Chapter 8
Plasmonic Exosome Biosensors for Medical Diagnostics

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Abstract This chapter provides an overview of plasmonic biosensor technology for the analysis of extracellular lipid vesicles that hold potential to serve as new type of biomarkers. In particular, it discusses detection of exosomes that are secreted to bodily fluids and become of increasing interest in clinical research. Plasmonic biosensor technology is pushed forward to provide new means for their sensitive and specific detection without the need of specialized laboratories. It offers a versatile optical toolbox for probing various biological species by tightly confined electromagnetic field of surface plasmons. These optical waves originate from collective oscillations of electron charge density at metallic thin films and (nano) structures. This chapter gives an introduction to surface plasmon photonics and its use in direct surface plasmon resonance and in fluorescence spectroscopy-based biosensors. It provides a brief summary of current state-of-the-art in exosome biomarker research and discusses current advances in exosome plasmonic biosensors for medical diagnostics of diseases, in particular cancer.

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1 Introduction

Over the last two decades, instrumentation and applications of various modalities of plasmonic biosensors were developed and gradually advanced. This technology became established in the field of biomolecular interaction analysis (BIA) and numerous surface plasmon resonance (SPR) biosensor devices become commercially available for e.g. drug development studies [1]. In parallel, SPR biosensors are pushed forward in order to provide a platform for rapid and sensitive detection of protein and nucleic acid biomarkers [2] that can be performed closer to patient without the need of centralized laboratory environment. Compared to currently used methods in clinical laboratories such as enzyme-linked immunosorbent assays (ELISA), research in plasmonic biosensors aims at providing faster and simpler analysis that can be performed by personnel that do not need specialized training. These advancements stem from research in plasmonics—a branch of nanophotonics that investigates confined electromagnetic field of surface plasmons occurring at metallic films and nanoparticles. For applications in the health sector, surface plasmons are employed to probe biological species that are bound at a metallic sensor surface. This type of optics is typically implemented for heterogeneous assays where biomolecular recognition elements (e.g. antibodies) are attached to the metallic surface in order to specifically capture the analyte of interest from analyzed liquid sample. The analyte binding events are converted to an optical signal by its probing with the confined surface plasmon field.

Only few years back, exosomes became an intense research area in medical sciences. These small lipid vesicles are secreted from viable cells to their cellular environment including bodily fluids and have been identified to play a key role in numerous biological processes. As they can pass across cellular membrane, they become a subject to research in drug development [3]. To fulfill their most important function as mediator of intercellular communication they carry proteins and nucleic acid molecules on their outer surface and inside as cargo. These molecules are distributed to the vesicles in a controlled way and are specific for their cell origin. Consequently some of these molecules can be used as general exosomal markers to discriminate exosomes from other lipid vesicles or cells and others as marker molecules identifying exosomes that originate from specific cell types. Currently, studies on differences in protein moieties found either at the exosomal membrane or inside due to various diseases are investigated. Cancer cell-derived exosomes are of special interest because the secretion of exosomes by cancer cells increases during tumorigenesis. Hence, next to the potential of molecules on and within exosomes as prognostic and predictive biomarkers, the exosome level itself can already be used as diagnostic marker.

SPR biosensors have been used for the observation of interactions of membrane proteins carried by small lipid vesicles with various ligands since early 2000 [4, 5]. However, only very recently this knowledge became translated into the analysis of exosomes in the context of medical diagnosis. This chapter focuses on such biosensor technology and introduces the reader to basics of surface plasmon optics

(plasmonics) and its implementation to optical biosensors that utilize direct as well as fluorescence-based detection formats (Sect. 2). Afterwards, the key characteristics of exosomes, their origin, and potential in medicine are discussed (Sect. 3). The final section provides an overview of current status in plasmonic exosome biosensors and discusses the possible future directions in this newly emerged research field.

2 Surface Plasmon Resonance-Based Detection Platforms

Surface plasmons are optical waves that can be resonantly excited with light at visible and near infrared wavelengths on surfaces of noble metals. They originate from coupled oscillations of charge density and the associated electromagnetic field which strongly confines energy of light. In plasmonic biosensors, surface of metallic layer or metallic nanoparticle is functionalized with recognition elements (such as antibodies or aptamers) in order to specifically capture target analyte from analyzed liquid sample. The captured analyte is probed by the confined field of surface plasmons to translate the binding events to detected optical signal. The capture of target analyte is associated with an increase in the refractive index which can be directly measured from induced detuning in the resonant coupling between a light beam and probing surface plasmons. This approach is exploited in technologies that are referred to as surface plasmon resonance (SPR) biosensors. In addition, the confinement of electromagnetic field by surface plasmons is accompanied by a massive increase of field strength which can provide powerful amplification means in spectroscopy techniques used for the analysis of molecular analytes. These include fluorescence (plasmon-enhanced fluorescence—PEF), infrared (surface-enhanced infrared absorption spectroscopy—SEIRA), and Raman spectroscopy (surface-enhanced Raman spectroscopy—SERS) [6–10]. Further in this section, key characteristics of surface plasmon modes are discussed and there will be shown how the field confinement (quantified by the penetration depth of surface plasmon field decaying away from the metal surface) can be tuned with respect to the size of target analyte. Afterwards, typical implementations of biosensors that take advantage of refractive index-sensitive SPR and PEF spectroscopy will be described.

As Fig. 1a illustrates, propagating surface plasmon (PSP) modes travel along a continuous metallic film and probe the adjacent medium with an electric field intensity $|E|^2$ that exponentially decays from the interface as $\exp(-2x/L_p)$, where x states the distance from the metal surface. The probing depth is typically quantified by a half of penetration depth $L_p/2$. For instance, it is equal to about 90 nm for PSP at red wavelength of λ = 633 nm and gold surface in contact with aqueous medium. This distance can be changed by a design of the surface plasmon supporting metallic structure.

For example, the coupling of PSP modes at opposite interfaces of a thin metallic film gives rise to new plasmonic modes with tunable characteristics. This coupling

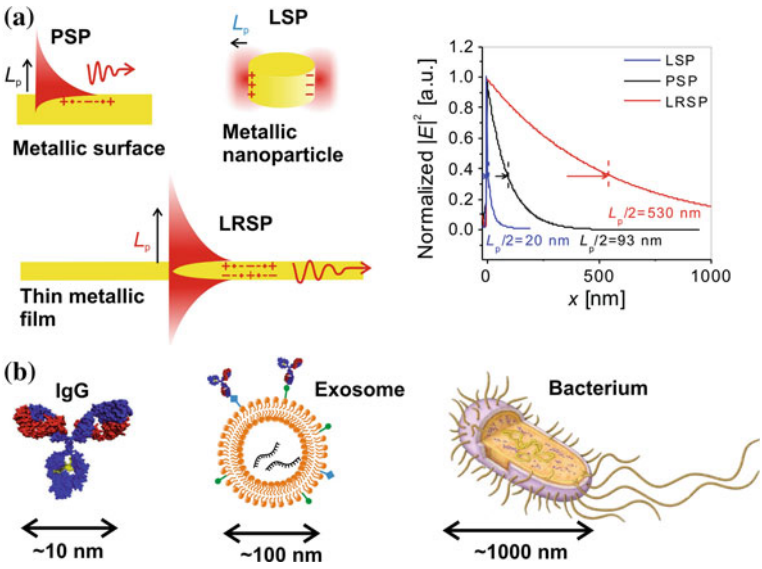

Fig. 1 **a** Comparison of the field profile $|E/E_0|^2$ of propagating surface plasmon (*PSP*), long range surface plasmon (*LRSP*), and localized surface plasmon (*LSP*). The profiles were simulated for gold SPR-active metal at wavelength of $\lambda = 633$ nm. The LRSP field was calculated for 20 nm thick gold film between water and Teflon AF. LSP field was obtained for a cylindrical gold nanoparticle with a diameter of 110 nm and a height of 50 nm in water. **b** Comparison with typical size of analytes that can be detected by plasmonic biosensors

