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Metal enhanced fluorescence (MEF) for biosensors: General approaches and a review of recent developments

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ABSTRACT

Fluorescence-based biosensor platforms have been intensively investigated not only to increase the sensitivity but also to improve the performance of biosensors. By exploiting metal from the macroscopic down to the nanoscopic surface, various architectures have been devised to manipulate fluorescence signals (enhancement, quenching) within near-optical fields. The interaction of a metallic surface with proximal fluorophores (in the range of 5–90 nm) has beneficial effects on optical properties such as an increased quantum yield, improved photostability and a reduced lifetime of fluorophores. This phenomenon called metal-enhanced fluorescence (MEF) has been extensively used in biosensory applications. However, their applications for biological analysis practically remain challenging in biological microenvironments. Therefore, this review primarily provides a general overview of MEF biosensor systems from the basic mechanism to state-of-the-art biological applications. The review also covers the pros and cons of MEF biosensor as well as discussions about further directions in biological perspectives.

1. Introduction

There is no doubt that the application of biosensors, an important spectrum technology, to fluorescence has become promising due to their great versatility, simplicity, sensitivity, non-invasive measurement, and multi-analyte detection (Hacia et al., 1996; Nguyen-Ngoc and Tran-Minh, 2007; Ai et al., 2008). However, one of the fundamental questions in fluorescence-based detection is how to enhance the fluorophores' quantum yield. The question came from the drawbacks – low quantum efficiency, photobleaching, autofluorescence, and so on – preventing fluorescence-based detection from achieving high sensitivity. In this respect, the use of fluorophores for biological applications requires high fluorescence intensity and photostability, which are two important criteria. Furthermore, there must be more versatile and robust architectures for a wide range of related applications.

Biosensor platforms, which produce amplified fluorescence signals, have been intensively investigated. Among these platforms, metallic nanostructures and metallic colloidal nanoparticles are an effective way to improve the optical properties of fluorophores (Willets and Van Duyne, 2007; Stewart et al., 2008) as they can interact with proximal fluorophores and produce an increased quantum yield with improved photostability at an optimal distance of 5–90 nm. This phenomenon, called metal-enhanced fluorescence (MEF) (Geddes and Lakowicz, 2002), originates from plasmon coupling between the metal and fluorophores. Recently, MEF-based methods have been applied to the biosensor system to improve the sensitivity of fluorescence detection to detect molecules at ultra-low concentrations (Lee et al., 2011; Xu et al., 2017). Along with signal enhancement, this promising technology allows sophisticated biological analysis of specific biomarkers and bioimaging on an adequate design.

Though MEF has a long scientific history, its application remains in its infancy regarding biological approaches. This might be from the fact that the sensitivity cannot be guaranteed because a variety of parameters must be considered in a biological environment. To resolve the issues in MEF based biosensors, in turn, a more fundamental approach is needed to apply MEF phenomena to biosensors. There are excellent published reviews and book chapters that illustrate the potential for plamonic platforms in which plasmonic sensors are well described (Dong et al., 2015; Spackova et al., 2016; Kumar et al., 2016). Instead, this review focuses on a general view of MEF based biosensor systems and considers the advantages and limitations from a biological perspective. In addition, given its importance in research, we briefly

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highlight the corresponding cutting-edge applications and future biological outlooks for MEF studies.

2. A general view of biosensors

A biosensor is defined as a small device involving biological sensing elements capable of detecting a chemical or biochemical, and has numerous applications in fields such as medical research, bioprocess monitoring, and biotechnology (Grieshaber et al., 2008). A typical biosensor is comprised of three parts: i) a bioreceptor, which is a biorecognition element that specifically binds to the analyte, ii) a transducer, which is an interface architecture that converts the signals where a specific biological event occurs, and iii) processing systems (detector, display), where the signal is detected and converted to other signals, such as electric signals, using the appropriate system.

The development of biosensors has involved integrating different fields such as materials science, molecular engineering, chemistry, and biotechnology with new research to design better sensors (Liu, 2014; Jayanthi et al., 2017). Since the first generation of biosensors was reported by Clark (1956) with the advent of the oxygen electrode, incredible progress has been made using such integrated strategies to improve biosensor performance. In general, biosensors can be categorized according to the basic principles of signal transduction and biorecognition. There is much variety in how biosensors work and which component serves as the main transduction element. Thus, biosensors with different transducer types (e.g., electrochemical, optical, piezoelectric, mass-sensitive, acoustic, thermal, or other) show different features and have their own pros and cons (Hoa et al., 2007; Ahuja and Kumar, 2009).

A new biosensing platform development for an effective transducer is also important for achieving higher sensitivity and lower detection limits. In principle, these systems can effectively convert bioanalytical signals into measurable physiochemical signals, which in turn quantify the amount of analytes. Transduction, which is a signal conversion, can be accomplished via a wide variety of methods based on numerous detection schemes. However, despite the rapid progress in analytical methods, developing inexpensive, robust, and versatile platforms for biosensing remains challenging.

3. MEF and its correlated optical biosensors

As shown in Fig. 1(a), optical biosensors typically consist of a biorecognizer, (optical) transducer component and signal amplifier (Grieshaber et al., 2008). Optical biosensors exploit the interaction of the optical field combined with a biological sensing element such as proteins, aptamers, and subcellular components (Borisov and Wolfbeis, 2008). These biosensors commonly use light absorption, luminescence, fluorescence, Raman scattering, reflectance, the refractive index, and other techniques. Before discussing what MEF is and introducing its applications to biosensors, fluorescence and surface plasmon resonance (SPR) associated with MEF should be considered. This is because these two optical spectroscopies in the near field regime of nanostructures would be closely connected to MEF phenomena in terms of the near-field optical interactions between fluorophores and metallic surfaces. Sometimes, MEF has been called plasmon-enhanced fluorescence (PEF) by certain scientists (Li et al., 2015). We observe a blurry boundary between MEF and PEF to define which factors primarily affect fluorescent enhancement due to the lack of a fundamental foundation. Of course, there are other possible optical interactions to MEF, but above all, it is paramount to illustrate two optical principles and common sensing features to explain MEF in detail.

The MEF process relies on several critical factors to produce desirable effects and subsequently, to introduce a new direction in fluorescence detection. Regarding the efficacy of biosensors, it is fundamentally important to use brighter and more photostable fluorophores to achieve a high level of sensitivity, as photodegradation in conventional fluorophores occurs while the fluorophore is in the excited state. The presence of metal near the fluorophore increases the rate of excitation and emission by opening additional electron configurations of fluorophores. Though MEF cannot occur if the quantum efficiency of the fluorescent materials is already 100% (Khurgin et al., 2007), it has been reported that MEF occurs with all types of fluorescent materials including organic fluorophores (Lakowicz et al., 2008), and small nanoparticles (e.g., quantum dots (Ray et al., 2006b), carbon dots (Li et al., 2012a), and upconversion nanoparticle (Feng et al., 2015)). This MEF process excels with regards to increasing the brightness, photostability and sensitivity at the same time. In addition, MEF platforms provide an advantageous route while fabricating a sensing platform. The design that couples with optical transducer and signal amplifier would make sensors simpler than traditional fluorescence-based sensors that require additional steps for signal amplification (Fig. 1.b). Thus, these features explain why MEF is beneficial for fluorescence-based detection and the robust platform based on MEF is a promising tool for producing effective biosensors.

3.1. Fluorescence based detection for optical biosensors

Fluorescence detection offers numerous important advantages over other methods. One of the reasons for the huge increase in fluorescence-based technology is probably the distinct features – i.e., fluorescent materials can act as excellent sensing probes to change some intrinsic property (fluorescent intensity variation, fluorescent shift) and the specific affinity for the ligand. Such fluorescent probes have become effective transducers that transfer biorecognition events into fluorescence signals evaluated using a variety of detectors. Hence, this principle of fluorescence based detection is most relevant and developed in biosensor applications. With a relatively simple detection process, the advantages of fluorescence for biosensors are summarized in the following manner:

i) high versatility (sensitivity, specificity, simplicity, and speed),
ii) a non-destructive way of tracking or analyzing biological molecules,
iii) allowing multiple analytes to be detected using different emission wavelengths.

The need for reliable and sensitive tools for efficient biosensor development has become vitally important in the field. In this context, fluorescence-based techniques have proved to be valuable tools. Present approaches to fluorescence-based biosensor development can be primarily classified into two different strategies: sensing components (new fluorescent probe and label designs) and transducing components (useful new schemes for sensor arrays, platforms, or architectures). The rapid advance of nanotechnology could contribute to the development of more versatile materials and facile devices.

By working with the plethora of new nanomaterials, a new class of fluorescent materials will bring biosensor systems to a higher level. Most nanomaterials with very small size ranges have fascinating and useful optical properties. New nanomaterials used as sensing components (e.g., similar to molecular fluorescent probes) mostly presented better and far more functional fluorescence behavior, providing numerous unprecedented possibilities to overcome the inherent drawbacks associated with fluorescent dyes. For example, a great number of fluorescent nanomaterials (e.g., quantum dots (Wegner and Hildebrandt, 2015), carbon dots (Sun et al., 2006), upconversion nanoparticles (Chen et al., 2014), conjugated polymers (Thomas et al., 2007) etc.) have been developed, which shows the remarkable optical properties and great potentials in terms of brightness, photostability (fading and blinking prevention), and a tunable emission spectrum. These merits offered by nanomaterials make sensor devices very sensitive and reliable. Thus, the primary reason for using these types of nanomaterials instead of conventional fluorophores is to improve

