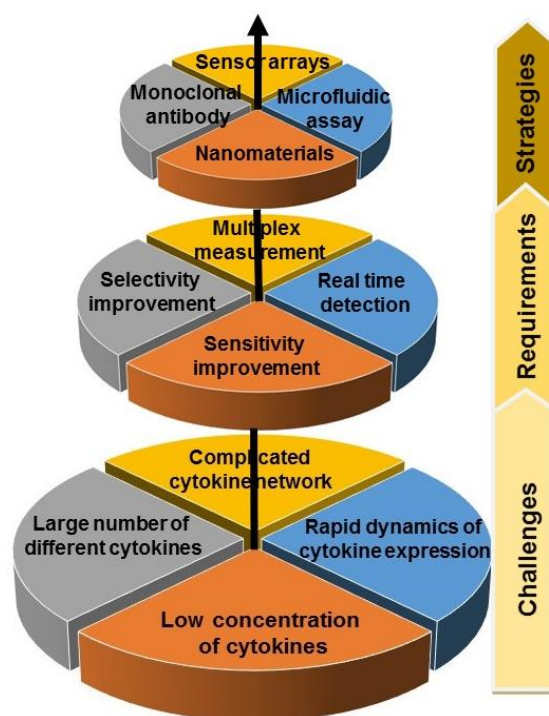

Figure 1 The scheme showing challenges, requirements and strategies for cytokine detection. The challenges include complicated cytokine network, large number of different cytokines, low concentration of cytokines, and rapid dynamics of cytokine expression. Correspondingly, cytokine detection methods requires multiplex

capability, enhancement in selectivity and sensitivity, and real time measurement. The strategies to address these challenges are proposed to be application of sensor arrays, monoclonal antibodies, nanomaterials, multifluidic system, and et al.

The most common approach for cytokine quantification is based on the idea of an immunoassay. Specific techniques include traditional ELISA assays,(Chiswick et al. 2012) enzyme-linked immunosorbent spot (ELIspot) assays,(Cox et al. 2006) antibody array assays(Schröder et al. 2010) and bead-based assays(Won et al. 2012). Traditional ELISA assays are reliable, but they are not rapid (6 h) and usually require a relatively large sample volume (100 µL). Generally speaking, all these assays require a long sample preparation time (> 6 h), and multiplexed approaches require a high level of complexity in the sample labeling. Some assay types require specialized flow cytometry infrastructure, and all are unable to monitor the cytokines in real time or in a dynamic manner. These limitations are the driving force for researchers to develop sensitive, selective, and rapid real time cytokine analysis platforms for comprehensive characterization and quantitative analysis of cytokines released in both healthy and pathological conditions.

The purpose of this review is to discuss recent advances in development of analytical approaches especially immunosensors for cytokine detection focusing on designing sensing interfaces to achieve high sensitivity, selectivity, stability, simplicity, and no sample matrix effects. This work is not intended to be a comprehensive review on cytokine detection, as several excellent reviews of analytical methods for measurement of cytokine proteins have been recently published.(Chikkaveeraiah et al. 2012; Rusling et al. 2010; Stenken and Poschenrieder 2015) Rather, we will examine the latest trends in cytokine detection based on immunosensing.

2. Principles of immunosensors

Immunosensors are immunoreaction-based affinity biosensors, which use

immuno-compounds as biological receptors. They usually integrate an immunoassay and a directly associated transducer in a single device (Figure 2). This device contains two essential components: biorecognition domain and signal transduction. The biorecognition domain should be a biological entity such as antibodies, peptides, proteins, or even whole cells. The integration of recognition elements (such as antibodies and antigens) with a signal transduction usually achieved by modifying the transducer surface with a chemical layer that enables sensitive and selective immobilization of recognition species.(Liu et al. 2012a) Thus, ideally, this biorecoginition domain should have high affinity (low detection limit), high specificity and selectivity (low interference), wide dynamic range, fast response time, long shelf life, and good generality for detecting a broad range of analytes with the same class of surface fabrication. Signal transduction elements are responsible for converting molecular recognition events into physically detectable signals such as fluorescence, colour, electrochemical signals, acoustic, or surface plasmon resonance changes.
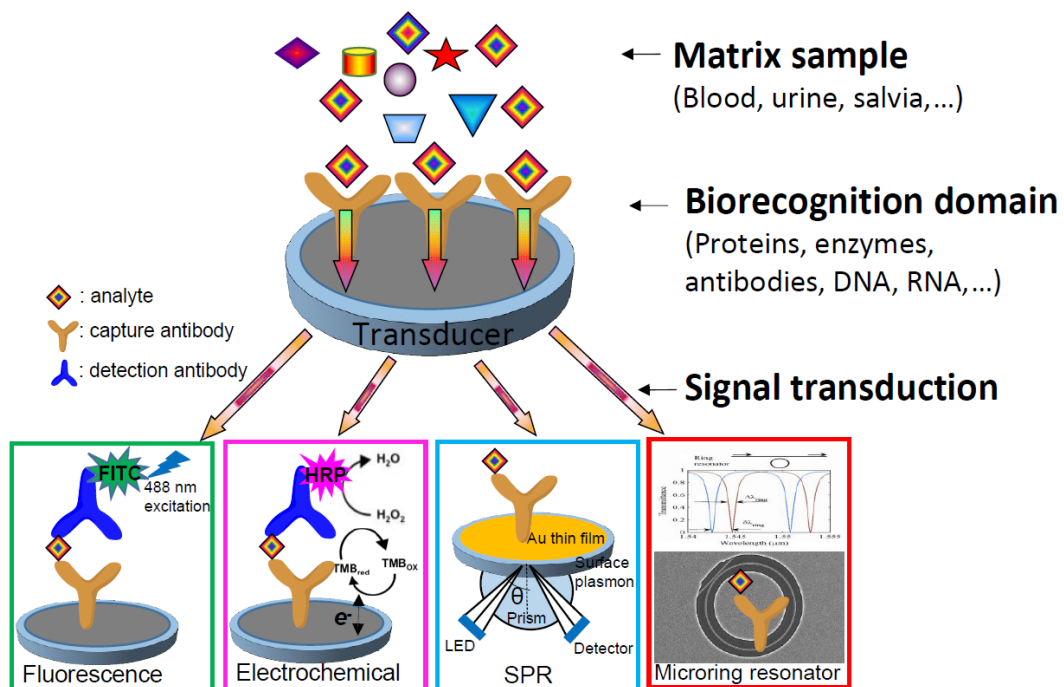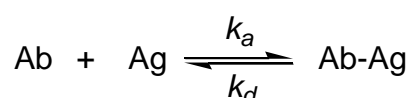
Figure 2 The general scheme of an immunosensor which includes matrix sample, biorecognition domain and signal transduction. Four important signal transduction approaches are schemed for cytokine detection, such as fluorescence immunosensirng, electrochemical immunosensing, surface plasmon resonance (SPR) based and microring resonator based immunosensing.