can occur when a thin metallic film is sandwiched between two dielectrics with similar refractive index. For probing aqueous medium that exhibit refractive index close to $n = 1.33$, such geometry can be realized by depositing a metal film (with thickness of few tens of nanometers) on the top of a low refractive index fluoropolymer such as Cytop from ASAHI Inc. ($n = 1.34$) or Teflon AF from DuPont Inc. ($n = 1.31$). Besides others, near field coupling of PSPs at opposite interfaces of a metal film gives rise to the occurrence of long range surface plasmons (LRSP) that exhibit a weakly confined field and probe deep away from the metal. An example in Fig. 1a shows that for a 20 nm thick gold film on Teflon AF surface, the aqueous medium is probed by extended LRSP field with a probing depth of $L_p/2 = 540$ nm at a wavelength of $\lambda = 633$ nm. Let us note that the probing depth can be tuned between that of regular PSPs and (virtually) infinity by changing thickness of the metallic film and by introducing a small perturbation to the refractive index symmetry [11]. Contrary to LRSPs, surface plasmons supported by metallic nanoparticles exhibit a more confined field that probes closer to the metal. At metallic nanoparticles with much smaller size than wavelength, so-called localized surface plasmons (LSP), with dipole charge distribution can be optically excited. For instance, a small gold disk with a diameter of 110 nm and height of 50 nm that is surrounded by medium with refractive index of $n = 1.33$ supports a LSP mode that confines the electromagnetic field at its curved wall at the wavelength close to

λ = 633 nm. The cross-section of the field profile in Fig. 1a reveals that the penetration depth is squeezed to about $L_p/2$ = 20 nm. Let us note that even stronger confinement of light energy is possible by metallic nanoparticles featuring narrow gaps or very sharp tips. It should be noted that the range of probing depths $L_p/2$ that is accessible with LSP, PSP, and LRSP modes matches dimensions of species that are used for medical diagnostics. For instance, the size of typical protein biomarkers is smaller than $\sim$10 nm compared to small lipid vesicle such as exosome with a diameter of around $\sim$100 nm and about micrometer sized bacterial pathogens (see Fig. 1b).

2.1 *Direct Detection Based on Binding-Induced Refractive Index Changes*

In order to optically excite PSP modes, a coupler needs to be used to efficiently transfer energy from a light beam to the PSPs. These couplers allow for phase matching of a light beam with PSPs travelling along the metallic sensor surface which is required for their coupling. In attenuated total reflection (ATR) method with Kretschmann configuration, the phase-matching is achieved by enlarging the momentum of an incident beam by using a high refractive index prism, see Fig. 2a. Alternatively, a periodic corrugation of the metallic surface can be used for the phase-matching by diffraction. As Fig. 2b illustrates, the interaction of the excitation light beam with surface plasmon modes is resonant and manifested as a narrow dip in the wavelength or angular reflectivity spectrum. At the dip minimum, the maximum energy is transferred from the excitation beam to surface plasmons. The condition under which the resonance occurs strongly depends on the refractive

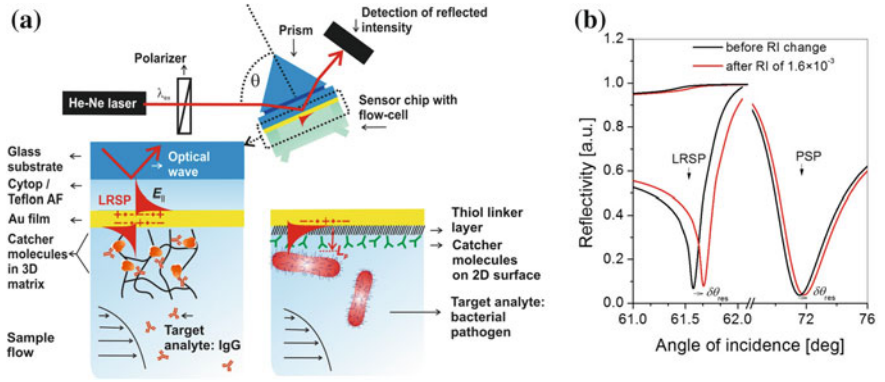

Fig. 2 **a** Schematics of coupling geometry that is used for the excitation of surface plasmon modes on a continuous metallic film utilizing attenuated total reflection (ATR) method. **b** Comparison of reflectivity spectra associated with the resonant coupling to PSP and LRSP waves for two refractive indices on the surface (adopted from [11])

index of the medium that the surface plasmons probe. For biosensors with angular modulation of SPR, a light beam at a fixed wavelength is made incident at varied angles θ and the reflectivity spectrum $R(\theta)$ is recorded. An increase of the refractive index (at the distance x up to L_p) induces a shift in the coupling angle θ_{res} to higher values (see Fig. 2b). In biosensors utilizing LSPs, surface plasmons at metallic nanoparticles are typically excited directly with an incident light beam. Changes in the refractive index in vicinity to the nanoparticles are observed as a shift of absorption band that is associated with localized surface plasmon resonance (LSPR) in the wavelength transmission or reflectivity spectrum [12].

Let us note that in typical SPR biosensors, detection of target analyte is performed in situ upon a flow of an aqueous sample along the metal sensor surface with immobilized catcher recognition elements (see Fig. 2a). The measured binding-induced refractive index changes occur due to the replacing of water by the target analyte at the sensor surface. The refractive index of protein biomolecules (typically $n = 1.45–1.5$) is higher than that of water ($n = 1.33$). The direct detection of other species such as lipid vesicles or even cells is more difficult due to the fact that these species contain a thin lipid membrane wall with mostly aqueous medium inside and thus exhibit low refractive index contrast. In order to amplify SPR response associated to weak binding-induced refractive index changes, assays where the refractive index change is enhanced by an additional binding step based on decorating the captured analyte by e.g. secondary antibodies attached to metallic nanoparticles are used [13].