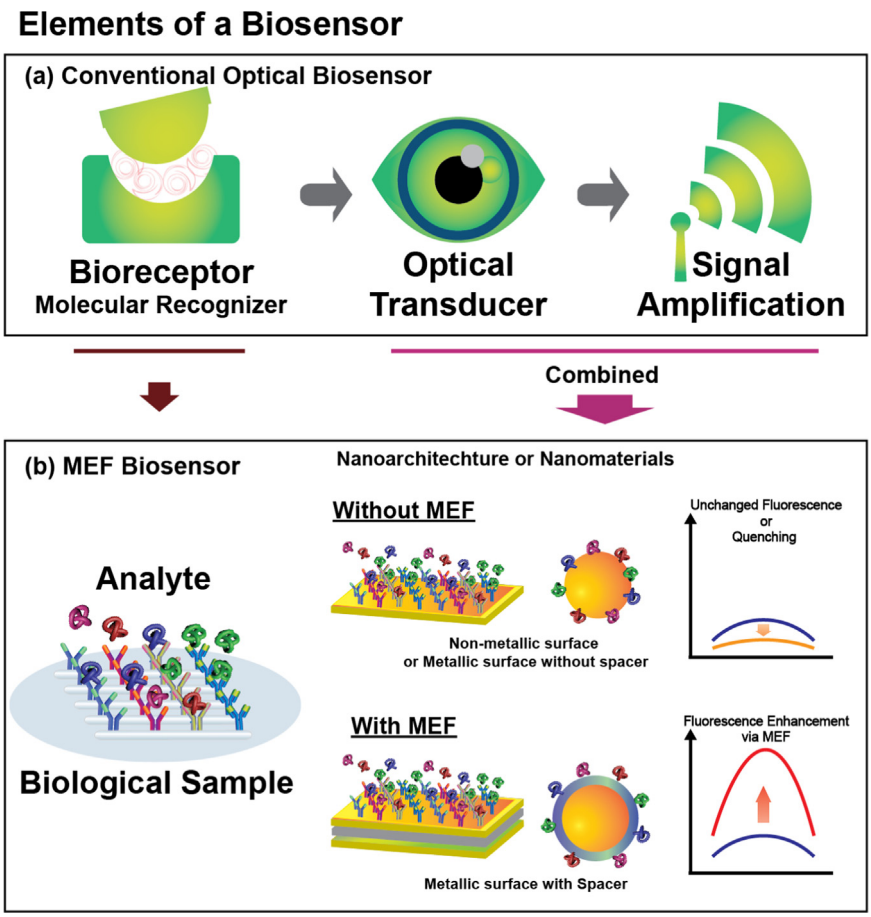

Fig. 1. The concept of optical biosensors, (a) Conventional optical biosensor and (b) its correlation to MEF platforms for optical biosensors.

optical response and the sensing mechanism. In certain cases, new fluorescent nanomaterials have specific properties with high selective sensitivity to target materials based on the inherent nature of nanomaterials (Zheng et al., 2015). Without adding any external labeling, these materials can perform as sensors for targeting specific molecules. However, except for a few cases, the development of new fluorescent nanomaterials as sensors mostly entails significant challenges due to the lack of specific molecular recognition elements for the analytes.

3.2. Optical biosensor based on SPR nanoarchitecture

Plasmonic nanostructures, referred to as SPR or localized SPR (LSPR) nanoarchitectures, have been used to develop various sensor platforms. This technology continues to gain significant attention from many scientists. The SPR sensors immobilize the transducers onto the nanoarchitecture surface (e.g., nanoarray, nanohole, nanoparticles etc.) (Wittenberg et al., 2011; Gomez-Cruz et al., 2018), which can interact with the analyte by producing an optical signal shift. This signal transduction can be advantageous, because it offers distinguishing characteristics (e.g., minimal interference, *label-free*, real-time monitoring) by varying the sizes and shapes of nanostructures (Chung et al., 2011). Experimental and theoretical biosensor research based on SPR or LSPR have demonstrated the detection of bioreagents including viruses (Bai et al., 2012), bacteria (Vaisocherova-Lisalova et al., 2016), DNA (Yuan et al., 2017b), and other biomolecules (Puiu and Bala, 2016; Lo et al., 2016).

By modifying the surfaces of these structures, it is possible to deliver information on the selective binding and detection of specific targets in contact with the metallic surface. Then, the signals are evaluated with their analytic spectroscopic methods (e.g., the wavelengths of the plasmonic absorption peaks for Raman scattering (Focsan et al., 2016)). Furthermore, the intense and confined electromagnetic fields induced by the LSPR can make a highly sensitive zone capable of detecting small changes in the surrounding dielectric environment of the nanostructures. Despite the advantages of SPR transduction, SPR-based biosensors have been applied to biomolecules to a much lesser extent due to many factors in biological studies. The major drawbacks should be considered due to non-specific binding and steric hindrance associated with the difficulty of immobilizing bioreceptors onto the sensor's surface. Hence, these constrain the accuracy of measurements in biological fluids. Even if theoretical approaches provided possible results that could detect bioreagents at the single molecule level (Aćimović et al., 2009), they still marked low sensitivity at plasmonic sensors in practical applications compared to other sensing platforms.

As a similar class of surface enhanced spectroscopies, the field of plasmonic sensors with nanoarchitectures has grown rapidly to investigate unique near-field phenomena in optical nanostructures. Among them, it seems that MEF-based sensors and SPR-based sensors are a subclass of plasmonic sensors. However, these sensors can be roughly classified differently based on surface enhanced sensing techniques. For example, fluorescence emission for MEF and Raman scattering for LSPR are competing phenomena. Although these phenomena have similar origins, the conditions in which they occur are very different. Therefore, it is important to keep in mind the specific conditions for MEF sensors compared to other surface enhanced spectroscopies.

3.3. When does MEF occur?

Experiments in fluorescence enhancement in the proximity of metal nanostructures have been reported since the 1960s. (Drexhage, 1970).

Along the same line, there have been various theoretical approaches and experimental reports on distance-dependent fluorescence enhancement on a metal surface. Generally, it is known that MEF occurs when an excited fluorophore is positioned near metals at a distance of 5–90 nm (Ray et al., 2006a; Ribeiro et al., 2017). Meanwhile, fluorophores are quenched at very close proximity (< 5 nm) or through direct contact with the surface of metals, in which the quenching effect overwhelms the enhancement effect. MEF can increase fluorescence intensity several hundred times. However, most of the reported results were inconsistent regarding the distance in which the maximum enhancement occurs. Several mechanisms for the enhancement factors have been suggested, but the precise mechanism is still somewhat debatable due to the complexity of metal-fluorophore interactions. This MEF phenomenon is a complex physical effect of surface plasmons and near-field optical effects, which lead to the modification of near- and far-field optical effects (Lakowicz et al., 2008; Deng et al., 2013). However, it definitely affects our thinking about conventional far-field fluorescence spectroscopy (Geddes, 2013).

3.4. Mechanism of MEF

Given the electrodynamic interaction, there are several factors responsible for the enhancement.

The first is the effect of local field enhancement generated near metallic structures (Fig. 2.(a)) (Li et al., 2015; Lakowicz et al., 2008). Metals can strongly interact with the incident light and produce concentrated electrical fields with a localized charge density oscillation. This is known as localized surface plasmon resonance (LSPR) around the surface within a nanometric scale, which modifies the optical properties of local fluorophores. The fluorescent molecules near the metal surface show an efficient coupling between the electromagnetic field and spatially confined free-electrons, which leads to higher emission intensity. Shape and size also play significant roles in determining the fluorescence enhancement, because the sharp corners and edges of metal nanostructures intensify the electric field where the fluorophore is located, enhancing the fluorescence intensity under resonance excitation (Zenin et al., 2015). Therefore, the strongly localized electrical field of a metal's nanostructure plays the role of an antenna where nearby fluorophores are exposed to greater light intensity.

Another factor is the plasmon-coupling effect mediated by a non-radiative interaction (Fig. 2. (b)) (Aslan et al., 2005). If the plasmon and the fluorophore are at an optimal distance, the energy transfer between them is dominated. This is explained by Förster (or fluorescence) resonance energy transfer (FRET), the mechanism of electron transfer through molecules (Govorov et al., 2016). To support this, there has been a reasonable consensus that a critical parameter is the metal-fluorophore distance. The non-radiative energy transfer between metal and fluorophore depends not only on the strength of the electric field but also on the degree of spectral overlap between the metal surface and the fluorophore (Lakowicz et al., 2008). Recent experimental and theoretical studies have shown that when the absorption spectra of metal nanostructures or nanoparticles overlaps the fluorophore's absorption, the excitation and emission rates of the fluorophore are enhanced. In addition, the fluorescence enhancement is more efficient (Tam et al., 2007; Abadeer et al., 2014). Thus, a possible explanation for the fluorescence enhancement may be through resonance energy transfer at a distance of ~ 10 nm and the Purcell effect at a longer separation of 10–50 nm (Li et al., 2015).

Finally, radiative decay engineering (RDE) is a potentially important effect (Fig. 2.(c)), the theoretical approaches of which are well described elsewhere (Lakowicz et al., 2008). In brief, the fluorescence intensity of fluorophores near metallic structures is also enhanced by changing the radiative and nonradiative decay rates, leading to an effective emission enhancement. In the near-field between excited-state fluorophores and the metallic surface, reciprocal interactions of plasmons created by fluorophores in the excited state and in the ground state occur (Ray et al., 2009). These interactions correspond to the absorption and scattering properties of metallic nanostructures that can increase the emission of fluorophores (decay rates, location, and direction). The radiative rate of fluorophores is modified in the presence of a metal and this modification decreases the lifetime of the fluorophore due to the increased rates of system radiation decay. These conditions can be explained by the molecular mechanism of feedback de-excitation; fluorophores near the metal surface can undergo more excitation-emission cycles before the fluorescence lifetime and the quantum yield change. In MEF, this effect increases the fluorophore quantum yield and the fluorophore's brightness while decreasing the lifetime.

4. MEF-based biosensor platforms

Most MEF-based biosensor systems have been using two general platforms: (1) a two-dimensional (2D) planar MEF platform or (2) metallic colloids MEF platforms.

4.1. Developmental stage of 2D substrate MEF biosensor system

4.1.1. Fabrication of 2D MEF substrate

The development of surfaces suitable for MEF has a long history. The 2D planar substrate platform has been extensively studied where fluorophores can be easily placed at a controlled optimal distance for MEF. Initially, metallic thin films were prepared on a 2D planar substrate (a homogeneous substrate) by simple fabrication techniques via electrochemical roughening (Geddes et al., 2004) and thermal evaporation (Kümmerlen et al., 1993; Aslan et al., 2008) (Fig. 3a). However, despite the simplicity in fabrication, the enhancement factors were relatively low. A facile technique for fabricating MEF substrates was required to achieve maximum enhancement.