The interaction of an antibody (Ab) with an antigen (Ag) forms the basis of immunosensors, which defines both specificity and detection limit of an immunosensor.(Mehrvar et al. 2000) The Ab-Ag interaction is characterized with an association and a dissociation reaction rate constant, k_a and k_d respectively.

$$Ab \ + \ Ag \ \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \ Ab\text{-}Ag$$

The ultimate detection limit of an immunoassay is determined by the antibody-antigen binding constant.(Moal and Bates 2012) The greater the binding constant of the antibody, the lower detection limit can be achieved. The affinity constant K_A, which varies in strength from 10^4 to 10^{15} M^{-1} (typically of the order of 10^8 to 10^{12} M^{-1}) depending on the nature of antigens and binding affinity of the corresponding antibodies,(Lee et al. 2014) can be described by:

$$K_A = \frac{k_a}{k_d} = \frac{[Ab-Ag]}{[Ab][Ag]}$$

Where [Ab], [Ag] and [Ab-Ag] are molar concentrations of antibody, antigen and antibody-antigen complex in solution, respectively. The transduction of such antibody and antigen biorecognition events either requires labels, commonly used in a myriad of immunoassay formats, or a method which can directly detect the change that occurs at the sensing interface. Most immunosensor devices reported to date perform indirect measurements by using labels such as enzymes,(Malhotra et al. 2012) fluorescent (Zhao et al. 2011) and chemiluminescent(Sardesai et al. 2013) probes that convert

affinity signal into a measurable response. Although indirect immunosensors are highly sensitive due to analytical characteristics of the label applied, the non-specific binding is a continuing problem.(Huang et al. 2015a) Therefore special methodologies to resist non-specific protein adsorption are critically required for sensing analytes in a complex matrix sample, such as blood or urine. Consequently, despite decades of effort it is still challenging to design a sensing interface with properties of high sensitivity, high selectivity, high stability, simplicity, and no matrix sample effect. The desire to have all of these properties simultaneously present at the same sensing interface drives research as well as commercial developments.

3. Immunosensors for cytokine detection

Given that cytokines are universal biomarkers implicated in the functioning of immune and other physiological processes, it is not surprising that cytokine detection is one of the hottest topics in immunosensing. However, publications reporting immunosensors for cytokine detection are limited due to some analytical challenges with cytokine detection (Figure 1). Table S1 lists representative immunosensors for cytokine detection based on different signalling strategies such as fluorescence immunoassay (FI), surface plasmon resonance detection (SPR), electrochemical-based methods (EC), silicon photonic micro-ring resonators (MR) and other methods. This section aims to generally describe the advantages and limitations with respect to each signal detection strategy, and a more detailed discussion of each of these different signalling strategies is reported elsewhere.(Stenken and Poschenrieder 2015)

3.1 Fluorescence based immunsensing

The fluorescence immunoassay (FI) is a method which monitors Ab-Ag binding based on changes in fluorescence signal (Figure 2), and recent publications outlining the principle of FI are summarized.(Wu et al. 2011) It represents the most widely studied methodology for cytokine detection due to its high sensitivity. In addition, fluorescent methods are simple, diverse and non-destructive, and can be integrated into

7

microfluidic devices for cytokine monitoring in real time.(Zhao et al. 2011) The wide abundance of different fluorescent labels makes FI technology capable of multiplexing. However, photobleaching of fluorescent dye labels and spectral overlap of reporter dyes may limit the degree of multiplexing, while luminescent background of sample matrix can interfere with the measurement and/or interpretation of results.(Campos et al. 2011) The detection limit of fluorescence based immunosensing ranges from fg mL^{-1} to ng mL^{-1}, so it is generally sufficient for many cytokines in physiological conditions.

3.2 Surface plasmon resonance based immunsensing

SPR is an important tool to monitor interactions between biomolecules.(Mayer and Hafner 2011) Changes of the refractive index after Ab-Ag biorecognition can be probed by exploiting special properties of electromagnetic waves at the metal surface (surface plasmons). Thus the interaction between analyte and a biospecific element on metal surface can be monitored by SPR biosensor without the use of extrinsic labels (Figure 2). SPR has wide applications on sensing and recent publications outlining the principle of SPR are summarized.(Guo 2012) SPR-based immunosensors for cytokine detection are attractive due to high sensitivity (~2 pg mL^{-1})(Jeong et al. 2013) and the absence of labels. However, a common challenge with SPR-based sensors is the issue of overcoming signals produced via non-specific binding events on the sensor.

3.3 Electrochemical based immunsensing

Electrochemical methods (EC) have been used for protein detection by immunoassays for quite some time.(Luo and Davis 2013) In this approach, the Ab-Ag bioreconginition is probed based on the electrochemical signal from redox probes labelled on detection antibody (Figure 2). The primary advantages of electrochemical methods are inexpensive equipment and high sensitivity particularly in amperometric based measurements. More recent applications of electrochemiluminescence have incorporated various nano-based or chip-based strategies with high sensitivity (10 fg mL^{-1}).(Sardesai et al. 2011) From Table S1 we can see electrochemical assays achieve

comparable detection limit to FL but with shorter analysis time. The scope of using EC for multiplexing is limited due to limited availability of redox probes for reporting the electrochemical signal.

3.4 Microring resonator based immunsensing and other approaches

MR represent a promising sensing platform (Figure 2) for real-time, label-free and multiplex detection of biomolecules due to their resonantly-enhanced sensitivity toward surface binding events between a target and antibody-modified micro-rings.(Washburn and Bailey 2011) Recent publications outlining the principle of MR sensors are summarized.(Amiri et al. 2015) The light coupled into the resonator via a waveguide is confined within the micro-ring cavity due to total internal reflections and high-Q resonant modes (Q~12000) are formed. Positions of these modes depend on the effective index of the resonant structure and thus get shifted when there is Ab-Ag interaction on the surface. This shift can be determined with high precision using the method of optical detection.(Qu et al. 2011) This is critical for field-based analytics and point-of-care diagnosis. For example, a silicon photonic micro-ring resonator was adopted for simultaneous detection of cytokines IL-2, IL-4, IL-5, and TNF-α with high accuracy in serum-containing cell media within only 5 min. (Luchansky and Bailey 2011) This report demonstrated that MR based sensing platforms have huge potential for multiplexed cytokine monitoring in complex immunological studies (Kindt and Bailey 2013) This approach also opened the possibility of performing real-time cells secretion measurements on single cells. Other new sensing platforms, such as, microarrays interferometric reflectance imaging sensors (IRIS)(Ahn et al. 2013) and arrayed imaging reflectometry (AIR)(Carter et al. 2011) are recently developed for cytokine detection with acceptable sensitivity (less than 10 pg mL^{-1}).

All these reported approaches aim to address one or more of detection challenges associated with cytokine detection: sensitivity (~10 pg mL^{-1} or better), selectivity, multiplexing, and real-time detection. Achieving high stability, simplicity, shorter

detection time and reduced sample volume are also driving the development of new cytokine immunosensing methods. The following sections are going to summarize strategies reported in literatures to meet the challenges of cytokine analysis including sensitivity, selectivity improvement, multiplex measurement, and real-time sensing.