2.2 *Plasmonic Amplification of Fluorescence Assays*

The resonant coupling of light to surface plasmon modes confines its power to a small volume above the metal surface which leads to a strong enhancement of the electromagnetic field intensity. As seen in Fig. 3, the field strength is typically quantified by the ratio of the electric field intensity of surface plasmons $|E|^2$ and that of the excitation beam $|E_0|^2$. Depending on the confinement and losses of excited surface plasmon modes, the field strength enhancement $|E/E_0|^2$ can reach values up to $>10^3$. In order to efficiently exploit the intense surface plasmon field for the amplification of fluorescence, the emitter (which is typically a fluorophore label attached to a biomolecule) is docked on the surface in close proximity to the metal. The amplification of fluorescence signal originates from three effects that can be combined. Firstly, the coupling of excitation beam with surface plasmons increases the emitter excitation rate that is (far from saturation) proportional to the field strength $|E/E_0|^2$ at the absorption wavelength λ_{ab}. Secondly, the emitter can be engineered to emit fluorescence light at the emission wavelength λ_{em} preferably by surface plasmons. These fluorescence-generated surface plasmons can be afterwards out-coupled to a specific direction towards a detector. Thirdly, the near field coupling of the emitter with surface plasmons allows improving the quantum yield

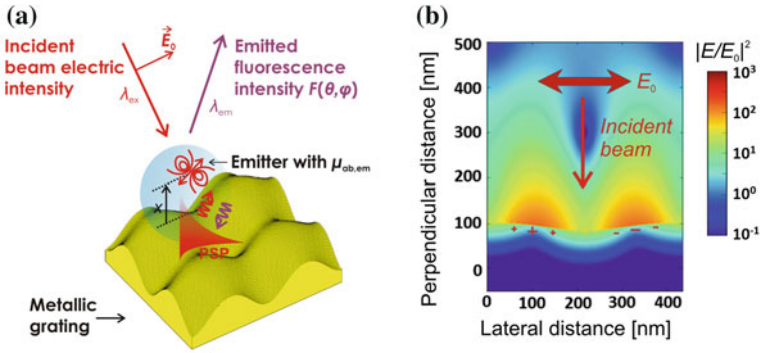

Fig. 3 **a** Geometry used for the grating-coupled surface plasmon resonance and **b** an example of the field strength generated on a gold grating surface in contact with water by diffraction coupling to PSP modes at the wavelength of λ = 633 nm (adopted from [14])

of emitter η (particularly emitters that are less bright and in solution exhibit a low quantum yield η_0).

The fluorescence emission characteristics strongly depend on the distance of the fluorophore from the metal surface x. As Fig. 4a illustrates, the emission from a gold surface at distance below $x < 10$ nm is strongly quenched due to the Förster energy transfer. At higher distance of around 20 nm, the majority of light is emitted via PSP modes travelling along the metal surface. When further increasing the distance, the emission via surface waves is suppressed (as the fluorophore moves out of the evanescent field of PSPs, see Fig. 1) and it emits directly to the far field. Let us note that the directly emitted light is isotropical and thus regular lens optics can typically collect only a small fraction (<1 %) of emitted fluorescence photons. However, the emission of fluorescence light via surface plasmons followed by their directional out-coupling to the far field allows to strongly enhance the yield in detected fluorescence light intensity. An example in Fig. 4b shows that a periodic corrugation of a metallic film allows to efficiently extract surface plasmon-coupled fluorescence emission from the surface by its beaming in the direction perpendicular to the surface. This feature can confine the emitted fluorescence intensity into an narrow cone that matches the maximum acceptance angle of used collecting optics and thus in combination with plasmonically enhanced excitation improves the sensitivity of fluorescence-based detection by a factor $>10^2$ [6, 15].

The employing of PSP or LSP waves for the amplification of fluorescence signal mostly relies on epi-fluorescence or total internal reflection fluorescence (TIRF) geometries depicted in Fig. 5a, b, respectively. In the epi-fluorescence sensors, an excitation beam at a wavelength close to the absorption wavelength of the used fluorophore label λ_{ab} is made incident on a sensor chip carrying plasmonic structures. The surface plasmon-enhanced field intensity at this wavelength probes the assay on the surface (see Fig. 5c) and excites fluorophore labels that report the target analyte binding events. The fluorophore emits fluorescence light at the

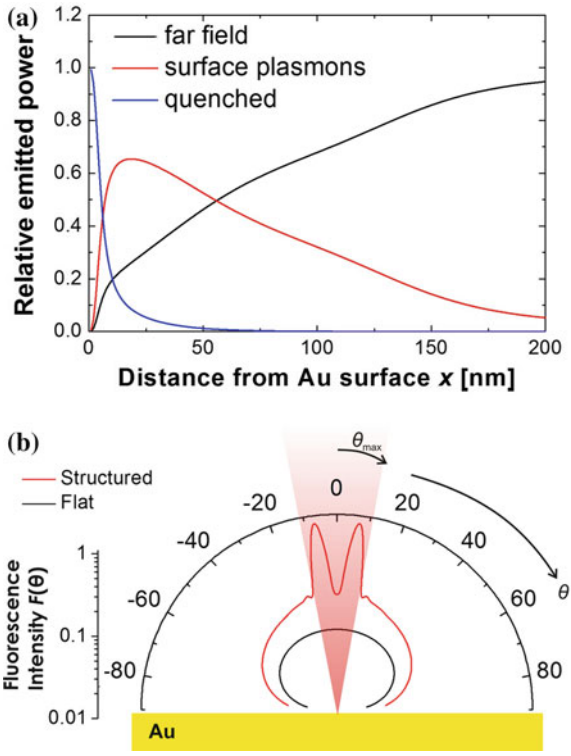

Fig. 4 **a** Distance-dependent coupling of fluorescence emission to surface plasmons at emission wavelength λ_{em} = 670 nm and **b** comparison of angular distribution of far field emitted fluorescence intensity from an emitter placed at a distance of x = 20 nm from a flat gold surface and corrugated gold grating surface that is in contact with water (adopted from [14])

emission wavelength λ_{em} directly to the far field or via surface plasmons that are subsequently out-coupled towards a detector (see example of angular distribution of surface plasmon-coupled fluorescence emission at Fig. 4b).

The TIRF-like geometry offers a possibility of combined SPR and fluorescence measurements based on ATR method with Kretschmann geometry (see Fig. 5b). In this implementation the resonantly excited surface plasmon waves on a thin metallic film allow for the observation of binding events by (i) changes in the resonant angle due to the binding-induced refractive index changes on the surface or (ii) by fluorescence light that is excited by the enhanced surface plasmon field intensity for assays utilizing fluorophore labels. The fluorescence light that is directly emitted perpendicular to the surface through the sample can be detected (as shown in Fig. 5b) or the directional surface plasmon-coupled emission by reverse Kretschmann configuration can be used for collecting of fluorescence light intensity [8].