While previous studies were simply conducted for metallic thin films prepared on the 2D planar substrate, a number of surface modified techniques have been developed by carefully controlling the properties of nanoparticle deposited on the substrate(Fig. 3.b) (Zhang et al.,

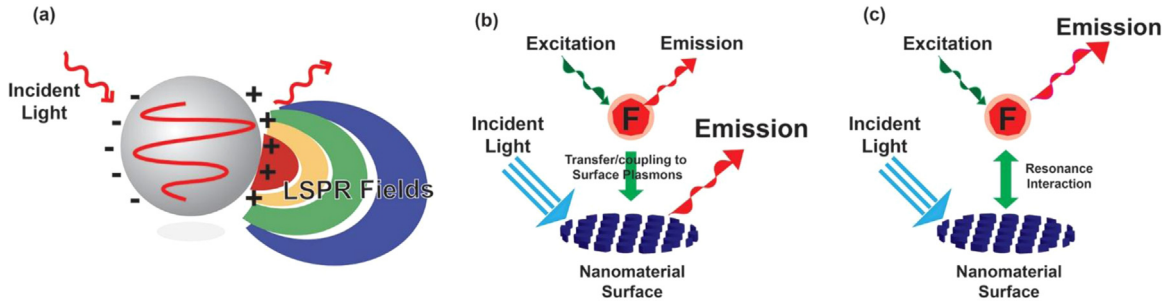

Fig. 2. Mechanism of MEF. (a) Localized surface plasmon resonance (LSPR). (b) Plasmon-coupling effect. (c) Radiative decay engineering (RDE). Adapted with permission from Refs. Li et al. (2015) and Aslan et al. (2005).

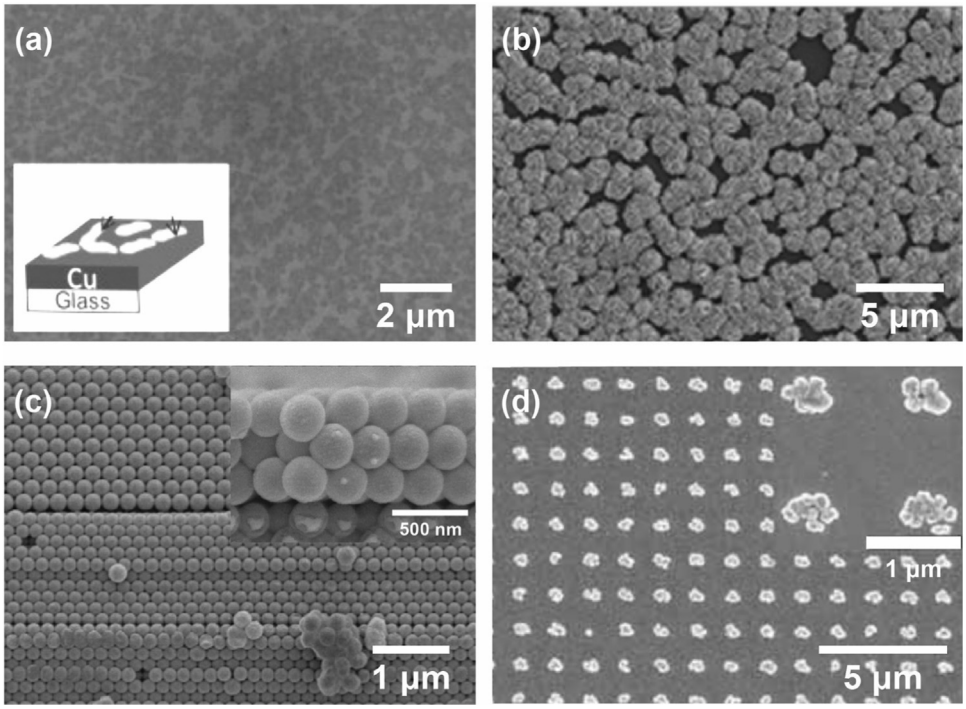

Fig. 3. Field emission scanning electron microscopy (FE-SEM) images of (a) metallic thin film, (b) roughened-nanoparticle deposition, (c) ordered nanoparticle array nanostructure, and (d) metal patterned arrays made by nanoimprint techniques for MEF 2D substrates. Adapted with permission from Refs. Aslan et al. (2008), Zhang et al. (2017), Li et al. (2011) and Yang et al. (2010).

2017). For example, aggregated nanoparticles (or a nanoparticle island) via metal colloid adsorption are applied for a fabricating tool used for 2D MEF platforms. Nanoparticles of varying dimensions can be readily formed by different parameters (i.e., the annealing temperature, the concentration of the colloidal solution). The ionic strength of a colloidal suspension varies according to its surface energy level, and the particles tend to make the aggregates on the smallest surface to reduce their surface free energy (Badawy et al., 2010; Stebounova et al., 2011). To reduce the interparticle separation, the ionic strength of particles increases gradually. At this time, if the metallic colloid has a high surface energy, it would be able to absorb effectively onto the substrate surface (Yamaguchi et al., 2007). In a similar fashion, various methods for controlling the self-assembly of nanoparticles on substrates have been reported (Grzelczak et al., 2010; Li et al., 2011). These techniques for attaching metallic colloids on appropriate substrates to construct a metal nanoparticle aggregate system have been performed up to the present.

In addition, applicable research using hierarchical and/or complex metallic 2D nanostructures have been reported. The representative examples are the use of flower-like, dendrite, or star-shaped morphology with a random distribution (Fig. 3.c) (Dong et al., 2012). A higher fluorescence enhancement by factors of up to a few hundred could be obtained for high nanomaterial adsorption densities (Zhang et al., 2017; Dong and Zheng, 2013). The aggregated or complex random formation of nanoparticles (and/or its islands) on a layer of dielectric substrate leads to the production of concentrated electrical fields due to the LSPRs of adjacent metallic nanostructure. Adjacent metallic particles (or structures) touch each other lightly so that fluorescence enhancement on these structures is much higher than those obtained with typical 2D metallic thin films due to surface enhanced effects, as with creating plasmonic nanogap structures – regions of high electromagnetic enhancement at the junction of adjacent nanostructures (Li et al., 2012b). However, these types of complex structures require more study.

Even if highly monodisperse or regular nanoparticles within the 2D MEF platforms are used, these complex platforms can compromise the reproducibility due to random distributions of metallic nanoparticles or structures. Disordered (heterogeneous) nanostructures can thereby hinder systemic approaches to clarifying the behavior of MEF on 2D substrates, typically resulting in an average fluorescence enhancement that is obtained from ensemble measurements of the entire system. In other words, the results have shown that the 2D MEF platforms optimized for bright emission in ensemble measurements with uncontrollable features of nanoparticles or nanostructures formed from various deposition methods.

To overcome these obstacles, several studies explored other approaches using more sophisticated patterning techniques (Fig. 3.d) (e.g., electron beam lithography (Corrigan et al., 2005), nanoimprint lithography (Yang et al., 2010), ion beam milling (Pang et al., 2017), and reactive ion etching (RIE) (Battista et al., 2017)). Each technique has its own advantages for precisely controlling the height, diameter, and density of the nanostructures. By combining the unique optical properties of nanostructured gaps and nanosubstrates (e.g., nanotriangle, nanocircle, nanooval, etc.) (Wang et al., 2012) consisting of a regular array presented for the tunable analytical method of LSPR substrates, these methods displayed a remarkable increase in fluorescence emission. Such methods are considered the most advanced for fabricating a miniaturized feature within a few nanometers. The investigations exploited different nanostructures by verifying the MEF, which can trigger large enhancement factors. However, the enhancement factors depend on the morphology of the pattern.

4.1.2. *Optimized MEF as controlling spacers*

Most importantly, one critical feature for MEF is a layer of dielectric (nonmetallic) material referred to as a spacer (Lakowicz et al., 2008; Deng et al., 2013). Generally, spacers are made of dielectric materials (e.g., SiO_2, TiO_2, Al_2O_3) or organic polymers (e.g., PEG–thiol/COOH–-thiol, polyelectrolyte, polyvinyl alcohol, Teflon). For a very short separation, the quenching effect is dominant. Thus, this MEF system must precisely adjust the thickness of the spacer to elevate close-range quenching within the enhancement distance. However, the sizes and shapes of uniform spacers with nanoscale thickness would be extremely difficult to accurately control with a fabrication process in the nanoscale range (Ray et al., 2015). Therefore, numerous techniques have been invented to control the thickness of the spacer.