4. Strategies for improving selectivity

4.1 Using monoclonal antibodies as the recognition reagent

Immunoassays are attractive for the detection of proteins due to their high specificity by introducing monoclonal antibodies. Two types of antibodies (capture antibody and detection antibody) are normally used in sandwich immunosensors (Figure 2). It is preferable to use monoclonal antibodies as capture antibodies since they provide high selectivity to analyte.(Zhang et al. 2012a) Although not all cytokines have commercially available monoclonal antibodies, monoclonal antibodies are used whenever possible, with the majority of immunosensors for cytokine detection. To transduce such biorecognition events either requires labels or a transduction method (such as SPR or MR) which can detect the change that occurs at the sensing interface. Thus detection antibodies are normally labeled with probes such as fluorescent molecules or particles, redox probes,(Bettazzi et al. 2013) or mass tags.(Ahn et al. 2013)

4.2 Using aptamers as recognition reagents

Aptamers are single strands of either DNA or RNA oligonucleotides that can be used to bind different analytes with higher selectivity and affinity than antibodies, and they are typically produced by selection from large combinatorial libraries.(Yüce et al. 2015) Using aptamers as recognition reagents in biosensors has been reviewed in references.(Deng et al. 2014; Iliuk et al. 2011; Kim et al. 2016) The current active research on aptamers as alternative molecular recognition agents and possible substitutes for antibodies, has further widened the application of immunosensing in chemical analysis.(Famulok and Mayer 2011) In particular, aptamers can be readily site-specifically modified during chemical or enzymatic synthesis to incorporate

particular reporters, linkers, or other moieties. Also, aptamer secondary structures can be engineered to undergo analyte-dependent conformational changes, which opens up a wealth of possible signal transduction schemes, irrespective of whether the detection modality is optical, electrochemical, or mass based. Another advantage of using aptamers for specific target capture is their higher stability compared to antibodies. Consequently they can be used in harsh protein denaturing conditions.(Famulok and Mayer 2011) The vast majority of aptamer applications include simply using an aptamer as a substitute for an antibody. Despite active interest in these potential antibody substitutes, the number of aptamers that bind different cytokines is limited.(Orava et al. 2012) To our knowledge, in the field of cytokine detection only aptamers against IFN-γ, PDGF, VEGF, IL-6, IL-32, IL-17, and TNF-α have been reported in literatures. Revzin's group has published several papers focusing on IFN-γ detection based on aptamer immunosensors.(Liu et al. 2012c; Liu et al. 2015; Liu et al. 2011; Tuleuova et al. 2010) The sensitive and specific aptamer immunosensors for PDGF detection have been developed by combining aptamers with nanomaterials.(Wang et al. 2015; Wang et al. 2012a; Zhang et al. 2015) In one of such reports the hairpin aptamer probes have been immobilized on a gold electrode through self-assembly.(Zhang et al. 2012b) In this design, the presence of IFN-γ opens the hairpin structure. With subsequent addition of hemin, the hemin/G-quadruplex peroxidase-mimicking DNAzyme is formed, which catalyzes the electro-reduction of H_2O_2 and amplifies the current response for IFN-γ detection at the sub-nanomolar level. This aptasensor shows high selectivity towards the target analyte by incorporating a specific DNAzyme sequence into the hairpin aptamer probe. Hence, aptamers are becoming widespread in analytical applications not only as alternatives to antibodies, but as unique reagents in their own right. More examples on hairpin aptamer probes for both selectivity and sensitivity improvement will be discussed in section 5.4.

4.3 Prevention of non-specific protein binding

Modification of sensing interfaces with molecules being able to resist non-specific

adsorption is another efficient way to improve sensitivity and selectivity of the immunosensing system.(Zhang et al. 2013) Nonspecific adsorption of proteins is generally minimized by masking surfaces with blocking agents such as bovine serum albumin (BSA). However, for a complex biological sample such as serum, which contains numerous proteins of different types, BSA blocking solution might not be sufficient. Recently zwitterionic polymers have demonstrated high resistance to non-specific protein adsorption in biological applications.(Schlenoff 2014) Gooding's group reported that zwitterionic phenyl layers have the anti-biofouling properties.(Gui et al. 2013) A hydrophilic layer such as poly(ethylene glycol) molecules (PEG) has also been explored to manage non-specific binding.(Liu et al. 2014a) Revzin and coworkers have been using modified PEG layers to improve the performance of aptamer immunosensors for cytokine detection.(Liu et al. 2011) PEG hydrogel has also been used to modify glass slide to resist the non-specific protein adsorption.(Seo et al. 2011) The application of polymer brushes in biomedical fields for resisting non-specific protein adsorption has been reviewed.(Krishnamoorthy et al. 2014) Hucknall and coworkers have designed a simple antibody microarrays on nonfouling poly(oligo(ethylene glycol) methacrylate) polymer (POEGMA) brushes (Figure 3) with femtomolar sensitivity for cytokines in serum and blood.(Hucknall et al. 2009) The nonfouling polymer brushes can efficiently resist protein adsorption from solution, and eliminate background non-specific signals in microarrays and lead to detection limits as low as 100 fg mL^{-1} (5 fM) in serum and 15 fM in whole blood. It is critically important to introduce molecules to sensing interface which can resist non-specific protein adsorption as the low detection limit depends on it. However, the problem of resisting non-specific protein adsorption has not yet been fully resolved. The management of non-specific binding remains one of the core challenges in cytokine analysis, and exploring reliable approaches to control non-specific protein adsorption needs further research.
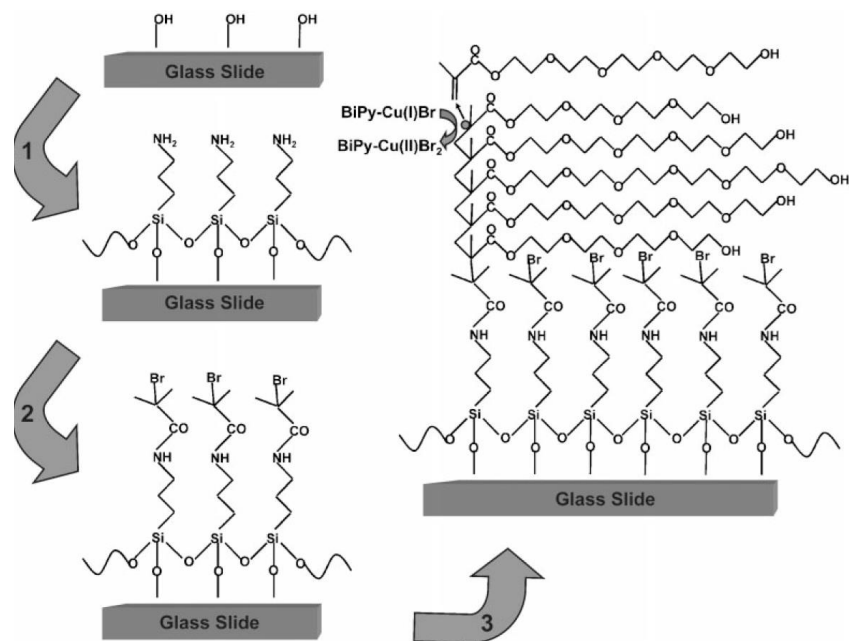
Figure 3 Synthesis of POEGMA brushes on glass via surface-initiated atom-transfer radical polymerization for management of non-specific binding. Reprinted with the permission.(Hucknall et al. 2009)

5. Strategies for improving sensitivity

Numerous strategies attempt to achieve sensitivity improvement through signal amplification, including nanomaterial-based approaches,(Lei and Ju 2012) DNA-labeling techniques,(Hocek and Fojta 2011) electrochemiluminescence,(Chen et al. 2012) and in situ hybridization methods.(Urbanek et al. 2015) Here we only focus on techniques for improving sensitivity of cytokine detection based on signal amplification taking place on sensing interfaces.

