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Anti Human Fibronectin–Gold Nanoparticle Complex, a Potential Nanobiosensor Tool for Detection of Fibronectin in ECM of Cultured Cells

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Abstract Specific protein detection by means of antibody-nanoparticle conjugates is a new field in medical nanobiotechnology. Among many nanoparticles used, gold nanoparticles show strong light-absorption properties which have been exploited in designing nanobiosensors. Fibronectin (FN) plays an important role in extracellular matrix (ECM) structure and function of normal cells; however, in conditions like lung carcinoma, its expression increases, especially in non small cell lung carcinoma (NSCLC). In this study, we conjugated gold nanoparticles to human fibronectin antibody (anti-hFN) to design a colorimetric nanobiosensor for detection of FN present in ECM of cultured cells. Three different cell lines, namely A549 (target cells), AGO-1522 (control cells), and Nalm-6 (negative control cells), were used to compare changes in color resulting from aggregation of gold nanoparticles due to higher amount of FN. Our construct was able to detect increased level of FN which was distinguishable visually by change in color and could be confirmed by spectrophotometer as well.

Keywords Fibronectin (FN) · Gold nanoparticles (GNPs) · Lung carcinoma · Nanobiosensor

Introduction

Broad applications of nanomaterials in the field of biology and medicine helped scientists to find a way to change many clinical and traditional diagnostic and prognostic methods [1, 2]. Cancer nanotechnology is a new area of medical nanobiotechnology research which has a great impact on the detection and diagnosis of cancer cells [1–3]. While accurate detection of cancer cells with the help of nanobiotechnology has various obstacles, optical detection paves a new road for a rapid and reliable method in this area [3, 4]. Numerous colorimetric nanobiosensors with specifically designed nanoparticles have the potential to detect specific cell types within the target organs [5]. To design colorimetric nanobiosensors, some metal nanoparticles (<100 nm) might be used to exhibit a strong shift in colors due to their surface plasmon resonance (SPR) peak in the visible range [6, 7]. Gold nanoparticles (AuNPs) are used for their specific characteristics like biofunctionalization, biostability, spectral properties, and surface plasmon resonance peak which occur in the visible range depending upon their size and shape [8]. AuNPs exhibit prominent distance-dependent optical properties which reveals when they come close to each other, their absorption and scattering properties are altered which leads to a change in color and shift in absorption spectra [9, 10]. Strong light-absorption properties of AuNPs is a distinguished feature which has been used in designing biosensors for diagnostic and therapeutic approaches and many techniques have emerged considering these properties which are all based on the aggregation of gold nanoparticles to identify target molecules [11–14]. Gold nanoparticle-based colorimetric biosensors have been increasingly applied for the detection of variety of analytes including cells, DNA, proteins, metal ions, and small molecules [15]. For example, in 2008, Medley and colleagues developed a colorimetric assay for direct detection of cancerous cells by using aptamer-conjugated gold nanoparticles [13]. In the context of DNA-specific sequence identification, Bao and colleagues applied a gene-specific oligonucleotide functionalized gold nanoparticle probes for single nucleotide polymorphisms (SNPs) identification in unamplified human

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genomic DNA [16]. In addition, Lee and colleagues detected mutation in the epidermal growth factor receptor (EGFR) of non-small cell lung cancer cells using selective aggregation of gold nanoparticles. They showed that addition of the mutant DNA which was hybridized with the complementary probe into the unmodified gold nanoparticle suspension at the optimal salt concentration leads to aggregation of gold nanoparticles and color change in solution [17]. However, in another study conducted by Choi and colleagues, they took advantage of gold nanoparticle-antibody complex to construct an ultra-sensitive surface plasmon resonance based immunosensor for prostate-specific antigen (PSA) detection in prostate cancer. This immunosensor enabled 10^3-fold enhancements in sensitivity with detection limit of 300 FM of PSA [11]. Beyond that, not only gold nanoparticle colorimetric biosensors can be used for diagnostic approaches, but also, they have significant applications in environmental monitoring and antibioterrorism [18]. In fact, colorimetric detection systems providing naked-eye readout signals have come to the fore in many detection methods as it is simple, rapid, and suitable for real-time and on-site detection [15]. These unique features may make colorimetric biosensors suitable for point of care and developing world applications [18]. However, conjugation of AuNPs to monoclonal antibodies with high affinity makes them a precious biosensor to detect the target protein in cancer cells [19].