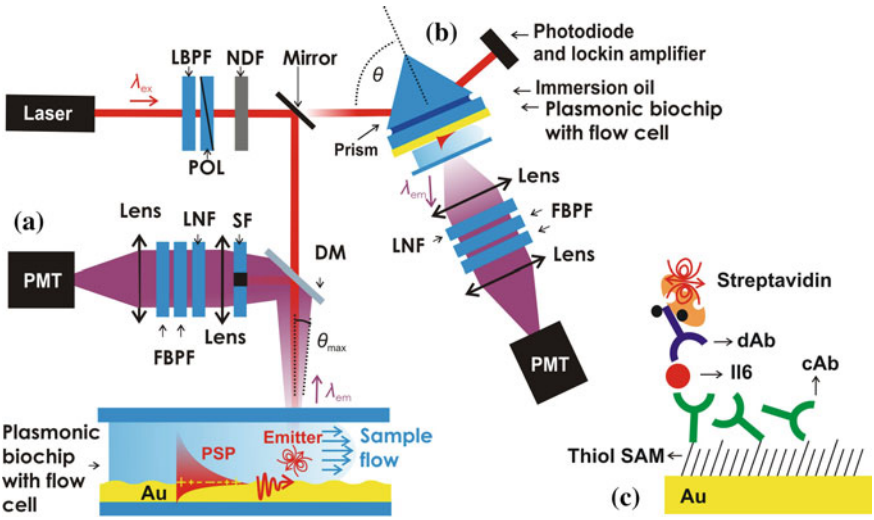

Fig. 5 Schematics of **a** epi-fluorescence geometry and **b** total internal reflection fluorescence (*TIRF*) geometry for the amplification of fluorescence signal by surface plasmons. Laser bandpass filter (*LBPF*), neutral density filter (*NDF*), dichroic mirror (*DM*), spatial filter (*SF*) and laser notch filter (*LNF*) are typically used. **c** Example of sandwich immunoassay on a metallic surface for the analysis of interleukin 6

3 Extracellular Vesicles as New Class of Biomarkers

Exosomes are small vesicles bordered by a lipid bilayer that are actively secreted by cells. They should not be confused with the "exosome complex" (or PM/Scl complex, often just called the exosome) which is a protein complex that is responsible for RNA degradation in the cell [16]. The small lipid vesicles were firstly accidentally discovered within the studies of Harding et al. who explored the fate of the transferrin receptor during the maturation of reticulocytes in 1983 [17]. At that point exosomes were misinterpreted as vehicles for the removal of unused proteins from the cellular membrane with the sole purpose of being a waste disposal system. It took another 15 years until exosomes came up again, but this time as active players of the immune system. Raposo et al. described that exosomes are secreted by B lymphocytes and MHC II proteins including antigens for presentation are exposed on their surface. Furthermore these protein-antigen complexes are not only present on the small vesicles, but also are fully capable of activating T lymphocytes and therefore an immune response [18]. These findings made exosomes extremely interesting for immunological research and possible use in immunotherapeutic applications.

20 years later research about these exosomes has opened a totally new field that combines disciplines from biology and medicine all the way to engineering. Particularly, the application of exosomes in the field of medicine became very wide

with some examples being vaccinations, gene delivery vehicles, (diagnostic) biomarkers for a variety of diseases, or therapeutic agents.

3.1 Characteristics and Composition

Exosomes belong to a class of extracellular vesicles that are actively secreted by viable cells. They are enclosed by a lipid bilayer membrane that holds a cytosol and cellular components. The size of exosomes ranges from 30 to 150 nm in diameter and they exhibit a density between 1.13 and 1.19 g mL^{-1}. Even though these numbers are generally accepted as specific characteristics of exosomes, more recent studies showed that within this density and size range there are still several distinct populations of vesicles [19].

Lipids, proteins, and nucleotides are the main components of exosomes. The bilayer membrane is built from different types of lipids including phospholipids (sphingomyelin, phosphatidylserine, phosphatidylinositol, phosphatidic acids, phosphatidylcholine, and bis(monoacylglycero)phosphate), cholesterol, and ceramides [20]. Some of these lipids can be used as exosomal markers, because they are enriched in the exosomal membrane compared to their parental cell membranes or other vesicles, like phosphatidylserine, sphingomyelin or ceramide. Bis(monoacylglycero)phosphate otherwise is, within cells, only present in the endosomal membrane and consequently incorporated into exosomes, but is not found on cell surfaces or vesicles that originate from the plasma membrane. Hence this lipid type can serve as a lipid marker specific for exosomes.

Proteins are found in the membrane of exosomes as surface molecules and inside exosomes as cargo molecules. The orientation of the transmembrane proteins in exosomes is the same as in the plasma membrane of cells, exposing the extracellular domains to the outside of the exosomes and the intracellular domains to the inside. Thus they can function in the same way as on their originating cell, e.g. interacting with other cells. Different families of proteins have been identified in exosomes including antigen presenting molecules (MHC class I and II), adhesion molecules (tetraspanins and integrins), membrane transport proteins (annexins, flotillins, and RABs), signal transduction molecules (heterotrimeric G proteins), endosomal sorting complex required for transport associated proteins (TSG101 and Alix), cytoskeletal proteins (actin, moeasin, and tubulin), enzymes (elongation factors and glyceraldehyde 3-phosphate dehydrogenase), and other cytosolic proteins (histones, ribosomal proteins, and proteasomes) [19]. All these molecules originate from the endosome, the plasma membrane or the cytosol of the cell, but proteins from other cellular compartments like mitochondria, nucleus, endoplasmic reticulum or Golgi apparatus are mostly absent. This is due to the biogenesis of exosomes, which is described in a later section. Some of the mentioned proteins are used as general markers for exosomes. These are the tetraspanins CD9 and CD63 and endosomal proteins TSG101, LAMP1 and Alix [19, 21]. In addition to these proteins that are more or less common to all exosomes, proteins specific for their cells of origin have

been identified in exosomes. Generally speaking exosomes derived from immune cells will expose and contain different molecules compared to those derived from cancer cells. Giving just one example, exosomes secreted by ovarian cancer cells can be specifically identified via membrane proteins EpCAM and CD24 [22].

The cargo of exosomes does not only include proteins, but also diverse RNA (messenger RNA [mRNA], micro RNA [miRNA]) and DNA molecules. Especially the RNA cargo of exosomes has been subject to many studies. Specifically the transfer of small RNA molecules can affect the gene expression pattern in the recipient cells. Moreover the nucleotides transported by exosomes of different origin are also varying. Thus it is possible to use the RNA molecules as specific markers for exosomal origin [19]. Again one example are ovarian cancer cell derived exosomes, because they contain a set of miRNAs that were shown to be capable of discriminating between serum samples collected from ovarian cancer patients and healthy donors [22]. These molecules are the cargo of exosomes which are supposed to be transported to other cells in order to fulfill their cell-to-cell communicative function. Hence the cargo of exosomes is pivotal for their function, which is discussed in more detail in the following section.

3.2 Biogenesis and Function

Within the last decades, exosome secretion has been identified in all cell types that have been examined including immune cells, epithelial cells, platelets, tumor cells, neuronal cells, and mesenchymal stem cells [21]. Furthermore exosomes are found in most bodily fluids including blood (serum and plasma), urine, bile, saliva, nasal secretions, semen, amniotic fluid, breast milk, cerebrospinal fluid, ascites, and others [23].

The biogenesis of exosomes takes place at the membrane of late endosomes as seen in Fig. 6. First vesicles are formed by budding of the endosomal membrane to the inside of the endosomes. Those endosomes are now containing smaller vesicles and are therefore called multivesicular bodies (MVBs). Finally the MVBs fuse with the extracellular membrane and release their cargo vesicles to the extracellular space. It is believed that for the budding of exosomes into the endosomes two distinct processes in the cell are required. One is responsible for the organization of the endosomal membrane and builds so called tetraspanin enriched microdomains within the membrane. At the site of these microdomains the tetraspanin proteins then attract other proteins leading to the budding procedure. The second process involves the so called endosomal sorting complexes required for transport (ESCRTs). The recruitment of these complexes is responsible for initiation and completion of the membrane budding [24].