5.1 Amplified transduction with nanomaterials

Generally, immunoreagents (such as antibodies) are immobilized on a transducer while the analyte (antigen) is measured through a label conjugated with one of the immunoreagents.(Pei et al. 2013) Nanomaterial-based fluorescent, luminescent, refractive index, light scattering and/or colorimetric labels have been integrated into analytical chemistry and used by large number of novel sensing techniques.(Scida et al. 2011; Shen et al. 2014) Nanomaterial-based sensing platforms can provide

13

advantages over traditional approaches in terms of sensitivity, stability, and capability for multiplexing and real time detection.(Chen and Chatterjee 2013) Importantly, nanomaterials can be functionalised to improve their ability to bind in a designated location, such as the surface of a cytokine secreting cell and signal the presence of proteins. They can be coated with antibodies, antigens, aptamers, enzymes specific to a protein, or receptors overexpressed on cell surface, or specific to cancer biomarkers. These nanomaterials can accommodate a large number of such targeting moieties due to high area-to-volume ratio, which makes nanomaterials attractive in biosensors.

For example, zinc oxide nanorod platform was reported for ultrasensitive fluorescence detection of IL-18 and TNF-α with the sensitivity of sub fg mL^{-1}.(Adalsteinsson et al. 2008) A gold nanoparticle (AuNP) based SPR immunosensor combined with a fluidic platform was developed for detection of TNF-α.(Oh et al. 2014) This cytokine secretion assay was sensitive enough to quantify intercellular-signaling proteins secreted by blood immune cells in 4-5 h which is 3 times shorter than in the traditional ELISA method. Surface Enhanced Raman Spectroscopy (SERS) based AuNP sensing platform has been developed for fast, wash-free, and multiplexed quantification of three cytokines, INF-γ, IL-2, and TNF-α, with the detection limit of 0.5 pM, 1.5 pM, and 0.3 pM, respectively.(Wang et al. 2013) By combination of the advantages of AuNP loaded graphene nanosheets, quantum dot based amplification, and heated electrode measurement, Zhang et al have proposed an ultrasensitive for the detection of IL-6 with detection limit of 0.5 pg mL^{-1}.(Zhang et al. 2011) In addition, AuNPs uniformly assembled on the surface of poly (styrene-acrylic acid) nanospheres have been also reported as a tool for detection of TNF-α with high sensitivity (0.01 ng mL^{-1}), stability and reproducibility.(Yin et al. 2011) Aptamer conjugated gold nanorods were used in a dual role as a label and a substrate to conjugate antibodies for a multiplex serum cytokine immunoassay detected by localized SPR in a microfluidic system.(Chen et al. 2015) The key achievement in this study is simultaneous detection of multiple analytes (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ) with high sensitivity

(5-20 pg mL^{-1}) from a 1 μL serum sample within 40 min.

Huang and coworkers have developed single molecule nanoparticle optical biosensors (SMNOBS) based on silver nanoparticles (2.6 ± 1.1 nm),(Huang et al. 2008) which were used for sensing and imaging of single TNF-α molecule. Once a single TNF-α molecule bound to a single monoclonal antibody molecule on single nanoparticle, the localized SPR of SMNOBS exhibited a large red shift of peak wavelength. These authors reported a dynamic range of at least 0-200 ng mL^{-1} TNF-α. A graphene oxide-based amperometric sensor was reported for a highly-sensitive detection of IL-6 with the detection limit of 4.7 pg mL^{-1}.(Huang et al. 2013a) An electrochemiluminescent immunosensor based on carbon nanotubes has been developed for detection of IL-6 in serum with detection limit of 0.25 pg mL^{-1} (Figure 4).(Sardesai et al. 2011) In this work carbon nanotubes were conjugated with the capture antibodies and the reporter silica nanoparticles with detection antibodies. An electrochemical immunosensor for measuring IL-6 in serum based on single wall nanotube forests and 5 nm glutathione-protected AuNPs were also developed.(Munge et al. 2009) Their analytical performance was approaching the physiological range for IL-6 (< 6 pg mL^{-1}) with detection limit of 10 pg mL^{-1}.
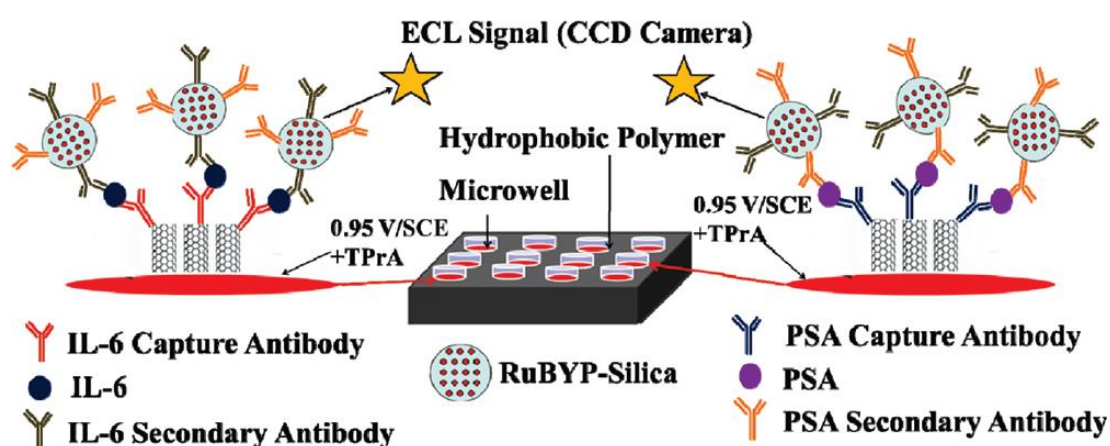

Figure 4 Design of microfluidic ECL array. Reprinted with permission.(Sardesai et al. 2011)

Another ultrasensitive immunoassay based on QDs-polymer functionalized silica

nanoparticle labels was developed for TNF-α measurement. (Yuan et al. 2011) Both electrochemical and electrochemiluminescence measurements can be used to quantify TNF-α with detection limit of 3 pg mL^{-1} and 7 pg mL^{-1}, respectively. The increased sensitivity of TNF-α was achieved by an increase of CdTe QD loading per immunoassay event due to a large number of surface functional epoxy groups on polymers. In addition to increase the loading number of capture antibodies, the application of specially designed nanomaterials on the electrochemical sensing interface can greatly increase electron transfer rate between biomolecules and transducer resulting in increased sensitivity.(Liu et al. 2014b) An ultrasensitive electrochemical microfluidic array optimized to measure a four-protein panel of cytokines (IL-6, IL-8, VEGF and VEGF-C) with detection limits in the 5-50 fg mL^{-1} range was developed by Rusling's group.(Malhotra et al. 2012) The sensitivity was improved by using off-line protein capture by magnetic beads carrying 400,000 horseradish-peroxidase enzyme labels and ∼100,000 antibodies. For enhancing the sensitivity, a strategy for detection of IL-2 which relies on silicon photonic microring resonator was reported by Bailey and coworkers.(Luchansky and Bailey 2011) In this study the signal arising from the initial binding event was amplified by employing a much larger secondary antibody due to significant change in mass, hence refractive index affecting resonance conditions.