Among the most abundant extracellular matrix components such as collagens, tenascins, proteoglycans, glycosaminoglycans, and laminin, fibronectin plays an important role both in extracellular matrix (ECM) structure and function [20, 21]. Fibronectin is a high molecular weight adhesive glycoprotein which is composed of two large monomers (250 kDa) that are linked by disulfide bonds at their carboxy terminal ends [22, 23]. After lung injury, the expression of cellular fibronectin is increased mainly due to promotion of fibroblasts proliferation [24, 25]. Fibronectin is expressed in several carcinoma cell types and has been implicated in carcinoma development. Fibronectin over expression has been found in lung cancer, specifically in non-small cell lung carcinoma (NSCLC) [26]. In a study addressing distribution of main integrins and ECM in patients with NSCLC (n=45), extensive (>50 % of section area) fibronectin and collagen type IV were reported in 22 and 55 % of tumors, respectively [27]. Similar studies on small cell lung carcinoma (SCLC) showed higher levels of fibronectin in all adult tissue samples as well [28].

In our study, by conjugating AuNPs to human fibronectin antibody (anti-hFN), a colorimetric biosensor has been designed to detect fibronectin (FN) which might be present in extracellular matrix (ECM) of human cultured cells. Aggregation of anti human fibronectin gold nanoparticles (anti-hFN–AuNPs) is based on targeting fibronectin through its recognition in the ECM of the cultured cells. Overlapping surface plasmon resonance of AuNPs due to their assembly in the ECM leads to a shift in absorption spectra and alteration in light scattering as well which causes signal transduction and change in color. In this study, our target cells was A549 lung cancer cells whereas cultured human skin (AGO-1522) and hematopoietic cell line (Nalm-6) were used as control and negative control cells, respectively.

Material and Methods

Gold Nanoparticle Preparation

Distilled and deionized water used for the preparation of all solutions. Gold nanoparticles (AuNPs) with a diameter of ~30 nm were prepared by citrate reduction of Gold III chloride hydrate (HAuCl4) which was purchased from Sigma based on the method introduced by Turkevich [29]. All glassware was cleaned in aqua regia (three parts HCL, one part HNO3). The synthesis of AuNPs was monitored by observing changes in the absorption band based on surface plasmon resonance of AuNPs.

Cell Culture

Three different cell lines namely human Caucasian lung carcinoma type 2 epithelial cells (A549), human skin (AGO-1522), and hematopoietic (Nalm-6) were used as target, control, and negative control cells, respectively, and all were obtained from the National Cell Bank of Iran (NCBI). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin, all purchased from Biosera. Cells were incubated at 37 °C in an atmosphere of CO_2/air (5/95 %) saturated in water. Counting of cells was performed to keep the cell number constant throughout the procedure.

UV-Vis Spectroscopy

Optical extinction spectra were recorded using digital UV-visible spectrophotometer (Ultrospec 4300 pro) in the 300–800-nm range and the graphs were analyzed using SWIFT II software.