Due to the diversity of exosome cargo molecules and surface marker composition, which is dependent on their cell of origin, also the functions of exosomes are diverse. Here we present some of the functions that have already been discussed in literature [26]. The first function that was attributed to exosomes by their

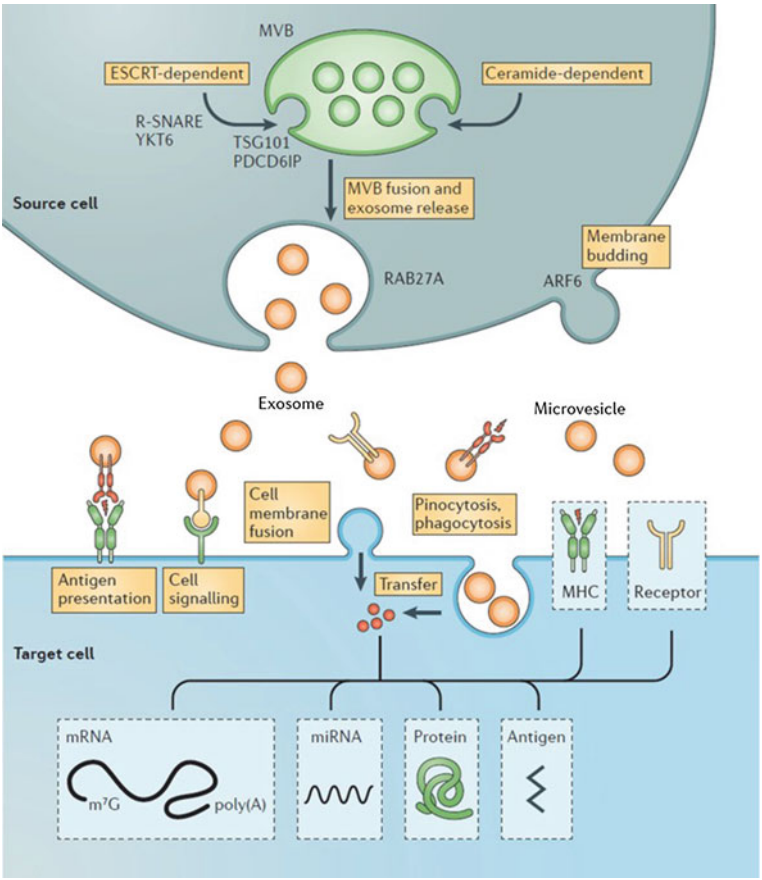

Fig. 6 Schematics of the biogenesis of exosomes. Reprinted by permission from Macmillan Publisher Ltd [25], copyright (2014)

discoverers was a mechanism to remove obsolete proteins from the cell. Nowadays this process is seen as alternate way of protein secretion, especially of proteins that lack specific signals for normal secretion.

The second and much more important function found to be fulfilled by exosomes is antigen presentation. As mentioned before, exosomes released by B-lymphocytes were able to stimulate T helper cells via antigen presentation with MHC II proteins on their surface. Since then many studies have demonstrated that exosomes are active players of the immune system and can activate but also suppress immune response, depending on their cell of origin. For example in contrast to the aforementioned B-cell derived exosomes that stimulate the immune system, tumor derived exosomes were found to induce T-lymphocyte apoptosis or impair dendritic cell development. On the other hand, exosomes released by infected cells function as pro-inflammatory agents, because they display pathogen-associated molecular

patterns, which are required to come into contact with immune cells to initiate an immune response.

The transfer of RNA molecules between cells is another function of exosomes [27]. The mRNA and miRNA molecules that are found within exosomes can induce epigenetic changes in the recipient cell. For example mRNA molecules that were transported by exosomes were shown to be actively translated in their target cell after exosome uptake, hence exosomes can introduce new functions to neighboring cells. Transferred miRNA molecules can change gene expression of the recipient cells by intervening at the mRNA level in the cytosol or on regulatory sequences in the cell nucleus and thus regulating transcription and translation of proteins.

Moreover exosomes can be used by viruses to transport infectious agents. For example, HIV makes use of the exosomal biogenesis pathway via MVBs for their own secretion. Hence the viral components are shuttled by exosomes to recipient cells without recognition by the immune cells, because the pathogens are hidden in unsuspicious vesicles. Also viruses can modify the cargo of exosomes in a way that recipient cells are more susceptible for viral infection.

3.3 Medical Applications of Exosomes

Exosomes are secreted by cells not only in normal or healthy conditions, but also in pathological state. In their role as intercellular signal transporter the small vesicles were found to be associated with several pathological processes like spread of viruses or pathogenic agents (e.g. HIV, EBV, and prions), tumorigenesis, formation of metastases, propagation of neurodegenerative diseases (Alzheimer's and Parkinson's disease) or immune activation and suppression [25]. This fact combined with their composition that is specific for their cells of origin give reason for their emerging role in the medical field. At present their application in the field of diagnostic, prognostic or predictive biomarkers is studied in depth for many diseases. Additionally exosomes are of great interest for therapeutic applications as new drug-delivery systems or as immunostimulating agents. With regard to their functions in tumorigenesis or viral spread they can also be the target of therapeutics themselves.

Concerning the therapeutic field, exosomes holds potential for several possible applications, with some of them being still ideas and others already in clinical trials. As exosomes play an important role in disease development or spreading, it is easy to understand that the vesicles are potential new targets of therapy. There are four different approaches to achieve exosome load reduction or function inhibition. One is to target their biogenesis. For example enzymes required for the exosome's formation can be inactivated or the production of necessary molecules can be inhibited. Another way is to block exosome's release from the cell by, for instance, suppressing the fusion of MVBs with the plasma membrane. A third strategy aims to inhibit the vesicle uptake by recipient cells. This might be performed by inactivation of exosomal membrane components that otherwise would bind to the

recipient cell. The fourth approach is to block those components on exosomes that are responsible for the pathogenic process they are promoting. Though these new therapeutic approaches are very promising, it has to be taken into account that interfering with exosome biogenesis or function could result in severe side-effects that are at present state of research not completely foreseeable [25].

Taking their immune stimulatory and suppressive activity into account, exosomes are interesting for a wide range of applications in the immune therapeutic field being the therapeutic agent themselves. The capability of immune cell derived exosomes to present antigens to the immune system via the MHC molecule enables them to stimulate an immune response in the body. Consequently such vesicles would be perfect candidates for vaccines. Cells could be exposed to a certain pathogen and antigen presenting exosomes could be isolated from the cell supernatant for consecutive administration to patients. Even more, it was already successfully shown that such vesicles have anti-tumor effects. Furthermore usage of exosomes, developed by bacteria or parasite infected macrophages, in treatment or prevention of infectious diseases is intensely studied. In contrast to their ability of triggering immune response, it was demonstrated that exosomes can induce immune tolerance. Clinical applications taking advantage of this feature are immune suppression while and after organ transplantations, treatment of autoimmune diseases, as for example rheumatoid arthritis, or of allergies. Some immunotherapeutic approaches to treat cancer are already in clinical trials of phase I and II [25, 28].