The simplest way to control the distance between fluorophores and

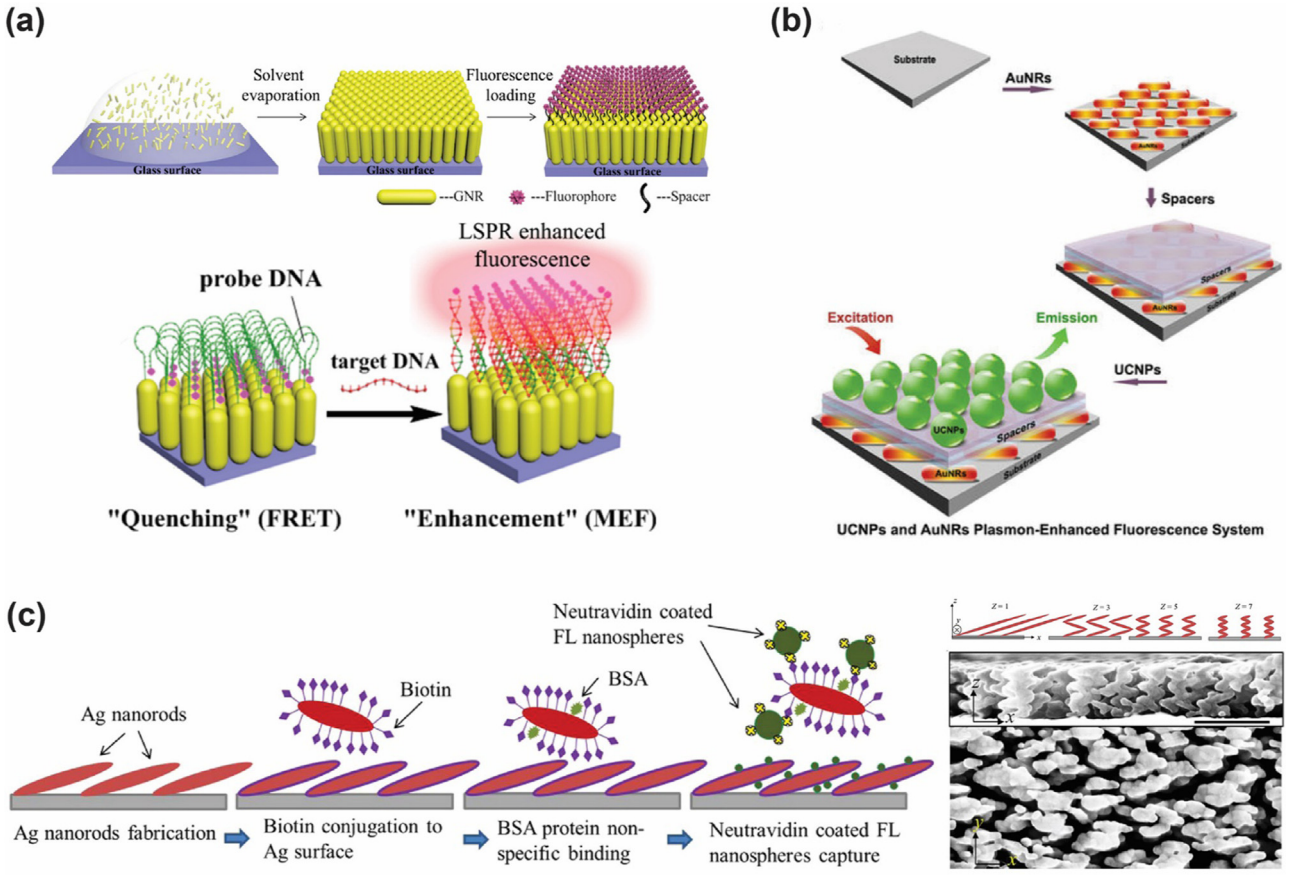

Fig. 4. Examples of MEF control via layer-by-layer (LbL) deposition. (a) The ordered gold nanorod (GNR) array chip for DNA detection upon hybridization (b) Fluorescence enhancement of upconversion NPs (UCNPs) using polyelectrolyte multilayers deposition. (c) MEF of zigzag Ag nanorod arrays. Adapted with permission from Refs. Feng et al. (2015), Mei and Tang (2017) and Ji et al. (2016).

a metallic surface is layer-by-layer (LbL) deposition of polymeric spacers, dielectric spacers, or hybrid composite spacers. Thereby, most studies related to methods for modifying the surface layers showed their use of organic polymers, inorganic dielectric materials, and hybrid composites by balancing fluorescence enhancement and undesired quenching. In addition, new structural studies revealed that multilayered films with hybrid nanocomposites have generated much higher MEF, leading to geometric and dimensional effects (Zhang et al., 2010; Jang et al., 2014). Using an LbL approach, various functional materials such as polyelectrolytes were used as building blocks to construct a series of functional multilayered thin films for MEF sensing. The multilayered film was composed of sequentially stacked functional materials such as DNA probes (Fig. 4a) (Feng et al., 2015), small nanoparticles (Fig. 4b) (Mei and Tang, 2017) and fluorophores positioned to the last layer. If multilayered films with stimuli-responsive (temperature, pH, ionic strength, light, etc.) materials acting as a spacer are used in the LbL assembly process, the obtained multilayered films yield unique characteristics corresponding to the external stimuli to control the structural changes and functions of MEF substrates (Ma et al., 2011; Wang et al., 2014). Moreover, a new type of structure, MEF of Ag zigzag nanorod (ZNR) arrays (Ji et al., 2016), made by oblique angle deposition (Fig. 4c), was studied to determine whether it is suitable for MEF applications. By changing the fold number – the morphology of the Ag zigzag shape – a 14-fold enhancement factor for biotin-neutravidin detection was obtained.

4.1.3. MEF control by SPR spectra

At a particular frequency of light, gold and silver exhibit strong extinction bands in the visible spectrum due to a combination of absorption and scattering (Jain et al., 2006). One important parameter for MEF, which has often been proposed, is the spectral overlap between the plasmon and fluorophores. To control the plasmon spectrum overlap, some studies have focused on the use of gold and silver nanomaterials with a variety of shapes such as rods (Niu et al., 2016b), prisms (Chen et al., 2007), and cubes (Liang et al., 2012). This is likely due to the dependence of spectral wavelength variations on the shape of nanoparticles that exhibit significant sensitivity to their local refractive index. A very recent study coincided with the maximal enhancement results when the plasmon is resonant with excitation and emission spectra (Khatua et al., 2014). In one case, a peak shift in the elastic scattering resonance occurs. For example, if nanocubes were deposited on the planar substrate as a patterned array, the LSPR peaks of nanocages can be easily shifted from the visible region to the NIR region by controlling the deposited thickness (Fig. 5.(a)) (Camposeo et al., 2015). In other studies associated with plasmon bands, various metallic species such as aluminum (Chowdhury et al., 2009), copper (Sugawa et al., 2013), and gold-silver alloy (Zhou et al., 2013) were used and deposited on the substrates to study MEF by changing the LSPR wavelength. Furthermore, an ordered array of copper (Cu) nanostructures showed significant LSPR generation and changes in LSPR wavelengths, resulting in enhanced fluorescence signals by appropriately thick interlayers (Fig. 5.(b)) (Sugawa et al., 2013).

To date, a variety of advanced 2D MEF nanosubstrates have been proposed using physical and chemical methods (e.g., thermal evaporation, chemical deposition, electrochemical deposition, chemical-thermal deposition, photoinduced deposition, lithography, dealloying, and adsorption of metal colloids on the substrate) (Dutta Choudhury et al., 2012). Furthermore, using various geometric and dimensional parameters in the MEF substrates including patterned (periodic) and non-patterned (randomized) metallic nanostructures, many

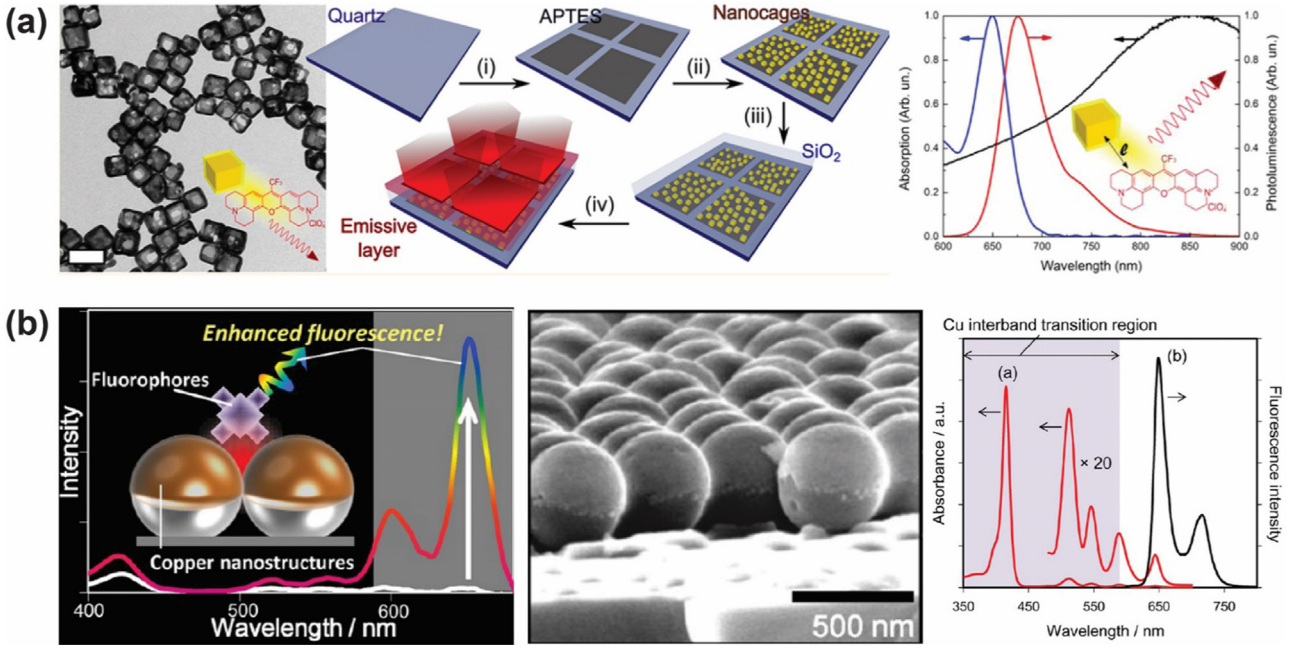

Fig. 5. Research examples of the spectral shift and overlap between the plasmon and fluorophores by controlling SPR spectra: (a) Au nanocages and (b) an ordered array of copper (Cu). Adapted with permission from Refs. Camposeo et al. (2015) and Sugawa et al. (2013).

investigators have proposed a significantly enhanced fluorescence of more than several orders of magnitude for single molecule fluorescence detection, compared with single layered metallic substrates. However, it is important for high-end applications to note that these techniques still need an easy and economic fabrication approach, because they are too time-consuming and complex to be cost effective in real practical applications.

4.2. Challenges of 2D MEF substrates in biosensing applications

Solid substrates might cause other critical problems, which place limitations on biological studies for 2D substrates. First, the amount of biomolecules such as proteins, antibodies, and peptides that can be attached to the rigid flat-substrate is limited. Biorecognition (or molecular recognition) that occurs between the rigid substrate is central to all biosensing platforms. However, in such cases, a limited binding of biomolecules on the rigid substrate will decrease the sensitivity.