Fabrication of Anti-hFN–AuNPs Conjugate

Monoclonal anti human fibronectin (F0791) was purchased from Sigma Aldrich. To fabricate anti-hFN-AuNPs conjugate, coagulation test was carried out ahead in order to find out the optimal concentration of antibody solution which minimizes the disturbance of the nanoparticle-antibody complex. Twenty-five microliters of 30 nm AuNPs were mixed well with 50 μg/100 μl of anti-hFN. The mixture was incubated for 5 min and then, to remove the excess antibody, the solution was centrifuged at 140,000 rpm for 20 min at 4 °C and

resuspended in storage buffer (HEPES 20 mM) for further use. The prepared anti-hFN-AuNPs conjugate was stored at 4–8 °C. Conjugation of AuNPs took place by non-covalant interactions (electrostatic coupling). In this process, there are three types of interactions that may take place; hydrophobic interactions, ionic interactions, and dative binding. Hydrophobic interactions are due to attraction between hydrophobic parts of the antibody and the metal surface, resulting in the formation of a non-covalent bond. Positively charged groups are abundant in antibodies, i.e., positively charged amino acids and the N-terminal is present. Ionic interactions are formed between these groups and the negatively charged surface of the particles. Dative binding is the formation of a covalent bond between the gold particle and free sulfhydryl groups of the antibody [30]. Conjugation of AuNPs with anti-hFN antibody was performed at room temperature (RT) and the pH was adjusted to 7–9 (optimum pH) based on the results gained from different pH examination (from 5 to 10) to address optimum pH for anti-hFN-AuNPs conjugate stability.

Detection of Fibronectin in Cellular Matrix by Anti-hFN-AuNPs Complex

For antibody-nanoparticle (anti-hFN-AuNPs) treatment, the cells were grown in 12-well tissue culture test plates (from Orange Scientific Company) and counted before adding anti-hFN-AuNPs and analysis of aggregation process. After 72 h of cell culture, the medium was initially removed gently and then washed with buffer (HEPES 20 mM). Then, 500 μl of the anti-hFN-AuNPs conjugate was added to each well and the plate was gently shaked and incubated for 30 min.

Results and Discussion

Construction of Anti-hFN-AuNPs Complex and Detection of Fibronectin

A monoclonal anti human fibronectin (anti-hFN) was conjugated to 30-nm gold nanoparticles (AuNPs) to confirm selectivity, specificity, and colorimetric aspects of the experiment. In Fig. 1, the spectra of (a) and (b), which are gold nanoparticle and gold nanoparticle conjugated to anti human fibronectin at 530 nm and 700 nm respectively show almost complete conjugation state of nanoparticle and antibody complex based on the report by Thobhani and colleagues [31]. The color of two solutions (a) and (b) was pinkish (Fig. 1b, a). The constructed nanoparticle-antibody was used to examine its recognition and binding to the fibronectin present in the ECM of cultured target cells (A549) (Fig. 1a, c). Comparison between two spectra, (b) and (c), shows a drastic change at 530 and 700 nm which confirms alteration in absorption and