Mesenchymal stem cell derived exosomes have, just as their parental cells, tissue regenerative activity and therefore find great application in regenerative medicine. While the establishment of mesenchymal stem cell based therapy, where stem cells are transplanted to the injured tissue and subsequently replace damaged cells, it was hypothesized that the stem cells secrete some soluble factors that lead to tissue regeneration. Nowadays it is confirmed that some of these so-called paracrine effects are mediated by exosomes. Consequently the administration of exosomes could substitute stem cell transplantation and thus overcome safety concerns associated with cell transplantation. The positive effect of exosome treatment on tissue regeneration has been proven in models of myocardial infarction, kidney injury, and skeletal muscle repair [25].

Finally these biological vesicles have the inherent capability of crossing biological membranes and delivering cargo from one cell to another. Therefore the vesicles can be used as new drug delivery system. Especially their innate ability to transfer RNA species among cells makes them perfect for packing specific oligonucleotides in their inside and designing their surface in order to target and treat specific recipient cells. Studies already described the successful transfer of small interfering RNAs and the induction of RNA interference effects in recipient cells. Another extremely important and advantageous property of exosomes is their inherent ability to cross the blood-brain barrier, which is one of the most complicated barriers in the human body to cross for therapeutic agents. Other very important features that are advantageous for exosomes as drug delivery systems are their biocompatibility, their immunological inertness if derived from the appropriate cells, and the possibility of producing individual patient-derived exosomes [25].

As exosomes are circulating in bodily fluids and carrying molecules specific for their cells of origin, they have great potential as source of biomarker. Additionally, exosomes are released in increasing amounts during tumorigenic development, for instance in ovarian cancer patients [22]. Hence simply exosomal concentration has potential as biomarker. A few examples of diseases that were investigated in regard of exosomal biomarkers are neurodegenerative diseases, several types of cancer, and preeclampsia [29–32].

Summing up, exosomes have greatest potential for the translation to clinical use, but there are still many open questions that remain to be answered before exosome applications can be used in routine. Many properties and mechanisms have to be elucidated to assess possible bio-hazards, safety risks, or side effects associated with administration of exosomes to patients or with interference with exosomal biogenesis. An even more basic problem is the lack of standardized methods for production and analysis of exosomes, which is urgently needed to obtain comparable results and effects. At this state of research you can even find contradictory observations about exosomes of the same origin, which might be due to the missing standardization.

4 Analysis of Exosome Vesicles

4.1 Established Laboratory-Based Methods for Exosome Studies

Methods for isolation on basis of density and size are most commonly used in exosome studies. The "gold standard" is differential centrifugation where several steps of centrifugations with increasing speed are used for exosome separation. Firstly, low-speed steps are employed to remove large species such as cells and cell debris from liquid samples, like cell culture supernatant or bodily fluids. Afterwards, larger extracellular vesicles are separated at about 10,000–20,000 g and finally exosomes are pelleted at high speed in an ultracentrifuge at a force of 100,000–120,000 g. By the means of ultracentrifugation the purified exosome solution will also contain extracellular protein aggregates, as well as lipoprotein particles or other contaminants. To get a higher level of purification density gradient centrifugation can be used. This method makes use of a glucose density gradient, in which particles float upwards or downwards dependent on their density. With this procedure contaminating proteins can be removed from vesicles. Also vesicles of different densities can be separated. However, lipoprotein particles and small lipid vesicles of similar density as exosomes will still be co-purified. An important fact that has to be taken into consideration for physical separation methods is that also sample viscosity has great influence on the purification efficiency. For purification of viscous biological fluids, ultracentrifugation will not be as efficient as for mediums like buffer. Consequently many fluids are diluted with buffer solutions to

decrease the viscosity and enable concentration of exosomes at the bottom of centrifugation tubes. Some protocols also include a filtration step either in the beginning, in between or at the end of the centrifugation steps. Other size selective separation methods rely on filtration or chromatographic separation. However, loss of vesicles during these procedures might be very high due to adhesion to the filter material or chromatography columns.

Immunoaffinity-based isolation techniques make use of the presence of specific surface markers on exosomes (as mentioned above some transmembrane proteins have been reported to be present in exosomal membranes in high quantities and hence can be used as target for antibody binding). To isolate exosomes, antibodies specifically binding to such surface markers are immobilized on, for example, magnetic beads, which again can be easily separated from a liquid by magnetic means. The vesicles can be further analyzed bound to the beads or other antibody coated surfaces, or after a detachment process. This method leads to rather high purity in comparison to density-based methods but isolates only subpopulations of vesicles, because no general exosomal marker that is solely present on exosomes has been identified yet.

Polymeric precipitation is another approach to isolate exosomes. This procedure involves a commercially available polymeric precipitation mixture that is incubated with the sample solution and combined with a subsequent centrifugation step. Yields of RNA and protein from this procedure are high, but it is not totally clear, whether these molecules are solely from exosomal origin or from co-purified contaminants.

The first observations of exosomes were performed with electron microscopy (EM). This technique is well established and gives direct evidence of the presence of vesicles in a sample. Transmission EM and scanning EM are most commonly used and provide information about size, morphology and to a limited extent about concentration of vesicles. If nanoparticles coupled to specific antibodies are used for labeling of vesicle markers, EM can also be used for the detection of molecules on exosomes in order to classify them based on the moieties exposed at the outer wall [33]. Atomic force microscopy (AFM) is also used for exosomes assessment, presenting the advantage of analyzing vesicles in solution.

Two other methods rely on detection of light scattering caused by vesicles in solution. One is flow cytometry (FC) that is an established method for cell counting and measures the side and forward scattering of one particle crossing a laser beam. At the current state of technology sensitivity of FC is not high enough to detect exosomes, because the limit of detection for lipid vesicles even with an especially dedicated flow cytometer for extracellular vesicle analysis is at 150–190 nm in diameter [34]. That means that FC is only applicable for detection of bigger vesicle classes. However, sensitivity can be enhanced by triggering the detection of small objects on the basis of a fluorescence signal instead of a light scattering signal as performed in conventional flow cytometry [35].

The other analysis method using light scattering is nanoparticle tracking analysis (NTA) where particles are made visible by the scattered light from a laser beam and then counted by a CCD-camera. Besides the concentration also the size distribution

of vesicles can be determined with this method based on the observation of Brownian motion of particles. The main disadvantage is the unspecificity of this method. Every particle, including other small liposomes, protein aggregates or even dust particles or salt crystals, scatters light and thus will be counted by NTA. Additionally in non-purified solutions, like bodily fluids, the background signal is very high. These two problems can be overcome by usage of fluorescence labels attached to exosomes specifically. However, in this case only a subpopulation of vesicles can be examined. NTA has a high sensitivity with the capability of detecting down to 46 nm beads, but with high errors in comparison to the other methods. The minimum detectable vesicle size is 70–90 nm [34].

Resistive pulse sensing (RPS) is a completely different technique that analyses size and concentration of vesicles by measuring the changes in ionic current while vesicles are transported through a nanopore in a membrane. With this method vesicles down to 70–100 nm in diameter can be analyzed, if the right pore size is chosen [34]. The major downside of RPS is its dependency on pore stability, meaning that a difference in pore size, for instance caused by a too big particle being pushed through the pore and stretching it, changes the signal.