These examples above show enormous scope of nanomaterials offering for immunosensing of cytokines in regards to signal amplification. There is a similar scope to covalently attach multifunctional components (biomolecules and anti-fouling molecules) onto nanomaterials without steric hindrance and exploitation of these ideas for cytokine detection is likely to lead to further advances in sensitivity.

5.2 Microsphere-based amplified transduction

In addition to nanomaterials, functionalized magnetic particles have been commonly used for purposes such as manipulation of cells, (Xu et al. 2011) isolation of specific DNA molecules,(Fitzgerald and Grivel 2013) or detection of biomarkers.(Joo et al.

2012) The large surface of a microsphere makes it possible to increase the sensitivity because a large number of antibodies can be attached. Simultaneous detection of six cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α in equine plasma using fluorescent microsphere immunoassays (FMIA) has been reported.(Hall et al. 2015) Compared with ELISA, FMIA has a higher sensitivity (~41 pg mL^{-1}). An electrochemical bioassay for analysis of TNF-α as low as 44 pg mL^{-1} was developed by coupling magnetic beads with disposable electrochemical platforms.(Bettazzi et al. 2013)

5.3 Surface-based signal enhancement tools

Recently, surface enhancement approaches have been developed to increase fluorescence signals from the available number of fluorophore labels to improve the sensitivity of fluorescence based assays. This approach uses either photonic crystals (PC) or, more commonly, metal nanostructures to enhance the sensitivity of molecular detection. SERS based surfaces have also been intensely investigated and show particular promise for sensitive cytokine detection.(Cialla et al. 2012) PC surfaces can enhance sensitivity of cytokine through the use of narrow bandwidth optical resonances that are designed to occur at specific combinations of excitation wavelength and incident angle. An ultrasensitive immunoassay based on nanoparticles-assembled PC was developed for detection of human epidermal growth factor receptor 2 (HER, breast cancer biomarker) with a detection limit as low as 10 aM in less than 10 μL of serum-based sample.(Han et al. 2012) A PC surface was designed for multiplex cytokine detection which can improve the detection limit by a factor of seven.(Huang et al. 2011) Recently, embedding PC surface in the microfluidic chip resulted in 20 times fluorescence enhancement, which was applied for detection of TNF-α and IL-3 with 80 fM detection limits. (Tan et al. 2015) A fluorescence-enhancing microarrays on plasmonic gold films for multiplexed cytokine detection with up to three orders of magnitude higher sensitivity than on conventional nitrocellulose and glass substrates were developed.(Zhang et al. 2013) The approach was used for detection of VEGF, IL-1β, IL-4, IL-6, IFN-γ and TNF-α in a panel through a four-layer immunoassay approach. This work demonstrated a high throughput

multiplexed cytokine detection method with one order higher sensitivity (0.06 pg mL^{-1}) and two order higher dynamic range than the conventional ELISA.

Fiber-optic sensors are chemically passive, have small physical dimensions, and are able to access challenging environments.(Wang and Wolfbeis 2012) They offer an advantage of long interaction length which, in some situations can yield enhanced signals. Moreover, optical fiber biosensors can be used in combination with different types of spectroscopic techniques, e.g. absorption, fluorescence, phosphorescence, Raman, SPR. It is therefore not surprising that optical fibers were explored as an interesting platform for cytokine detection.(Huang et al. 2013b; Jeong et al. 2013) One of the published approaches was based on fiber-optic SPR for detection of IL-1, IL-6, and TNF-α in a buffered saline solution and a spiked cell culture medium.(Battaglia et al. 2005) In this study, the detection limit of IL-6 was reached to be 0.44 ng mL^{-1}. It has also been demonstrated that optical fiber based sensors has potential for real-time monitoring of biologically relevant molecules in complex biological fluids. A fiber-optic localized surface plasmon resonance sensor was fabricated for detection of IFN-γ using spherical AuNPs on a flattened end-face of optical fiber.(Jeong et al. 2013) The authors emphasized that the fabricated SPR sensor can be used for real-time label-free immunoassay, by the virtue of having a fast detection time (5 min), high resolution and sensitivity (2 pg mL^{-1} for IFN-γ).

5.4 Hairpin DNA probes based amplification strategy

Recently lots of research work on hairpin DNA probe based immunosensors for the ultrasensitive detection of biomarkers has been reported.(Ge et al. 2016; Gong et al. 2014; Guo et al. 2015; Yao et al. 2014; Zhang et al. 2014a; Zhang et al. 2014b) This topic has been symmetrically reviewed.(Huang et al. 2015b) As an example, a hairpin aptamer DNAzyme probe was used for sensitive and visual detection of IFN-γ based on an original quadratic amplification strategy (Figure 5).(Zhou et al. 2013) In this study, the addition of target IFN-γ resulted in two recycling amplification cycles with

assistance of Bst-polymerase and λ exonuclease to generate numerous G-quadruplex/hemin DNAzymes. Colorless $ABTS^{2-}$ is converted into to a green-color product $ABTS^-$ with presence of H_2O_2. This produces a dramatic color change of the solution which enables highly sensitive visual detection of IFN-γ (50 pM) with the naked eye. Using the similar amplification strategy as Zhou et al, Zhang and coworkers developed a method to quantify IFN-γ with two orders higher sensitivity (0.1 pM).(Zhang et al. 2014b) Recently, a new type of amplified fluorescence polarization (FP) aptasensor based on allostery-triggered cascade strand-displacement amplification (CSDA) and polystyrene nanoparticle (PSNP) enhancement for ultrasensitive detection of cytokines has been developed.(Huang et al. 2015c) The assay system consists of a fluorescent dye-labeled aptamer hairpin probe and a PSNP-modified DNA duplex (assistant DNA/trigger DNA duplex) probe with a single-stranded part and DNA polymerase. Two probes coexist stably in the absence of target, and the dye exhibits relatively low FP background. Upon recognition and binding with a target protein, the stem of the aptamer hairpin probe is opened, after which the opened hairpin probe hybridizes with the single-stranded part in the PSNP-modified DNA duplex probe and triggers the CSDA reaction through the polymerase-catalyzed recycling of both target protein and trigger DNA. Throughout this CSDA process, numerous massive dyes are assembled onto PSNPs, which results in a substantial FP increase that provides a readout signal for amplified sensing process. This newly proposed amplified FP aptasensor enables the quantitative measurement of $VEGF_{165}$ with a detection limit of 86 aM, which is about six orders of magnitude lower than that of traditional homogeneous aptasensors.
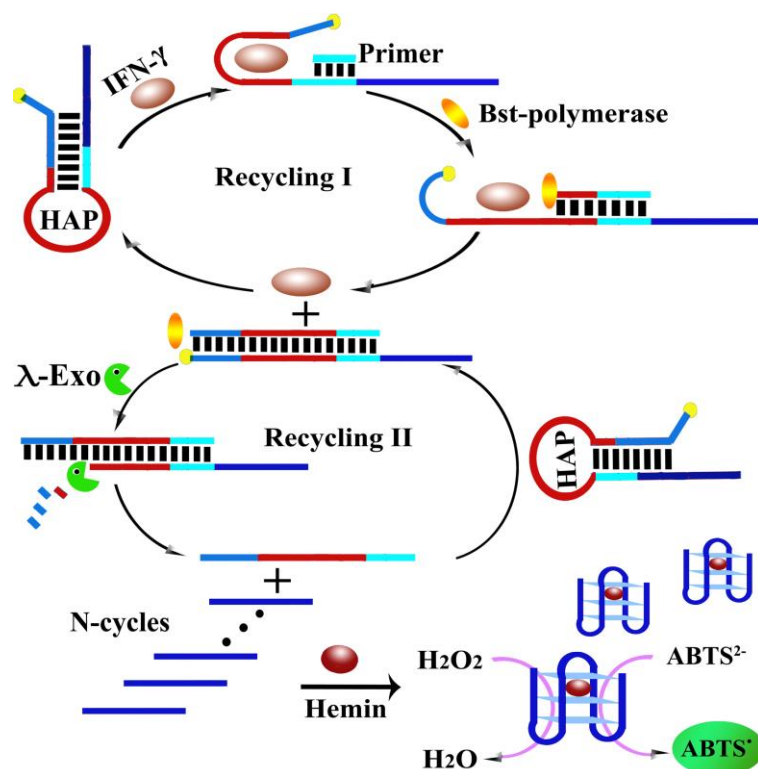
Figure 5 The principle of novel quadratic amplification strategy for highly sensitive visual detection of IFN-γ. Reprinted with the permission.(Zhou et al. 2013)