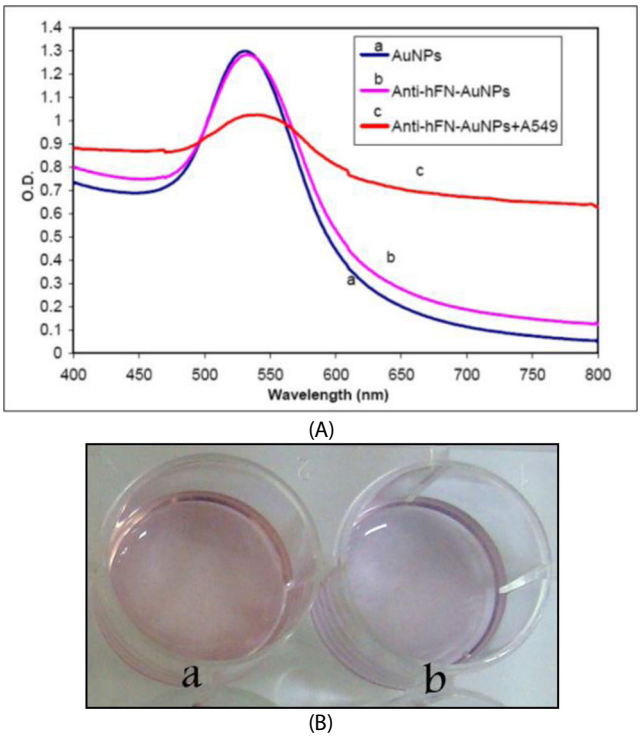

Fig. 1 **A** Spectra of the gold nanoparticle aggregation in the presence of fibronectin in target cells: *a* AuNPs, *b* conjugated AuNPs with anti human fibronectin, and *c* conjugated AuNPs with anti human fibronectin in presence of (A549). **B** Visual comparison of changing color in two wells. *a* The well containing anti-hFN-AuNPs with no cell (blank) and *b* the well containing anti-hFN-AuNPs with target cells (A549)

scattering of gold nanoparticles (Fig. 1a, b and c) and a prominent visual change in color from pink to violet (Fig. 1B, a and b).

Examining Constructed Antibody-Nanoparticle Complex by NaCl

Figure 2 demonstrates four samples which are: (a) anti-hFN-AuNPs, (b) anti-hFN-AuNPs + NaCl, (c) anti-hFN-AuNPs + A549, and (d) anti-hFN-AuNPs + A549 + NaCl, in which analysis of their results could eliminate any false positive/negative data.

The spectrum of anti-hFN-AuNPs (a) denotes higher range of absorbance comparing to the spectra of anti-hFN-AuNPs with target cells (c) which is due to the binding of anti-hFN-AuNPs to the fibronectin present in the ECM of the target cells. From a physical point of view, SPR is defined as the collective oscillation of electrons on the metal surface when they are excited by light at specific wavelengths [32]. SPR band intensity and wavelength depend on factors affecting electron charge density on the particle surface such as particle size, shape, and structure. In the aggregated form of gold nanoparticles (anti-hFN-AuNPs + A549), the conduction electrons near each particle surface become delocalized and are shared among neighboring particles which results in SPR

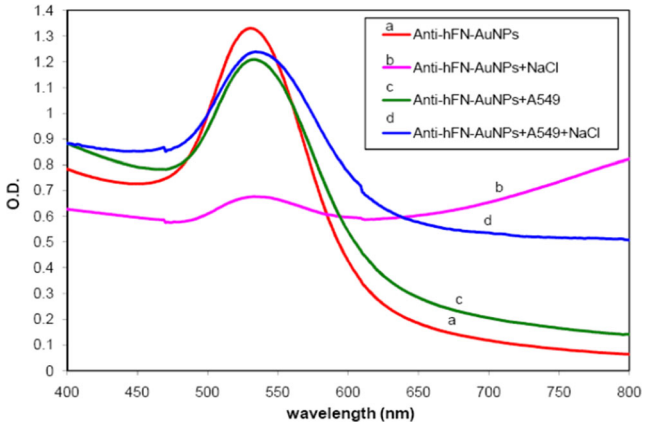

Fig. 2 Comparison of four different samples to confirm binding of constructed antibody-nanoparticle complex to the target cells (A549): *a* and *b* show human fibronectin antibody-gold nanoparticle complex and human fibronectin antibody-gold nanoparticle + NaCl, respectively. *c* and *d* show human fibronectin antibody-gold nanoparticle in presence of target cells and human fibronectin antibody-gold nanoparticle in presence of target cells + NaCl, respectively