Another limitation of the rigid substrate was discovered in biological fluids and caused by the direction of diffusion over the sensing layer wherein the geometry of a 2D flat-substrate restricts the diffusion of biomolecules. One-way directional diffusion occurs between the flat-substrate and biological fluids, whereas radial diffusion occurs at the 3D spherical substrates. Consequently, diffusion affects the sensitivity of 2D rigid sensor arrays.

Moreover, the denaturation and dehydration of biomolecules caused by a rapid evaporation of the aqueous environment may cause them to lose their biological activities. Their biological structures (i.e., tertiary structure) could also be modified or destroyed at the interface of the MEF substrates. This results in a relatively reduced sensitivity on the MEF substrates.

Last but not least, complex optical phenomena occur in the excited fluorophore state with SPR on the metal surface. Sometimes, fluorophores positioned at the MEF sensors require high energy excitation sources, which hinders the fabrication of a nanoscale multiplex analytical platform. This means that, regarding nanoscale architectures, these biosensors are strictly required for the durability of the sensing layer. Accordingly, much research has considered robust 2D planar surfaces that can monitor the kinetics of biomolecular interactions. However, the fabrication processes of nanostructure substrates in previous works are either complicated by expensive machines or unstable. It is still very challenging to commercialize the MEF substrates for biosensing application due to the problems associated with manufacturing costs and time as well as reproducibility.

4.3. Developmental stages of colloidal suspension MEF biosensor system

Since the first attempt to use a silver core and silica shell in a solution sensing platform (Aslan et al., 2004; Aslan et al., 2007), there has been a basic need to develop ideal substrates to better understand MEF in colloidal suspensions. Most related works for MEF study have been conducted using nanoparticles isolated by shells (so called core-shell structures). These structures are defined as consisting of an inner material with other shell layers. The formation of core-shell structures provides an easy way to avoid the quenching of the fluorophores using wet chemistry or bottom-up approaches (Alloisio et al., 2016). Furthermore, it has many features required for an ideal biosensing probe to detect particular components in a complex bioassay. Coating the inert metal shell on the surface can improve the dispersibility, stability, and biocompatibility, which are suitable for use as specific targeted probes with enhanced fluorescence in solution after surface functionalization (Hu et al., 2013; Dong et al., 2014).

Such metallic colloids in their own structure can be designed for different purposes and can effectively work as substrates to enhance fluorescence detection. These are typically observed up to several orders of magnitude fluorescence enhancement in suspension, accompanied by reduced lifetimes and improved photostability. As a consequence, these developments open up a broad range of possibilities for ultrasensitive and low-background fluorescence detection.

Generally, multiple steps (at least three) are needed to prepare MEF nanoparticles according to their methodologies. The preparation of metallic core nanoparticles is the first step towards the formation of the core-shell. Advances in new synthesis techniques have made it possible to fabricate various nanoparticles/nanocrystals, leading to a change in size and shape of metallic colloids (e.g., rods (Lohse and Murphy, 2013), triangle (Xue et al., 2015), cubes (Sun et al., 2016), and stars (Cui et al., 2013)). Moreover, it is important to fabricate nanoparticles with different metal species (gold, silver, copper, zinc, alloy, among others) (Zhang et al., 2016). This is primarily because the optical

properties of metallic colloids can be tuned by altering the synthetic parameters prior to designing MEF nanoparticles. For instance, the absorbance of Au nanoparticles is sensitive to the refractive index of the surrounding medium and the surface plasmon band is redshifted with increasing Au nanoparticle size (Link and El-Sayed, 1999; Okamoto et al., 2000).

After production of core metallic nanoparticles, the pre-synthesized metallic nanoparticles are stabilized against agglomeration through the formation of an appropriate dispersant layer, retaining their unique optical properties. At this stage, molecules or thick layers can be used to keep them dispersed in the aqueous phase to prevent coagulation and act as spacers to avoid fluorescent quenching. In this respect, the choice of shell materials is crucial for MEF among different types of spacer shells.

As briefly noted above in the section on 2D MEF substrates, the establishment of a spacer layer gives considerable attention to the type of spacer shells that can be used in designing MEF substrates and nanoparticles. Though there are numerous parameters for controlling the MEF efficacy for designing systems with a tunable MEF, spacers can be the most significant in achieving MEF effects. To date, a variety of spacers have been reported. Approaches for establishing a spacer layer are primarily classified into two categories based on the materials used for distance modulation: inorganic spacer (e.g., silica) and organic spacer (e.g., polymer, proteins, DNA, aptamer and others). However, neither the inorganic nor organic spacer is superior; instead, each has advantages and disadvantages as documented elsewhere (Cui et al., 2014). The purpose of this contribution is to provide a general viewpoint of MEF sensors from a biological perspective. Hence, we did not introduce properties that depend on the spacer type, but aimed for correlation with a biological facet.

4.3.1. Inorganic spacer for colloidal MEF nanoparticle

Looking at the inorganic spacer first, the inorganic spacer shell's primary advantage stems from the property of fixed and controllable thickness, which means that inorganic spacer shells can control the shell thickness. As depicted in Fig. 6(a), typically, silica shells have been used to represent the inorganic spacer shell on the surface of the metallic core due to many benefits such as biocompatibility, robustness, chemical inertness, thermal stability, and optically transparent materials (Gontero et al., 2017; Asselin et al., 2016). Silica plays two roles in the MEF structure: easing surface functionalization and controlling the separation distance between metal and fluorophore to obtain the optimal MEF effect. Actually, the surface chemistry of silica is well understood. The surface of silica shells can be easily modified with suitable functional groups, such as amino, thiol, or carboxyl, enabling further functionalization. Importantly, a major aspect of silica spacers provides the same level of thickness up to 90 nm in a controllable manner by adjusting the silica precursor concentration during the synthetic processes (Bardhan et al., 2008). As a result, there have been many reports of silica coated metal nanoparticles and their hybrid composites (Yang et al., 2011; Kim et al., 2009), showing the precise distance modulation for obtaining maximal MEF efficacy.

Moreover, the outer silica shell can incorporate almost any fluorescent material by coating nanoparticles with fluorophores (covalent attachment) or by trapping the fluorophores inside outer shells (simple doping). Through these relatively simple methods, the fluorescence molecules are at a suitable distance for enhancing fluorescence, which leads to the in-depth study of structural properties for MEF. For these reasons, the silica shells are suitable for designing systems with a tunable MEF and allow experimental and theoretical access to the MEF phenomenon.

Notably, the formation of inverted core-shell structures was reported using metallic nanoshells (Fig. 6.(b)) (Zhang et al., 2012a). This inverted structure could be fabricated through different routes and showed different optical properties. Based on theoretical calculations, the metal shells can have uniform electrical fields inside the core

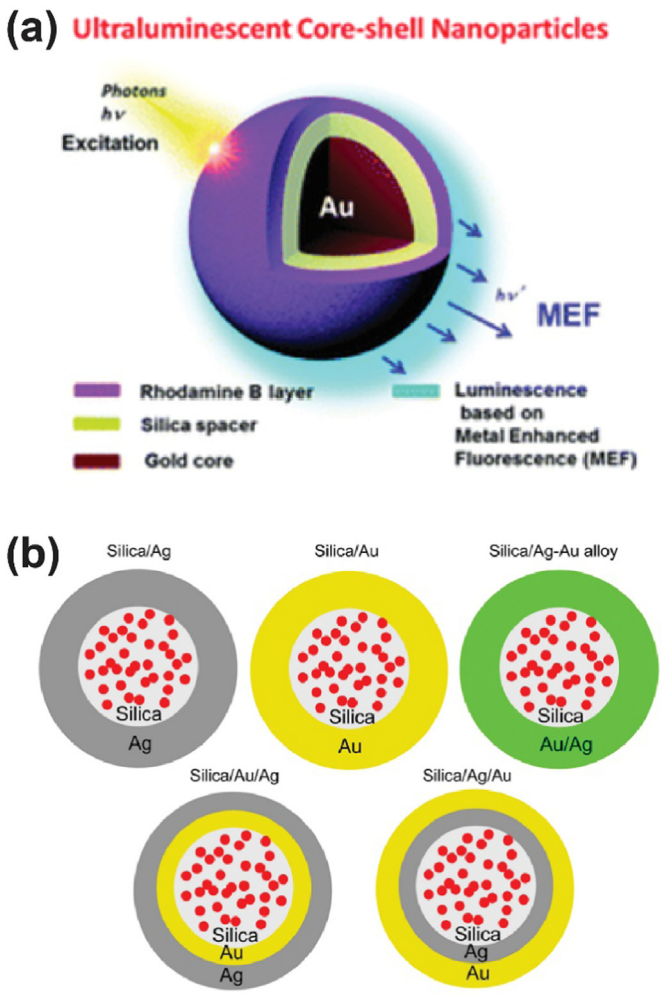

Fig. 6. Two inorganic types of core-shell MEF NPs: (a) the core-shell structure and (b) the inverted core-shell structure. Adapted with permission from Refs. Gontero et al. (2017) and Zhang et al. (2012a).

(Moores and Goettmann, 2006). When the fluorophores are within the metal shells, they can efficiently interact with plasmon resonances. According to other reports, the metal shells formed on the silica sphere display dual or multiple plasmon bands that can interact efficiently with the fluorophores inside the silica core, particularly when heterogeneous (bimetallic) shells are used as spacers. Thus, it is possible to use structural modification strategies such as the inverted structure for rational MEF design (Soulé et al., 2013).

4.3.2. Organic spacer for colloidal MEF nanoparticle

Compared to inorganic spacers, organic spacers can be exploited using different soft materials to design MEF hybrid composites. This method is commonly used as a linker and as a spacer to control the distance between metal nanoparticles and the fluorophore through covalent bonding or electrostatic attraction. The primary advantage of this approach is that it combines the characteristics of the metallic core and organic shells. Initially, it appears that this approach can control the MEF effect, but there are drawbacks due to the flexibility of organic spacers. Because organic materials are not rigid enough to obtain accurate distance control, specified experimental conditions (solvent, temperature, pH, light etc.) are required to preserve organic molecules and their properties in MEF enhancement (Gilbert and Martin, 2015). In other words, their relative stability is inferior compared to inorganic spacers, resulting in decreased sensitivity and unexpected results in biological fluids.