6. Multiplex cytokine detection

Cytokines act in concert to function in network of effectors. In clinical studies, a multianalyte profiling approach provides more information on the cytokine network than a single-analyte measurement, as several cytokines need to be tested in each sample, ideally in real time. The quantitation of multiple analytes by multiplexed immunoassays offers the advantages of specificity and reduced sample and reagent volumes with implications for the cost-effectiveness of assay. The most popular methods for multiplex cytokine detection are based on color-coded beads and biochip assays.

6.1 Colour-coded beads

In high-throughput sensing technologies, the encoding microbeads and nanoprobes with a unique code is widely used to identify the attached ligand molecules. Blicharz and co-workers combined the advantage of microsphere based suspension array with

the use of a fluorescence microscope for the analysis of inflammatory cytokines in saliva.(Blicharz et al. 2009) The multiplexed antibody array in the study achieved simultaneous detection of ten cytokines associated with pulmonary inflammatory diseases in saliva. Only 100 μL sample was used and the total assay time was 2.5 h. Another bead-based platform reported in the literature to exploited ECL for simultaneous detection of antigens VEGF, IL-8, and TIMP-1 by imaging fluorescently encoded microbeads individually located in a microwell array.(Deiss et al. 2009) The multiplexed ECL platform in this case was an electrode prepared from etched fiber optic bundles coated with gold (Figure 6). The ECL from the array was viewed with a microscope by performing a cyclic voltammogram. This work was the first demonstration that individual sensing bead can be imaged by ECL in a multiplexed sandwich immunoassay.
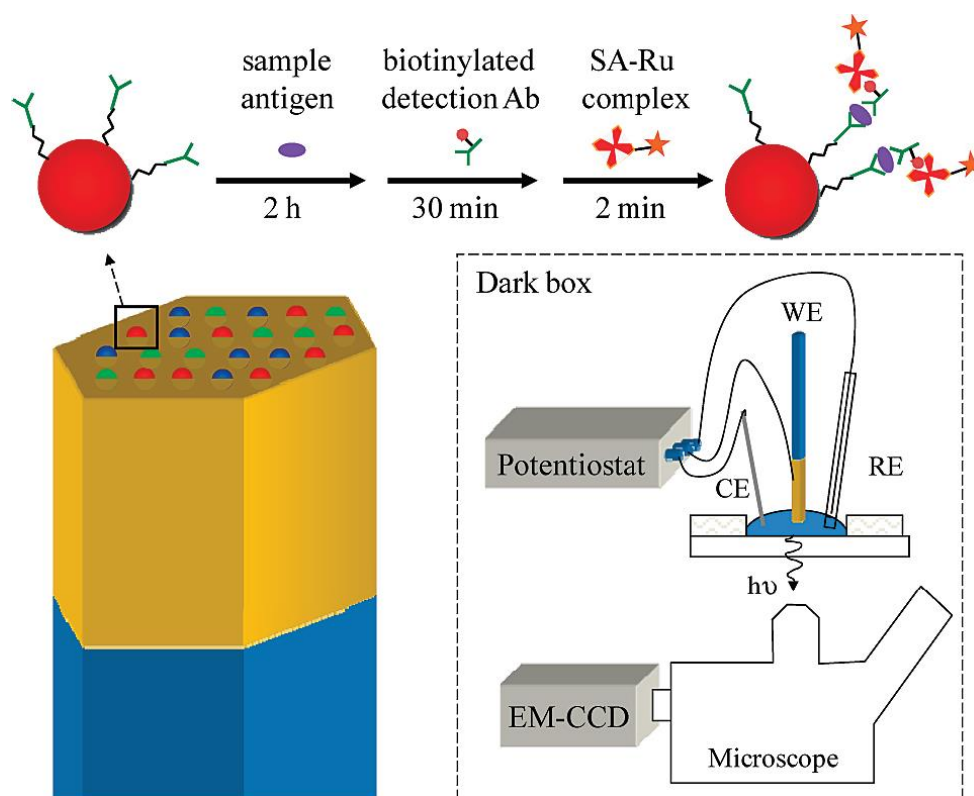

Figure 6 The sandwich immunoassay procedure. The beads are loaded into microwells created in an etched gold-coated fiber-optic bundle which acts as the working electrode (WE) for ECL. Reprinted with permission .(Deiss et al. 2009)

6.2 Sensor arrays

Color coded beads (suspension arrays) are popular choice for multiplex cytokine detection, but there are limitations on the number of distinguishable codes in the same array. To overcome this obstacle, a new encoding approach was developed by the combination of QD and magnetic NPs with nanosphere structure which ensures a greatly enlarged encoding capacity by tuning the magnetic field.(Song et al. 2014) This approach has applied to detection of IgG to demonstrate the reliability of NPs as encoded carriers in multiplex immunoassays.(Stoeva et al. 2006) The result is that NPs conjugated with specific antibodies have bound only to the corresponding positive antigen immobilized on the substrate. In addition, the reproducibly obtained detection limit of IgG was low as 1 fM. The very bright and spectrally narrow NPs Raman tags using SERS provide new opportunities for the optical encoding systems,(Wang et al. 2012b) and are expected to revolutionize high-throughput bioanalysis where multiplexing at high levels is needed. With tunable optical waveguides, silicon photonic MR has been demonstrated great potential for multiplex cytokine detection.(Sloan et al. 2013) In addition, using the multiplicative effects of optical resonant coupling to the PC in increasing the electric field intensity experienced by fluorescent labels and the spatially biased funneling of fluorophore emissions through coupling to PC resonances, PC enhanced fluorescence can be adapted to increase the sensitivity (pg mL^{-1} level) towards multiplex cytokine detection.(George et al. 2013)

7. Real time cytokine detection

7.1 Microfluidic system

Dynamic changes in analyte concentration are difficult to be measured in real time and in many cases this can only be done with special microfluidic devices.(Singhal et al. 2010) Microfluidic devices process volumes of fluids on the order of nanoliters and are capable to achieve multiplexing, automation, and high-throughput screening. The coupling and integration of a sensing system in a microfluidic device has

successfully been applied for real-time analysis with a small amount of sample.(Konry et al. 2013) Quasi-real time cytokine detection has been realized by combining immunoassays with a micro fluidic device.(Nie et al. 2014) For example, a simple lab-on-a-chip biosensor was developed to perform near real-time diagnostics of clinically relevant analytes such as cytokines and antibodies.(Cohen et al. 2015) In this work, the reagent volumes were reduced to 0.5 µL (nearly three orders of magnitude less than in a conventional assay), and the washing steps required in standard immunoassays were eliminated by the same chip. In addition, the detection process could be accomplished in seconds (nearly in real-time) in the flow through incubation channel.