4.2 *Emerging Plasmonic Biosensor Platforms for the Exosome Detection*

As summarized in the previous Sect. 4.1, the research in exosomes mostly relies on sophisticated tools that are deployed in specialized laboratories and can only be operated by highly-trained personnel. In order to exploit the potential of exosome biomarkers for disease diagnosis in clinical applications, new technologies that can be used in simpler manner should be introduced for their analysis. In addition, such technologies have to provide high sensitivity and specificity in order to avoid using tedious pre-concentration steps (e.g. ultracentrifugation) which are impractical to implement in regular clinical laboratories. Only very recently, the potential of plasmonics-based biosensors for the analysis of exosomes, considering their moieties exposed on their outer membrane, was recognized. This section provides an overview of currently achieved results in this newly born field.

Höök's laboratory demonstrated that direct detection format of SPR can be used for the measurement of concentration of exosomes in liquid samples by monitoring their affinity binding kinetics (see Fig. 7) in the mass transfer-limited regime in conjunction with an appropriate model [36]. They showed a label-free detection of exosomes with a mean diameter of 234 nm that were secreted by human mast cells cultivated in vitro. The surface of the sensor was modified with an antibody against tetraspanin membrane protein CD63 on the exosomal membrane which represents one of the exosome markers. Obtained results were confirmed by NTA analysis of exosomes in liquid samples and they revealed that SPR is suitable for direct detection of exosomes at low pM concentrations.

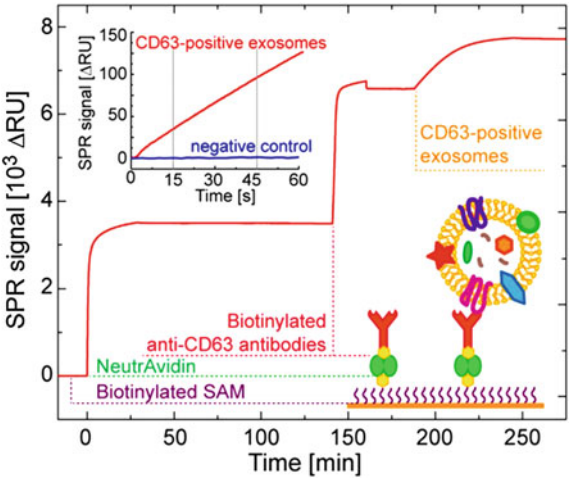

Fig. 7 SPR observation of affinity binding of exosomes (secreted from human mast cells) to the sensor surface functionalized with antibodies against a specific epitope on its outer membrane wall (*CD63*). Reprinted with permission from [36], copyright (2014) American Chemical Society

In order to enhance sensitivity of heterogeneous assays for the detection of small lipid vesicles, the SPR biosensor was combined with fluorescence-based detection in our laboratory. The surface plasmon-enhanced fluorescence spectroscopy was implemented by using an optical arrangement utilizing an ATR coupler as showed in Fig. 5b. The sensor performance was tested with lipid vesicles exhibiting a mean diameter of 72 nm (measured by NTA) that were prepared from phosphatidylcholine and phosphatidylserine at a ratio of 4:1. The sensor surface was functionalized with a chimeric protein made of a 1/1 complex between streptavidin and annexin V, which recognizes the phosphatidylserine moieties that are often present on exosomes [33]. As Fig. 8 shows, a sandwich assay was used for the detection of lipid vesicles.

Firstly, a volume with known concentration of vesicles (calculated from lipid concentration and confirmed by NTA) was suspended in HEPES buffer and flowed over the sensor surface for 10 min followed by a 5 min rinsing step with pure buffer. Afterwards, a solution with annexin V that was labeled with a Cy5 dye was flowed through the sensor for 10 min in order to bind to the captured lipid vesicles. As can be seen in the inset of Fig. 8a, the injection of the Cy5-labeled annexin is accompanied with a rapid increase in the fluorescence signal originating from the molecules present in the bulk solution. Then, a gradual increase in the signal is observed due to the affinity binding to captured vesicles at the surface. After the subsequent rinsing of the surface with buffer, the fluorescence signal rapidly drops (as there are no labeled molecules present in the liquid sample) to a level that is higher than before the flow of labelled annexin V. The difference in the fluorescence signal ΔF before and after the flow of annexin V-Cy5 conjugate is ascribed to the binding of the protein to the captured vesicles. The respective calibration curve showed in Fig. 8b indicates that the analysis of lipid vesicles at concentrations below 100 fM is possible by surface plasmon-enhanced fluorescence spectroscopy. The sensor specificity was checked on a control surface that was not carrying

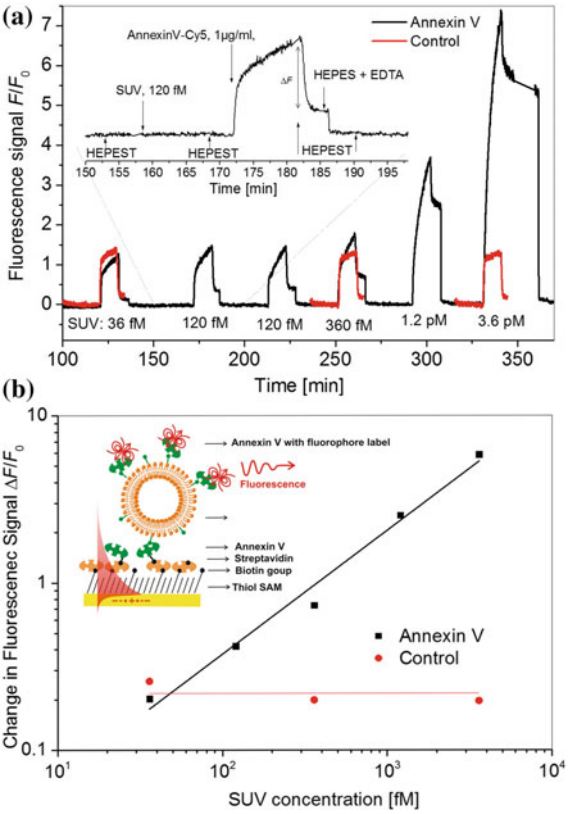

Fig. 8 Sandwich assay for detection of small unilamellar lipid vesicles (*SUV*) exposing phosphatidylserine, a model system for exosomes. Annexin V was used as a ligand and the plasmonically enhanced fluorescence signal was measured by using an additional binding of Cy5-labeled Annexin V to captured lipid vesicles

annexin V catcher molecules. It should be noted that the sensor surface can be easily regenerated for repeated experiments on the same chip by removal of calcium from the solution and by usage of EDTA.