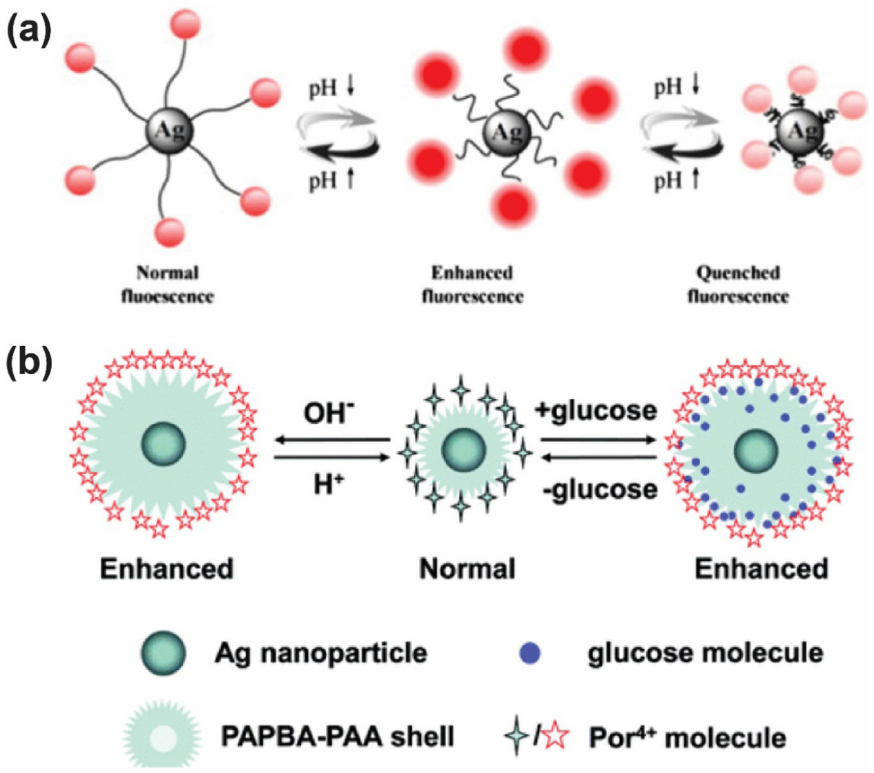

Fig. 7. An MEF system with stimuli responsive shells: (a) polyacrylic acid (PAA) and (b) poly(3-acrylamidephenylboronic acid-*co*-acrylic acid) shell (PAPBA-PAA). Adapted with permission from Refs. Ma et al. (2015) and Zhang et al. (2012b).

In certain cases, however, its conformational flexibility is useful for meeting the diverse application requirements. For example, stimuli responsive polymers on the surfaces of metallic NPs have been introduced for organic polymer spacers (Fig. 7a, b) (Ma et al., 2015; Zhang et al., 2012b). The MEF effect could be tuned by external stimuli such as temperature, light, and pH. Among different thermosensitive polymer families, poly(N-isopropylacrylamide) (PNIPAM) or related copolymers have been by far the most commonly used (Tang et al., 2011b). PNIPAM or its copolymers presents a lower critical solution temperature (LCST) of approximately 32 °C, which allows the shape and structure of metallic colloids to be tailored for the MEF effect. Moreover, with the behavior of pH sensitive poly(acrylic acid) (PAA), a number of hybrid MEF nanocomposites bearing a PAA spacer shell have been reported by changing the pH of the medium in MEF enhancement (Yuan et al., 2017c). Using the stimuli responsive behaviors, the metal-fluorophore distance can be controlled through the presence of stimuli sensitive units. A typical project based on this concept is the development of an activatable MEF system for fluorescence sensing and/or imaging in specific regions. In addition to the use of stimuli responsiveness, many other research groups have described similar procedures to address functional soft materials – DNA, proteins, aptamers, etc. – as effective linkers and spacers incorporated into tunable MEF systems.

After the construction of inorganic spacer onto the surface of nanoparticles, functional organic materials can be combined in a way similar to the methods used on 2D substrates (Jang et al., 2014). These strategies have been extended to manipulate the surface functions by integrating advanced technologies (e.g., multilayered core-shell nanoparticles) (Sun et al., 2016). To exploit this concept, a solution based biosensing platform was designed for the highly sensitive detection of biomolecules. For example, by combining a core-shell geometry with oligonucleotide hybridization probes, so-called molecular beacons (Fig. 8) (Wang et al., 2009; Pang et al., 2015), fluorescent biosensing techniques have been demonstrated in various applications for the multiplexed detection of miRNA, RNA capture assays, and the kinetics of DNA hybridization.

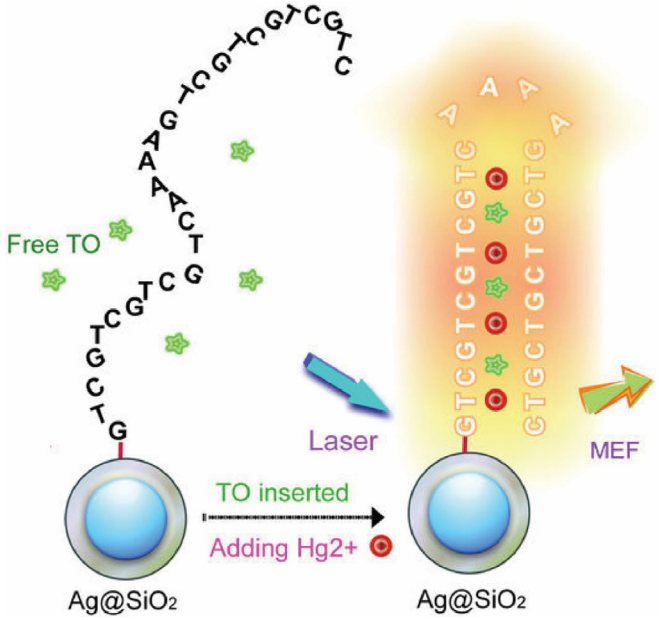

Fig. 8. The MEF-based aptamer-Ag@SiO2 sensor, which applied oligonucleotide hybridization probes. Adapted with permission from Ref. Pang et al. (2015).

4.4. Challenges of colloidal MEF system in biosensing applications

As discussed in the section on 2D substrates, one of the fundamental problems is non-specific absorption, which in turn entails decreased sensitivity and an influence on colloidal stability. The selectivity is

typically determined from the outer functionalized layer on the metallic surface (i.e., external labeling) to overcome unspecific binding of the analyte caused by biological complex solutions. However, external labels may change the surface interactions, which definitely limits plasmonic coupling approaches in sensor applications. This issue of the plasmonic type of nanosensors is always a dilemma. In addition, even though MEF colloidal nanoparticles tend to aggregate in biological fluid in the absence of targeted molecules, this yields misleading signals and increased background signals. In most cases, aggregated colloidal nanoparticles are irreversible and cannot be reused. Thus, the production of robust colloidal sensors for analytical purposes is highly required for synthetic stabilities with high selectivity and sensitivity. The lack of reusability in almost all colloidal platforms needs to be resolved for practical use.

The MEF efficacy in colloidal systems is quite low due to absorption and scattering with other nanoparticles in colloidal suspensions, in contrast to 2D MEF substrates. In this context, other problems have been newly addressed to discuss increases in the low enhancement factor under the biological fluidic condition. Inconsistent experimental enhancement for colloidal MEF nanoparticles was attributed to artifacts produced by experimental procedures (Ribeiro et al., 2017). This study demonstrated that experimental artifacts might cause light scattering and/or absorption by the particles when soft or porous spacers are used. To explain this effect, most studies related to plasmonic phenomena involving MEF or SERS in colloidal nanoparticles describe inner filter effects (Ameer et al., 2012), which refer to light intensity attenuation caused by the light absorption and/or scattering of the nanoparticles. However, there still are no exact grounds for explaining which factors will be affected in the colloidal system. There also needs to be theoretical and experimental elucidation for effective MEF colloidal systems.

Most importantly, MEF systems always require strict experimental conditions to preserve their properties for fluorescence enhancement. In most biosensing systems, the proof-of-concept has been reported by providing the results under controlled and restricted experimental conditions. As a consequence, validation of these results in real samples needs to be addressed.

5. Cutting-edged bio-applications for MEF

As described in the previous sections, MEF systems for biosensors are available in a variety of forms such as 2D nanosubstrates, colloidal nanoparticles and combined multiplex forms. Due to the unique fluorescence properties of MEF, this technology offers many opportunities to introduce wide-ranging applications for adjusting nanomaterials and/or nanostructures. Importantly, though there are numerous examples for biosensors from the wide-ranging applicability of fluorescence in bio-related fields, most have been introduced in other review articles (Lakowicz et al., 2008; Dong et al., 2015). In the next section, we selected more relevant and practical applications for biosensing as a multifunctional substrate for MEF, reflecting on the biological limitations discussed in this article.

5.1. One-dimensional (1D) MEF nanowire

The research on MEF of one-dimensional (1D) metallic nanowires is of great interest for biosensing applications. Compared to zero-dimensional (0D) or two-dimensional (2D) nanomaterials, 1D nanomaterials exhibit unique optical advantages for sensing from the dimensionality (a nanoscale transverse dimension versus a microscale or semi-nanoscale longitudinal dimension) (Burt et al., 2005). For decades, the 1D hybrid nanocomposite has been used as a building block for potential applications such as multifunctional atomic force microscopy (AFM) probe (Jing et al., 2006). Along with the principle of LSPR, a fluorescence enhancement sensor that uses hybrid 1D nanowire sensing platforms can be systemically designed for the facile, highly sensitive, and selective detection of target molecules.