In another example demonstration of this approach, IFN-γ released from an individual T-cell was detected by immunsensors integrated into a microfluidic chip.(Zhu et al. 2008) In this study, cell purification and cytokine detection were performed on the same microdevice which was able to significantly reduce the detection time to 1 h and sample blood volume 3 µL (Figure 7). The microfluidic chips are also compatible with multiplexing. For example a multi-analyte aptasensor for rapid detection of cytokines has been developed.(Liu et al. 2011) In this study, IFN-γ was labeled with anthraquinonoid (AQ), and TNF-α was labeled with methylene blue (MB) redox reporters respectively. Once the cytokine conjugated with the corresponding aptamer, the now modified conformation of the aptamer resulted in decreased redox current. These microfluidic devices were integrated with the aptasensor by standard soft lithography. The cytokines released from T-cells or monocytes were monitored on the same electrode by use of square wave voltammetry.
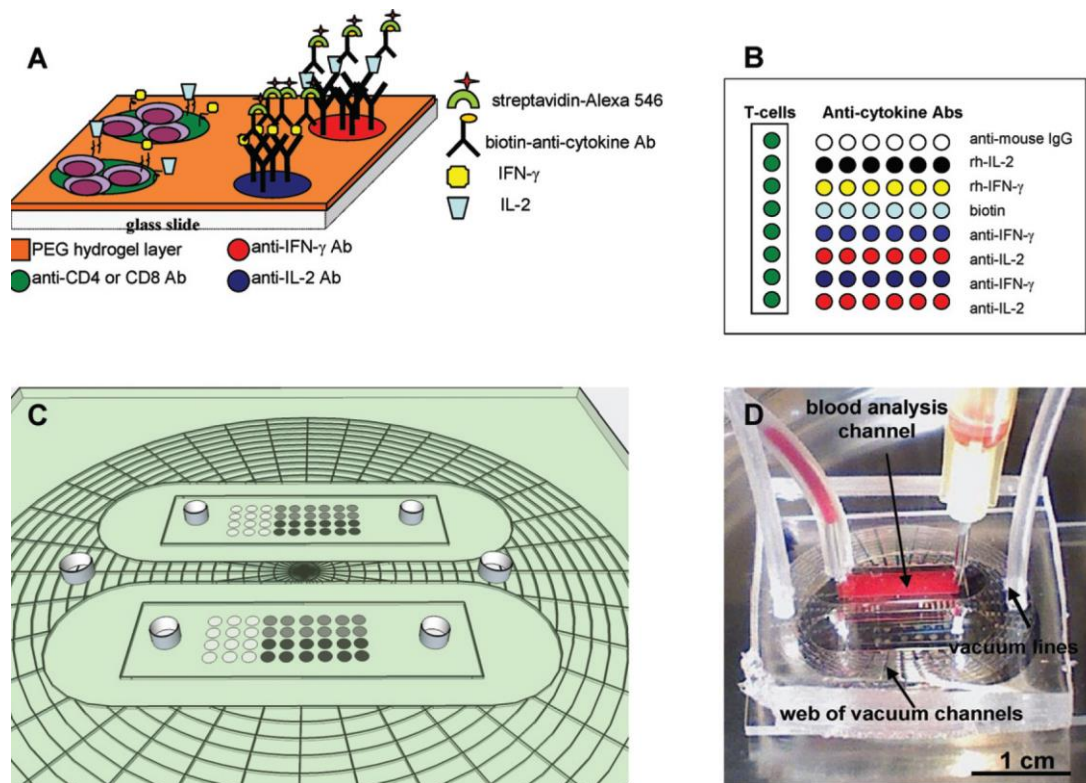
Figure 7 (A) The conceptual design of microarrays for detection of T-cell-secreted cytokines. (B) A map of the 8×20 microarray for capturing T-cells and detecting T-cell-secreted IL-2 and IFN-γ. (C) Design of a microfluidic platform employed for integration with Ab microarrays. (D) An image of a PDMS microdevice employed for T-cell capture and cytokine detection experiments with one reaction chamber filled with unlysed whole blood. Reprinted with permission.(Zhu et al. 2008)

Another multiplex method based on EC was developed using a silicon chip technology for real time detection of IL-1β, IL-10, and IL-6 which were secreted in acute stages of inflammation after implantation of a surgical device into the patient.(Baraket et al. 2014) The fabricated silicon chip incorporated an array of eight gold microelectrodes which allow simultaneous detection of different cytokines through electrically addressable diazonium-functionalized antibodies. The interactions between cytokines and corresponding antibodies were monitored by electrochemical impedance spectroscopy. This design was highly sensitive towards three cytokines in a concentration range of 1-15 pg mL^{-1} where acute inflammation was observed. An

24

aptasensing surface combined with microfluidics was employed by other authors for detection of IFN-α and TNF-γ which were released from immune cells.(Liu et al. 2012c) In the study, anti-IFN-α DNA aptamers and anti-TNF-γ RNA aptamer were thiolated and fuctionalized with the methylene blue redox reporter (Figure 8). The microdevice consisted of two parallel microfluidic channels, each channel containing four cell capture/sensing sites. Upon mitogenic activation, the secreted IFN-γ and TNF-α molecules were captured by aptamers, and monitored by performing square wave voltammetry at different time points at individually addressable electrodes. The detection limit of IFN-α and TNF-γ was found to be 0.06 nM and 0.58 nM, respectively.
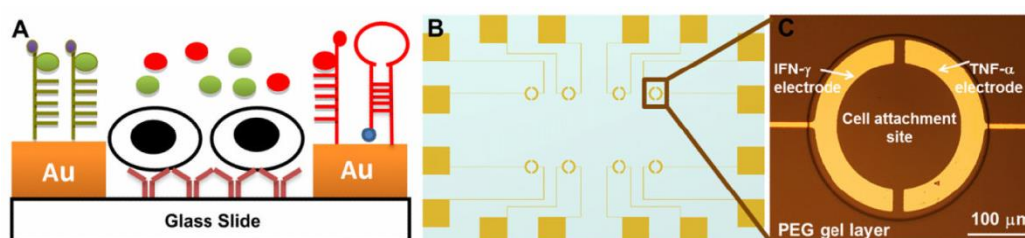

Figure 8 (A) Schematic of a pair of half ring-shaped Au electrodes were modified with different cytokine-binding aptamers. (B) Electrode layout. (C) 300 µm diameter of PEG wells are used to capture approximately 400 cells inside one well. Reprinted with the permission.(Liu et al. 2012c)

Microfluidics, aptasensors, and surface micropatterning were also combined to detect local IFN-γ released from the captured CD4 T cells from a heterogeneous cell sample in real time.(Liu et al. 2011) The sensing mechanism is based on a change in hairpin conformation due to binding of cell-secreted cytokine molecules. To this aim, sensing electrodes were packaged in PEG so as to define cell attachment sites in the proximity of each electrode. These attachment sites were modified with anti-CD4 Ab to promote binding of CD4 positive T-cells. Upon infusing the sample (red blood cell lysed blood) into fluidic channels, leukocytes were captured next to sensing electrodes and stimulated to produce cytokines. The IFN-γ released by cells was then detected at the neighboring sensing electrodes by using square wave voltammetry. This method

makes it possible to detect local concentration of IFN-γ released from live cells in real time, and the signal appear as early as 15 min after when as few as 90 T-cells are stimulated.