shifts to lower energies, causing the absorption and scattering peaks to red-shift to longer wavelengths [33]. Our results were in accordance with the results of another study which used aptamer-conjugated gold nanoparticles (ACGNPs) for the detection of cancerous cells. In this study, shift in optical spectrum of particles and color change took place as a consequence of ACGNPs assembly around the cancer cell surface [13]. Similarly, in another study using unmodified gold nanoparticles and oligonucleotide probes for diagnosis of mutations in EGFR in lung cancer cell lines they reached to comparable results. It was well demonstrated that at optimal salt concentration, in the presence of mutant DNA, oligonucleotide probes hybridize with the complementary mutant DNA and substantial aggregation of unmodified nanoparticles occurs leading to shift of UV-Vis absorption spectrum to longer wavelength (from 520 to 600–700) [17]. Moreover, a nanoprobe was developed for the diagnosis of *Mycobacterium tuberculosis* in suspected clinical samples in another research, by functionalizing gold nanoparticles with *M. tuberculosis*-specific oligonucleotides. In this study, addition of high NaCl concentrations, which disturbed the ionic charges of the nanoprobe solution, to the *M. tuberculosis*-negative sample, induced nanoprobe aggregation and turned the solution purple. While, in the event of specific probe hybridization to complementary DNA of *M. tuberculosis*, no nanoprobe aggregation observed after addition of NaCl and solution remained red [34]. In a similar manner, we added 100 μl of NaCl 1 M to the samples to determine the binding of antibody-gold nanoparticle complex to the fibronectin of the ECM of cultured cells, as in absence of fibronectin NaCl disturbs the ion charge balance of the solution due to the most aggregated condition of AuNPs (b), whereas anti-hFN-AuNPs (a) without NaCl shows the highest pick absorbance. The pick spectrum of anti-hFN-AuNPs + A549 +NaCl (d) was very close to anti-hFN-AuNPs + A549 (c) even after addition of NaCl which indicates almost complete and selective binding and relatively the most aggregated condition in the solution when the target cells are present. Based on the results obtained from spectra of different samples, it was clear that anti-hFN-AuNPs was bound selectively to fibronectin present in the ECM of the cells and gathering of the anti-hFN-AuNPs in the matrix of the target cells leads to alteration in the absorption and scattering of the gold nanoparticles. NaCl is mostly used as a negative confirmatory test as it disrupts the electric change of the AuNPs and it causes the most aggregated state of the AuNPs, the large-scale assembly of the particles, and change in their spectral properties. Comparison of two spectra, anti-hFN-AuNPs + target cells and anti-hFN-AuNPs + target cells + NaCl were relatively no different mainly due to selective binding of the anti-hFN-AuNPs to the fibronectin, aggregation of particles and ultimately elimination of the possibility of any more aggregation by NaCl.

Usage of Antibody-Nanoparticle Complex with Target and Non Target Cell Lines

As fibronectin generally is present in the ECM of the most attached cell lines, binding of the Anti-hFN-AuNPs to these attached cell line was expected but it was important to differentiate between ECMs with much higher amounts of fibronectin.

Figure 3 shows the results of using Anti-hFN-AuNPs with different cell lines. Spectrum (a) is anti-hFN-AuNPs solution without any cell with the plasmon resonance peak at 530 nm with the absorbance of 1.4. However, a closer look in the spectra b, c, and d shows decrease in the level of absorbance at 530–550 nm. The assembly and selectively embedding of the Anti-hFN-AuNPs in and around the ECM of the AGO-1522

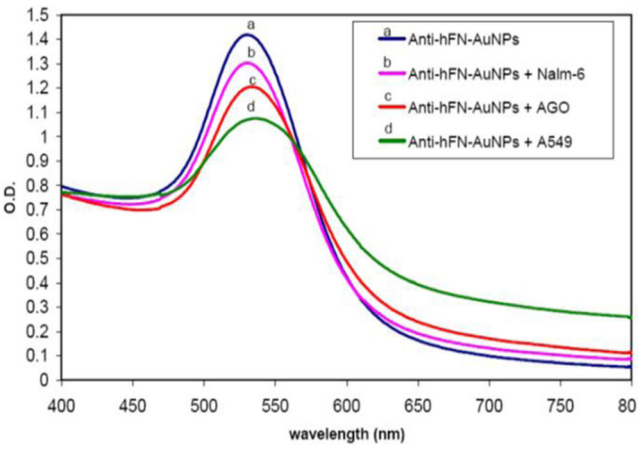

Fig. 3 Spectra of nano conjugate with target and non target cell lines: *a* Anti-hFN-AuNPs (no cell), *b* Anti-hFN-AuNPs + Nalm-6(negative control cell line), *c* Anti-hFN-AuNPs + AGO-1522(control cell line), and *d* Anti-hFN-AuNPs + A549(target cell line)