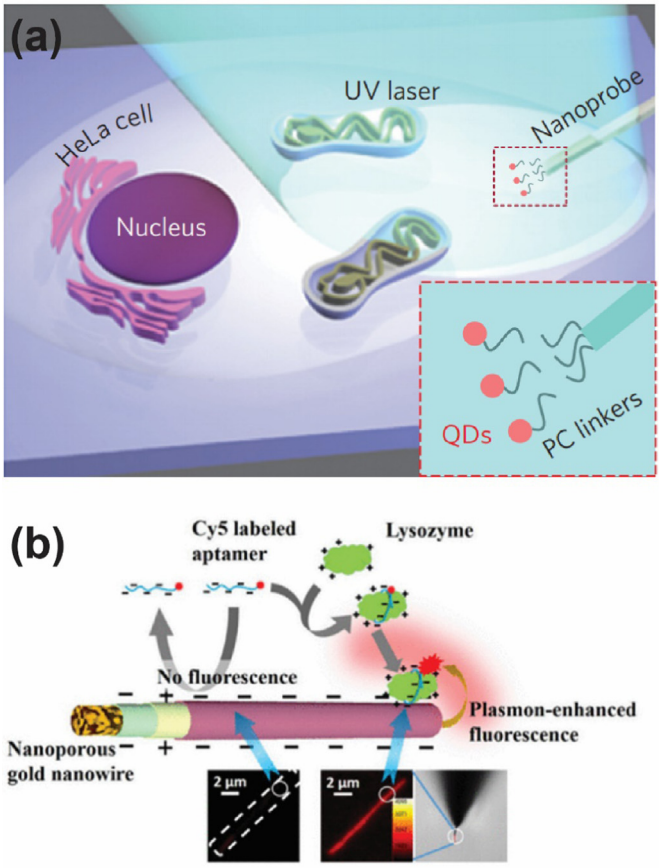

Fig. 9. (a) Schematic illustration of a single cell endoscopy and (b) the nanoporous biosensing nanowire to the aptamer/aptamer-lysozyme complex. Adapted with permission from Refs. Yan et al. (2012) and Yuan et al. (2016a).

Based on its dimensionality, when the fluorophores are at the ends of nanowire structures (the edges or corners of metallic structures), strongly localized electric fields can be intensified to show the increased fluorescence enhancement. At the two ends of nanowires, higher fluorescence enhancements have been achieved via the MEF approach (Yuan et al., 2016b). However, certain reports demonstrated that fluorescence enhancement on the 1D hybrid nanowire structure was lower than that on other nanostructures. Thereby, artificial defects on the 1D nanowire structure have been made wherein a myriad of nanoscale gaps act as strong SPR regions to further enhance the fluorescence properties of MEF. The creation of nanoporous structures for metallic nanowires allows the LSPR wavelength of the nanowires to be adjusted according to the fluorophore spectrum (Niu et al., 2016).

An interesting proof of concept for a single cell endoscopy was proposed by combining nanowires with the plasmonic-coupling effect (Fig. 9a, b) (Yan et al., 2012; Yuan et al., 2016a). At the cellular level, it is notably paramount for realizing biological events inside single cells to identify when or where chemical/biological events occur. The experimental results herein show that the 1D nanowire hybrids will be potentially very useful in MEF-based intracellular sensing and imaging. The MEF effect could provide much improved sensitivity to the rational design of MEF based sensing tips. Therefore, the single-cell endoscope is expected to be a promising technology for high-resolution fluorescence imaging when combined with the MEF effect.

5.2. Immuno MEF sensor chip

An immunoassay (e.g., immunohistochemistry, immunocytochemistry) is a representative fluorescence analytical method used in biological research and the medical field (Taylor and Levenson,

2006). Fluorescence-based immunoassays use simple binding of fluorescence labeled antigens or a sandwich format in which second antibodies have been labeled with fluorophores. However, biosensors that include imaging and detecting based on immunoassays also suffer from certain limitations such as a low quantum yield, photodegradation due to long-term exposure time, and a low signal-to-noise ratio due to interference from non-specific binding or autofluorescence. This might lead to an equal enhancement not only in the fluorescence intensity but also in the background signal noise during detection and imaging. Therefore, in recent years, a number of new analytical methods combined with the MEF effect via 2D nanoarchitecture use the fluorescence amplification scheme (Matveeva et al., 2004; Matveeva et al., 2007). MEF immunoassays for biosensors achieve high fluorescence enhancement using a multilayered slide glass (Deng et al., 2013; Jang et al., 2014).

The immunoassay can be extended to a variety of wide-ranging applications for biological research. Immunohistochemistry (IHC) is a valuable tool for studying the localization of specific molecules by fluorescence in cells or tissues. An interesting development regarding IHC is a type of silicon-supported silver-island plasmonic chip (Ag@Si chip) (Fig. 10) (Yuan et al., 2017a), that has the sandwiched structure designed for use in cell/tissue applications. The Ag@Si chip showed enhancement measurements on the fluorescence of IHC-labeled tissue sections with an Ag@Si chip covering. The enhanced signal intensity of fluorophores on the membranes of cells or tissues could be obtained via the MEF effect by simply covering the fluorescent-labeled cell and tissue samples with the chips. The prototype chip should be improved for real use in a lab or other technology fields. It is obvious that this simple strategy of integrating into other assays or sensing platforms is highly beneficial.

5.3. Hydrogel MEF substrate

We already discussed that, at the air-sensor substrate interface, biomolecules can suffer denaturation and oxidation reactions. Hence, it is important for biosensor systems to impede non-specific binding on the surface of biosensors without losing their biological nature. Considering the need for a protective layer to solve this problem, the use of hydrogel substrates for biosensing platform construction is promising. Platforms can work well in aqueous environments (Mateescu et al., 2012) because hydrogels can contain water due to their stability and softness in aqueous media. In addition, the hydrophilic nature of hydrogels could often enable minimized non-specific interaction with biomolecules such as proteins or with cells. Hydrogel is a suitable matrix for sensor surface protection, but hydrogel layers might not be ideal candidates for MEF applications due to the technical challenges in controlling the hydrogel layer thickness with spacers.

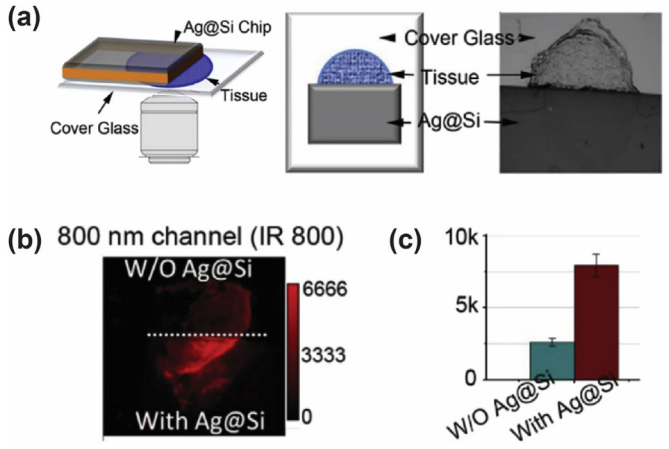

Fig. 10. (a) A schematic view of the fluorescence immunosensing chip, (b) the scanner images of the tissues obtained with/without Ag@Si coverage, and (c) the fluorescence intensity distribution, which was acquired by averaging the fluorescence intensities with/without Ag@Si coverage. Adapted with permission from Ref. Yuan et al. (2017a).

Instead, the hydrogel sensor entrapping silica coated silver nanoparticle was developed by combining the 2D substrate of hydrogel platforms with a particle-based MEF platform (Fig. 11) (Jang et al., 2015). The hydrogel layer in this design can absorb biological fluids containing proteins within the 3D polymeric matrix. The MEF effects can be observed due to entrapped Ag@SiO_2 nanoparticles. To support the feasibility of this biosensing system, the results demonstrated that the amount of Ag@SiO_2 incorporated within the hydrogel matrix can affect the fluorescence enhancement. When the concentration of Ag@SiO_2 increased, a large portion of fluorophores were positioned near Ag@SiO_2 NPs, which resulted in the maximum enhancement factor. This study was conducted to make applications for biosensors more practical using a microfluidic device incorporated within the hydrogel microarray system.

5.4. Non core-shell formation in MEF nanoparticles

To produce MEF nanoparticles, most studies have focused on the integrated structures (i.e., the formation of core-shell structures), which should be constructed in at least three steps: preparing a metallic core, coating with an optimally thick dielectric layer for the MEF effect, and binding fluorescent materials onto the outer surface. These processes have been considered useful for fabricating MEF nanoparticles, so they are virtually formulated to study MEF nanoparticles and wide applications based on such structures (Tovmachenko et al., 2006; Planas et al., 2016). For some special cases, there are different types of MEF nanoparticles in colloidal suspension. Among various factors, the most relevant is the interparticle distance between the fluorescent emitter and metal nanoparticles. When meeting this condition, fluorescence enhancement was observed. Thus, we introduced two examples associated with non core-shell formation: heterodimer MEF NPs and polymeric spherical MEF NPs.

The first method for fabricating non core-shell MEF NPs is through the use of heterodimer nanoparticles – comprised of two adjacent nanoparticles – which have great potential in multimodal applications due to the two exposed and accessible surfaces. Aside from these features, one type of heterodimer nanoparticle for MEF was designed by combining silica nanoparticle encapsulating fluorescent carbon dots and metallic nanoparticles (Liu et al., 2015). As shown in Fig. 12(a), the silver nanoparticles were positioned on the surface of a mesoporous silica nanoparticle that incorporated luminescent carbon dots. The fluorescent enhancement was observed to increase nearly 3.4-fold due to the interaction of silver nanoparticles and luminescent carbon dots.

The other way is to fabricate a polymeric spherical structure incorporating metallic nanoparticles (Fig. 12(b)) (Tang et al., 2011a). In the previous section, we discussed stimuli responsive MEF nanoparticles wherein external stimuli responsive polymers (e.g., PNIPAM, PAA) were used to control the MEF effects. In contrast, hybrid polymeric spheres incorporating silver nanoparticles were reported using poly(N-isopropylacrylamide-*co*-acrylic acid) (PNIPAM-*co*-PAA). In this study, by in situ thermal reduction of Ag+ to Ag, a pH- and thermo-responsive hybrid microgel was prepared. Interestingly, the hybrid microgel reacted in response to external stimuli, which can lead to significant changes in volume. As a consequence, the MEF effects can be manipulated.

Compared to reported MEF NPs with the core-shell structure, these strategies are relatively simple and effective on some occasions depending on how these MEF nanoparticles were designed. However, a critical drawback under these formations might be that systemic approaches are impossible for determining the main factors of MEF effects.