Bhavsar and co-workers developed a gold electrode electrochemical impedance immunosensor for label-free and sensitive detection of IL-12 in physiological fluids with the detection time of 90 s.(Bhavsar et al. 2009) An amperometric sensor was reported by Huang and coworkers for detection of IL-6 with the sensitivity of 4.7 pg mL^{-1}.(Huang et al. 2013a) This research provides a promising starting point for future development of highly-sensitive, real-time cytokine detection.

7.2 Biochips

Designing suitable biochips is another approach to achieve dynamic and local monitoring of cytokine expression. This is an active area of research and recent trends in protein biochip technology has been reviewed.(Reddy Jr et al. 2015) Several groups are employing antibody-modified surfaces in conjunction with detection technologies, such as SPR to monitor cytokine secreted from cells in real time.(Milgram et al. 2011; Valentina et al. 2015)   This is a promising direction for dynamic, label-free sensing, but the problem of expensive instrumentation will need to be addressed and detection of specific cell-secreted cytokines has yet to be demonstrated. In a few instances the biochip technology has been combined with the microfluidic system to realize the real time detection.(Jokerst et al. 2010)

8. Practical considerations in cytokine immunosensing

For designing an immunosensing device, stability is a crucial factor besides the sensitivity, selectivity, and other factors reviewed in above sections. Among various methods for binding, covalent coupling is one of the best functionalization approaches because it results in a strong and stable attachment of desired biomolecules on the substrate.(Liu et al. 2014b) The traditional self-assembled monolayer (SAM) of thiols are widely used in many studies, but this approach has many limitations.(Civit et al.

2010) Recently, aryldiazonium salt chemistry-based surface functionization has attracted many researchers because it can overcome the disadvantages of gold thiol chemistry while keeping its advantages. In addition, aryldiazonium salts with different terminal groups can be grafted on various materials to form stable covalent bonds, which significantly widens its application in sensing.(Mahouche-Chergui et al. 2011) Our team has reported many immunosensing interfaces based on the stable aryldiazonium salt chemistry.(Liu et al. 2014a; Liu et al. 2012b; Liu et al. 2014b) Recently Arya *et al*. presented a new platform based on 4-fluoro-3-nitrophenyl grafted gold disk electrode for label free quantification of IL-2 with detection limit of 1 pg mL^{-1}.(Arya and Park 2014) Besides formation of stable organic layers on interface, the stability of antibody on sensing interface is another important factor that can significantly affect sensor performance.(Ahn et al. 2013) The desorption of antibodies reduces the surface density of capturing molecules which leads to false negatives.(Ahn et al. 2012) Although, stability is essential for the sensing device development, it is surprising to find that the stability of sensing interface is infrequently discussed in the context of cytokine detection. Hence, there is plenty of scope for improvement in this area.

Reproducibility is another important property of a successful biosensor. Only a few studies focus on this topic for cytokine detection.(Agalliu et al. 2013; Hosnijeh et al. 2010) To our knowledge, the reported methods for cytokine immunosensing mostly concentrate on the increase in sensitivity and decreased sample volume but not on reproducibility, which might be due to the challenges of cytokine detection as we summarized in Figure 1. Thus this review will not be able to satisfactorily cover this topic.

9**. Commercial cytokine detection assays**

A number of commercial cytokine kits based on different platforms are available and listed at Table S2. Despite these active activities on cytokine kit development, the

precision and reproducibility of these new approaches have not been well defined. In a study of multianalyte bead-based (Luminex) kits, the World Health Organization (WHO) cytokine standards were assayed at the same expected concentrations as the standards provided with each kit, but the WHO and kit standards often yielded very different absolute concentrations.(Nechansky et al. 2008) For example, the IL-4 standard from Linco was greatly (more than 1 log) underestimated in the Linco kit compared to the WHO standards although the read-out obtained with the IL-2 standards provided by all kits were in accordance with the WHO standard. In addition, multiple studies have compared standard-sensitivity multiplex assays with each other.(Breen et al. 2011; Moncunill et al. 2013; Richens et al. 2010) These comparison studies have shown variable agreement among assays and have indicated that absolute cytokine concentrations differ across testing platforms. Such variability is not unique to multiplex assays, as proficiency testing has demonstrated that absolute concentrations of cytokines measured by a single-analyte ELISA can vary widely from lab to lab, although a similar rank order of cytokine concentrations between samples is often preserved. Differences in the number of samples detected in accurate range and reproducibility were observed depending on the method used and even the cytokine detected, although Luminex-based kits were found to be highly reproducible and reliable.(Berthoud et al. 2011) Hence, the cytokine amount measured was critically influenced by the actual kit used. The quantitive determination of cytokines and therefore their use as biomarkers in serum samples have to be interpreted with specified conditions.

10. Conclusion and future perspectives

Cytokine immunosening approaches provide powerful tools for future of infectious disease diagnosis and drug screening.(Zhou et al. 2012) Hence, there is continuing demand for cytokine detection by immunosensing. The significant cytokine detection challenges can be met by designing functional sensing interfaces with improved performance. For example, engineering nanomaterials applied to the sensing interface or using surface enhancement techniques can greatly increase detection sensitivity.

Introducing monoclonal antibodies, aptamers, or anti-biofouling molecules to the sensing interface provides a pathway to improve selectivity. In addition, conjugation detection antibodies with different labels helps realize multiplex cytokine detection. More importantly, it is possible to achieve real time cytokine detection by integrating microfluidic devices, new nanotechnology tools and different transducer methods. Selected exciting technologies for cytokine analysis proposed and developed by various groups, have been outlined in this review.

What about the future for cytokine detection? Regardless of multiple recent developments, ELISA is likely to remain the standard workhorse for cytokine detection. The ability to measure multiple cytokines simultaneously and in real time is extremely important in a variety of physiological conditions, because the concentration fluctuation of one cytokine often induce changes in other networked cytokines. Thus more efforts need to be invested in finding improved labels which are able to report the optical or electrochemical signals efficiently. Together with fibre-optical based sensors, colour-coded beads combining with microfluidic devices will have great potential for real time in vivo multiplex cytokine detection in the future.(Revzin et al. 2012) Moreover, aptamer based biosensors will be the next hot topic for cytokine detection because of their high stability, while aptamers against more cytokines will be required. Therefore, research in the area of cytokine immunosensing is in its early stages, and will continue to grow. Its further development will have significant effect on cytokine biology and early diagnosis of a range of diseases.

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