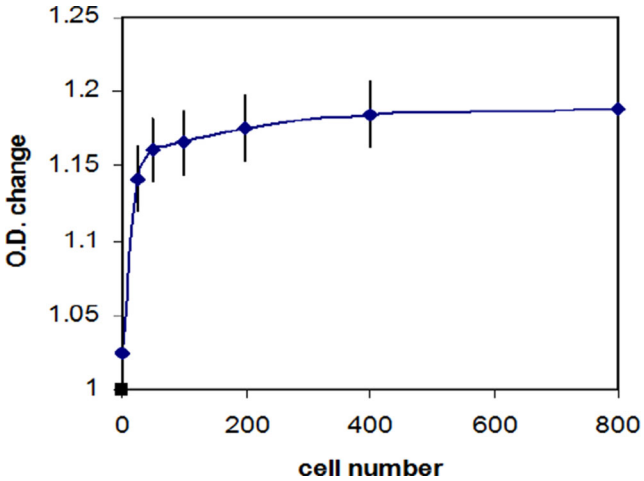

Fig. 4 The cell number effect: the cell number in wells of the plates were designed to contain from 0(no cell) to 800,000 cells

and A549 cell lines due to the present of fibronectin leads to alteration in the light scattering and absorption properties of the gold nanoparticles. To be realistic, it is difficult to reach definite selectivity regardless of the molecular marker used, and nonselective binding to the suspended cells devoid of ECM and fibronectin may occur which is highly unlikely to be to an extant to assure false positive results.

However, in Fig. 3, the comparison of the spectra of the target cells (A549) (d), control and negative control cells (AGO-1522 (c) and Nalm-6 (b)) shows a clear difference in shift at 600 to 750 nm (Fig. 3b–d) and towards the low-energy of the absorption spectrum area, near IR. High absorption is achieved with the target cell line (A549) containing larger particle aggregates which are due to higher level of fibronectin in its ECM.

It is noteworthy to mention that A549 cell line, adenocarcinomic human alveolar basal epithelial cell line is known to express fibronectin in higher amounts. A review article by Jeffrey D. Ritzenthaler eand colleagues in 2008 clearly discussed about alteration of fibronectin expression in lung carcinoma and higher levels of fibronectin in both non small cell lung carcinoma (NSCLC) and small cell lung carcinomas (SCLC) [22].

Incubation Time

The effect of incubation time of anti-hFN-AuNPs with the target cells on the rate of aggregation was studied and the results demonstrated that aggregation of anti-hFN-AuNPs takes about 60 min to complete (data not shown). However, there was a change in color from pink to violet within 30 min. At longer times, there was no change in color beyond what was seen in 30 min.

Effect of Cell Number

The effect of increase in cell number on aggregation rate and level of optical density is shown in Fig. 4. The results show that the rate of aggregation is cell number independent and increase in cell number did not change the rate of aggregation. This might be due to the fact that increase in cell number does not necessarily leads to accumulation of more ECM and its fibronectin beyond certain limits. We also found that the