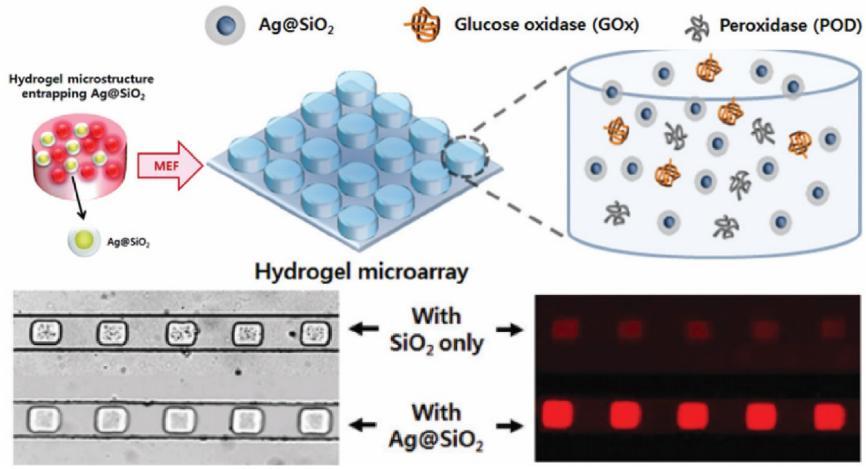

Fig. 11. A hydrogel microarray entrapping silica-coated silver nanoparticles ($Ag@SiO_2$). Adapted with permission from Ref. Jang et al. (2015).

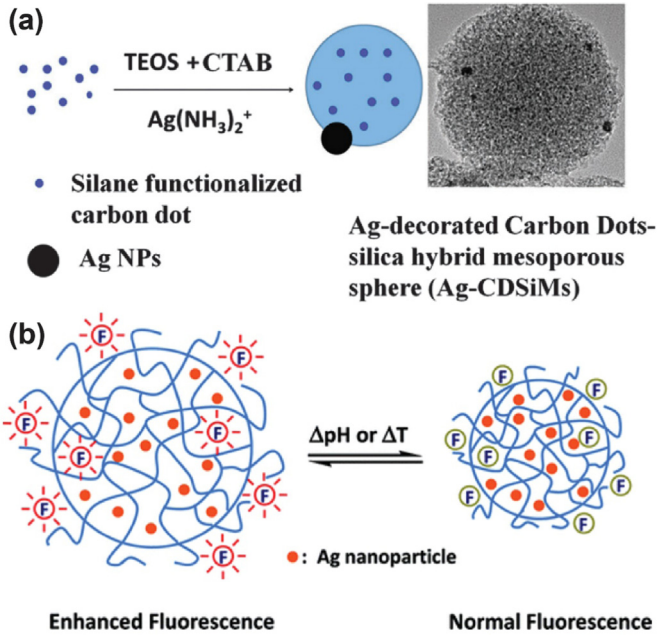

Fig. 12. Non core-shell MEF NPs: (a) heterodimer type and (b) polymeric spherical type. Adapted with permission from Refs. Liu et al. (2015) and Tang et al. (2011a).

6. Challenges and future perspectives

6.1. General challenges

Given the electrodynamic nature of plasmonic coupling within near optical fields, it is reasonable to question which factors cause fluorescence enhancement. Despite all the efforts discussed in previous sections, there has been no consensus among researchers regarding a generalized model in MEF studies. Maximizing the MEF highly depends on the optimal range of distances separating fluorophores from metal, but there are several essential factors and variables to consider: (i) the chemical composition of the metallic surface, (ii) the structural dimensions, geological pattern and aggregation state such as size and shape for large excitation cross-section and amplified localized surface plasmon resonance, and (iii) the degree of overlap between the plasmon band and the fluorescence excitation-emission spectrum.

Aside from the optimal distance for MEF, researchers have tried to demonstrate the effect of other parameters on fluorescence enhancement (e.g., materials, shape, size, spacer thickness, and spectral overlap) (Chen et al., 2007; Mishra et al., 2013; Ayala-Orozco et al., 2014; Reineck et al., 2013). For instance, one of the lesser known studies endeavored to verify the effect of the aggregation state in a solution–based sensing platform (Gunawardana et al., 2015). At high concentrations, most conventional fluorophores usually undergo self-quenching. In contrast, the results showed approximately 100–200-fold fluorescence enhancement compared to the free state of the fluorophores by aggregating a high concentration of MEF colloidal nanoparticles. Furthermore, a study with gold nanorods was conducted to validate the correlation between two variables: distance and plasmon wavelength (Niu et al., 2016). Therefore, a series of these less known studies are needed for a systemic approach to the dependence of MEF on other parameters such as fluorophore concentration and MEF nanoparticle aggregation states.

6.2. Future MEF perspectives for in vitro and in vivo sensing

Many researchers have proposed the use of metallic colloids in aqueous solution, because this would be more desirable when applied to biosensing and related research intended for live cells for the tissue or in vivo model (Lavis and Raines, 2008; Chinen et al., 2015). The basic idea of combining colloidal MEF platforms and fluorophores provides an opportunity for many potential advantages to creating ultra-bright fluorescent probes beyond the conventional organic fluorophores' limitations (Fig. 13). A nano-sized particle (or probe) referred to as a sensor – to detect and communicate a particular biological event at the same time – serves as a recognition element and a signal transducer simultaneously (Maysinger et al., 2015). Thus, these

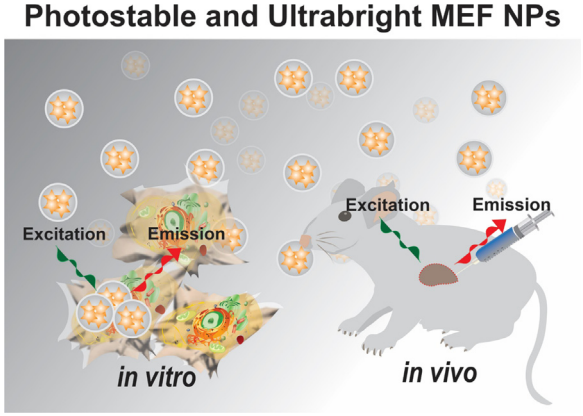

Fig. 13. A cartoon of a promising in vitro and in vivo biosensing tool using MEF colloidal NPs.

nanoparticles with versatile functionality, such as targeting moieties to specific markers, are particularly attractive tools for biological sensors in medical fields.

However, we have witnessed an extremely small number of MEF platforms applied in colloidal suspensions with regards to the in vitro analytical system. In these examples, no advantage could be observed using MEF phenomena, compared to in vitro cellular detection using other nanomaterials. There was no guarantee to its effectiveness in a cellular environment, even if suitable biosensor structures could be designed and synthesized. In addition, it is unclear which of them would be best matched to analytical applications based on MEF. To the best of our knowledge, there is no in vivo analytical system applied to MEF sensing platforms. Assuming certain situations in the physiological and pathological processes, the MEF phenomena can notably provide great opportunities for the development of highly sensitive sensors from the cellular level to the organ level. Nevertheless, few present-day MEF based assays in colloidal suspension are suitable for assessing target molecules in vitro and in vivo.

A decade ago, in vivo imaging already suggested the development and use of metallic colloidal conjugated fluorescent dyes in medical imaging (Geddes et al., 2003), but more extensive research was needed to apply the MEF phenomenon. We think one of the reasons might be the same as described above, i.e., difficulties in preparing suitable nanostructures in biological solutions. Such requirements for in vivo studies certainly present much higher technical challenges. When administered into biological fluids, MEF nanoparticles will come into contact with several thousand proteins. The outer layers consist of bound proteins called protein corona (Tenzer et al., 2013). The formation of protein corona in biological circumstances would be affected not only by the physiological properties of nanoparticles (size, surface charge, shape, etc.) but also by physiological environmental conditions (temperature, pH). Ultimately, it is not possible to expect that MEF phenomena will work in biological fluids.

6.3. Label free MEF biosensors

Label-free sensitive methods at plasmonic sensors are increasing in popularity. The need for developing quantitative *label-free* detection methods is paramount to determining the relative measurement of biomolecules binding to plasmonic surfaces without additional surface modification and functionalization. Nonetheless, MEF biosensors should not be fabricated for *label-free* methods because signal transduction relies on fluorescence mediated by plasmonic resonance, which differs from the detection mechanism of peak shift of SPR or LSPR. Thus, for most MEF sensors, external labeling is indispensable except in very specific circumstances. If a targeted analyte is an inherently natural fluorescent material, a new tool of *label-free* MEF sensor would possibly be constructed and designed. Under a controlled circumstance, when targeted fluorescent molecules bind to the MEF structured surface, the fluorescence intensity of target molecules would increase. However, in this type of *label free* biosensor, the selectivity of targeted molecules would not be guaranteed without external labeling. As a consequence, this causes another dilemma. Most biomolecules have very low intrinsic fluorescence and are considered virtually non-fluorescent material. Thus, for designing MEF biosensors, specific labeling using fluorescent molecules becomes necessary. Hopefully, significant progress in recent interdisciplinary approaches with nanotechnology has paved the way for developing *label free* biosensors to apply to MEF phenomena.

7. Summary

This paper provides a general overview of MEF systems from the basic mechanism to bio-applications, which are being developed intensively around the world. Two major approaches using either 2D substrates or colloidal suspensions for MEF studies and promising analytical tools have been introduced. However, as numerous efforts have been made to devise more relevant and practical MEF biosensors, a successful MEF technology in biosensors is still expected to achieve high sensitivity and selectivity with ideal fluorescence amplification. Even though recent investigations for MEF have demonstrated various significant advantages, developing them for a particular biological target is still challenging. In this regard, we addressed several challenges from a biological perspective. Therefore, future research in biosensors should focus on clarifying the interaction between MEF and biomolecules in biological fluids. In addition, well-structured interdisciplinary approaches involving nanochemists, engineers, physicians, and biologists must be conducted for more practical and affordable MEF biosensors.

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Conflict of interest

Authors have no conflict of interest.

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