In order to analyze multiple moieties exposed by exosomes in parallel, an SPR biosensor with array detection format was developed [37]. A technique referred to as SPR imaging was employed as seen in Fig. 9. In this method, an expanded monochromatic optical beam is made incident on the metallic sensor surface and the spatial distribution of the reflected intensity is imaged to a two-dimensional detector such as CCD [38]. By this means, the spatially resolved measurement of binding induced refractive index changes is possible. The SPR imaging was used for the detection of exosomes present in cell culture supernatant originating from a human hepatocellular carcinoma cell line (MHCC97H). The sensor chip was functionalized by arrays of antibodies specific to intra- and extra-cellular parts of widely reported exosome transmembrane glycoproteins including tetraspanins (CD9, CD63, CD82) and tumor-related proteins CD41b, E-cadherin, and EpCAM. This array was employed to the study of the correlation between the secretion of exosomes and metastatic potential of the cell lines. For this purpose the exosome

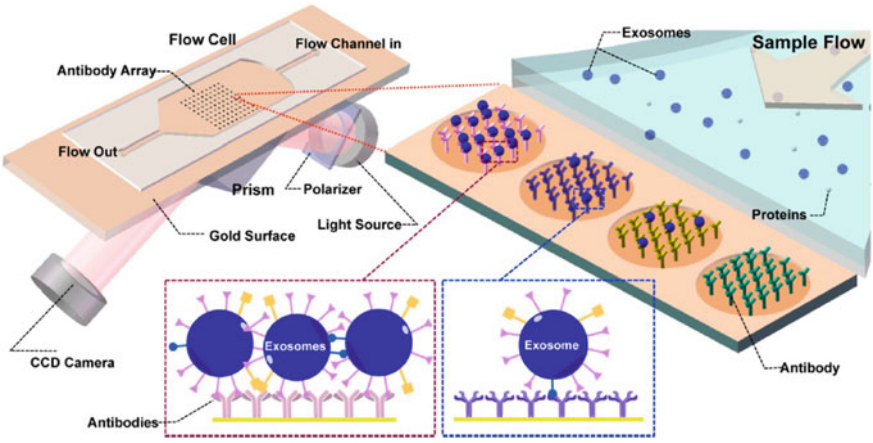

Fig. 9 Microarray detection format with the direct readout by using SPR imaging. Reprinted with permission from [37], copyright (2014) American Chemical Society

binding to the SPR imaging sensor of cell culture supernatant from the aforementioned cell line MHCC97H was compared to the signal from MHCC97L, which has in comparison to the first a lower metastatic potential. Results showed that binding was significantly increased when analyzing the cell culture supernatant of the higher metastatic cell line in comparison to the lower metastatic cell line, indicating that more exosomes were present and secreted by the higher metastatic cells. These results were further confirmed by the analysis of cell culture supernatants of two different metastatic mouse melanoma cell lines in the same manner. These results lead to the conclusion that exosome analysis could not only be used for diagnosis by examining the exosome's origin, but also for determining the stage of disease and as prognostic marker for disease progression.

Advanced sensitivity for high throughput SPR analysis of exosomes was reported by using a plasmonic chip with arrays of nanoholes in a thin metallic film that support localized surface plasmons [39]. The sensitivity of the developed sensor platform was tested for detection of exosomes secreted by the ovarian cancer cell line CaOV3 by using a biointerface with immobilized antibodies against CD63 (which is, as mentioned before, a characteristic marker for exosomes). The limit of detection of 670 aM was achieved by the amplification of exosome specific capture by additional metallic nanoparticles coupled to secondary antibodies. Afterwards, this sensor platform was used for the profiling of various extracellular protein markers for benign cell lines and ovarian cancer cell lines and in particular the levels of CD24 and EpCAM were used to distinguish between exosomes originating from ovarian cancer cells and those secreted by benign cells. This study reported promising data on the application of the plasmonic sensor for the diagnosis and therapy monitoring of ovarian cancer by the analysis of ascetic fluid collected from ovarian cancer patients which was demonstrated without any amplification of

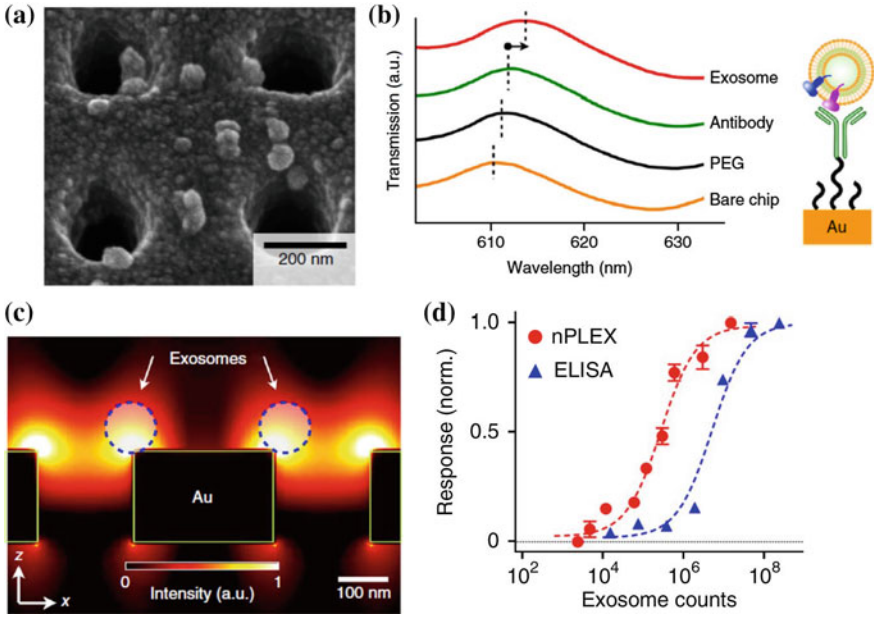

Fig. 10 Schematic of the nano-plasmonic exosome assay (*nPLEX*) for the diagnosis and therapy monitoring of ovarian cancer. **a** SEM image of the gold sensor surface with arrays of nanoholes and captured exosomes, **b** a detail of simulated localized surface plasmons probing the sensor surface, **c** the optical response measured in transmission wavelength spectrum and shift of LSPR due to the modification of the sensor surface and capture of target analyte, and **d** the comparison of calibration curve of plasmonic nPLEX sensor and ELISA with chemiluminescence readout for the detection of exosome secreted by CaOV3 cells. Adopted by permission from Macmillan Publishers Ltd [39], copyright (2014)

enrichment. Let us note, that the performance characteristics were compared to regularly used Western blotting and ELISA and the achieved sensitivity of the plasmonic biosensor was superior to these routinely used methods, see Fig. 10.

5 Summary

In the last few years, the rapidly developing research in exosomes was recognized as a potentially new application area for plasmonic biosensors. In the initial phase, this type of optical sensors was employed for the study of key characteristics of exosomes and their interaction with biofunctionalized surfaces in order to establish sensitive and specific assays for their detection. The reported results indicate that probing affinity captured small lipid vesicles such as exosomes by confined surface plasmons allows for the detection of trace amounts of such analytes (at concentrations below fM). This achievement establishes a basis for subsequent analysis of

exosomes in biological samples without the need of tedious purification and amplification steps. In the context of cancer diagnosis and therapy monitoring, the obtained results indicate that plasmonic exosome biosensors represent a promising technology for rapid and robust diagnosis without the need of complex instrumentation that is available only in specialized molecular biology laboratories. This new field can greatly benefit from advancements in SPR biosensor instrumentation, biointerfaces and assays that are continuously pursued for portable analysis of other already established biomarkers for disease diagnosis.

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