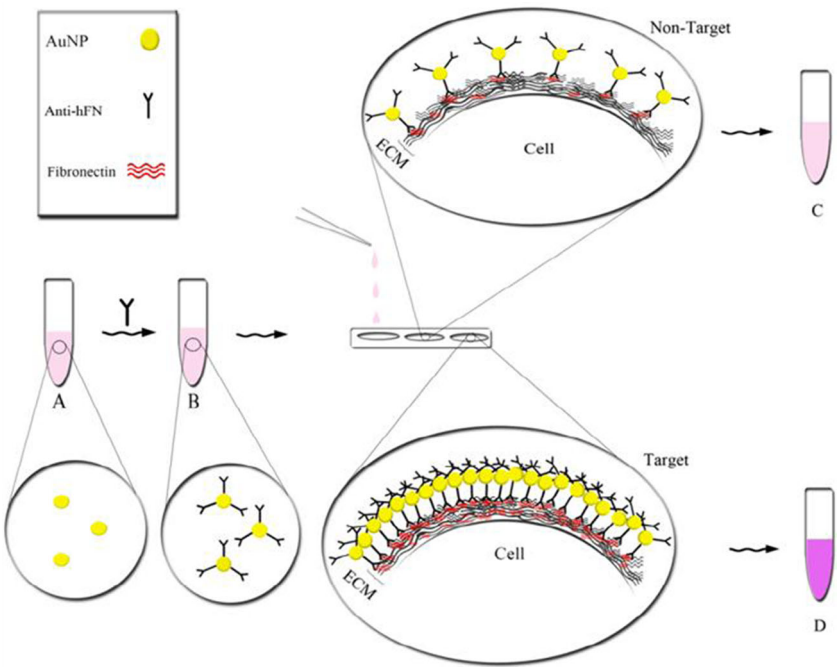

Fig. 5 Schematic illustration of antibody-nanoparticle complex with target and non target cells: steps involved in conjugation of gold nanoparticles with anti human fibronectin are shown in *A* and *B*. Aggregation and assembly of the antibody-nanoparticle complex in target and non target (control) cells are shown in *C* and *D*

maximum O.D. did not change as the number of cells was increased beyond certain limits.

In general, AuNPs colorimetric biosensors irrespective of the type of target molecules, recognition elements used (i.e., oligonucleotide probes, aptamer, antigen, and antibody) and AuNPs modifications have basic features and performance in common. They are generally simple, rapid, specific, user-friendly, and provide visual readouts and eliminate the needs for specialized and sophisticated instruments and equipment [11–18, 34]. In comparison, our biosensor was well qualified with comparable performance. It has shown great promise in accurate colorimetric detection of fibronectin in cancer cells taking advantage of inherent specificity of antibody–antigen interactions and unique characteristics of AuNPs. In fact, conjugation of nanoparticles with antibodies combined the properties of the nanoparticles themselves with the specific and selective recognition ability of the antibodies to antigens. Besides, construction of this biosensor was both simple and cost-effective as it used monoclonal antibodies which are commercially available owing to their broad applications [35].

In addition, this colorimetric biosensor facilitates real-time on-site detection of fibronectin over expression in ECM of cancer cells and may rival with current fibronectin detection techniques such as immunohistochemistry (IHC). IHC has tremendous potential to show exactly where a given protein is located within tissue by employment of specific antibodies that can be visualized by staining. Regardless of IHC extensive applications and advantages, its outcomes may present significant bias as efficiency of this technique is influenced by experience of hands that perform the procedures and also eyes that interpret results [36]. Another prospective application of this biosensor may be detection of fibronectin overexpression in noninvasive clinical samples such as sputum, as analysis of sputum cytology abnormalities has been associated with higher chance of early lung cancer diagnosis [37].

Conclusion

Detection of cancer cells with the help of a technique without any invasive procedure and sophisticated instruments is an ideal goal in diagnosis of cancer. In our preliminary study, we constructed a complex containing gold nanoparticles conjugated with anti human fibronectin (Anti-hFN-AuNPs) to bind specifically to fibronectin of ECM of cultured cells.

Anti-hFN-AuNPs complex selectively bond to fibronectin of the cells containing ECM; however, ECM of the cells with higher levels of fibronectin showed more prominent alteration in absorption and scattering due to more aggregation of gold nanoparticles. A schematic representation (Fig. 5) is designed based on our preliminary data which tries to explain the whole scenario. Based on the preliminary results, we conclude that this constructed complex has the ability to detect cancer cells with higher level of fibronectin like lung cancer visually, just by change in color.

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