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Cancer biomarkers detection using tracer nanoparticles and advanced nanoscopic scanning

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Abstract
Background: New advances in the field of molecular medicine and the discovery of novel diagnostic and therapeutic approaches to cancer diseases will depend on identifying biomolecular structures and behaviors. Using advanced nano-detection devices, nanomedicine can provide a new insight into obtaining single-molecule analysis of functionalized biomolecules or biomarkers in human malignant disorders. Because of the critical biological functions of small non-coding RNA oligonucleotides, accurate nanoscopic identification of miRNA-21 molecule was supposed to be performed.
Methods: To this end, gold nanorods (GNRs) as tracer particles with cationic surfaces were employed to label anionic miRNA-21 after normalization. Their successful interaction was approved by an average hydrodynamic diameter and surface zeta-potential test using dynamic light scattering (DLS). Then, the structural changes of the samples were investigated through scanning tunneling microscopy (STM) with a single atomic resolution.
Results: The DLS results to investigate whether GNRs could efficiently label miRNA indicated affinity of CTAB-coated GNRs to miRNA-phosphate groups in the complexes. The high qualities of STM 2-D and 3-D nanographs of GNRs and GNRs-miRNA complexes demonstrated significant differences in morphologies, placing arrangements, surfaces, sizes, and aspect ratios.
Conclusion: Following the observed direct effects of the targeted biomarkers on the tracer gold nanoparticles, we introduced a new insight into biomedical agent nanocharacterization of molecular confrontation using a newer nanoscopic application. These findings can develop nanodiagnostic and prognostic techniques such as miRNAs detection in cancer diseases via advanced scanning nanotechnology and nanocolorimetric assay.

Keywords: Gold nanorods, Cancer biomarkers, microRNA-21, Scanning tunneling microscopy.

1. Introduction
Nano-molecular medicine aims at using biological components via advanced nano-technique to identify nano-biomolecular characteristics and structure for which an innovative insight is required to perform single molecular or particulate analyses [1-4]. In addition, profound understanding and manipulation of biomolecule properties at nano-scale provide significant improvements of diagnostic methods such as cancer biomarker detection [5].
Recently, in molecular medicine, there has been growing interest in using molecular nucleic acid-based techniques [6, 7]. Regulatory non-coding RNAs (miRNAs) play key role in many critical biological functions. The miRNAs as robust biomarkers are applied in the emerging field of molecular medicine, especially in the cancer diagnosis and prognosis [8, 9]. Furthermore, these low-molecular-weight RNAs are effective on cellular proliferation, apoptosis [10], and down-regulation of pathogenic genes. In particular, miRNA-21 as the key oncomir is up-regulated in a variety of human cancers [10, 11], including breast [6, 7], lung [9], liver cancers [10, 12].
Gold nanorods (GNRs) as the tracking nano-particles with unique optical properties, positive surface charges and bio-interaction capabilities [13-15] can be used in the sequence-specific detection methods to identify specific biomolecules or biomarkers [16-18].
In scanning probe microscopy (SPM), the highest throuput is achieved via is scanning tunneling microscopy (STM), which is associated with high atomic resolution of two and three-dimensional imaging for single particles or molecules [19, 20]. A detailed topographic map of surface changes can be precisely scanned at the nano-level to measure electrical signals of biomolecular bonds through STM [21].
This research is focused on utilizing electron tunneling effects to reach an ultra-resolution detection of miRNA-21 as a cancer biomarker at nanomolar concentrations. In this study, we would like to introduce a novel nanocharacterization method to develop diagnostic and

therapeutic nanomolecular approaches for MicroRNA with regard to structural changes of tracer nanoparticles such as GNRs.

2. Materials and Methods

2.1 Chemical reagents and analytical instruments

The rod-shaped gold nanoparticles in the forms of colloidal suspension (cat no: 716812; 10 nm in diameter) were produced from Sigma-Aldrich, Germany. In the current study, nano-topography was carried out using scanning tunneling microscopy (NAMA STM SS3-L1) and highly ordered pyrolytic graphite (HOPG) surfaces from NATSYCO, IRAN. Surface potential and size analysis were investigated through dynamic light scattering (Nano-ZS analyzer; 633 nm, Malvern Co., U.K).

The most effective sequence of human miRNA-21 (MIMAT0000790) as one of the most common cancer biomarker (5'- UAGCUUAUCAGACUGAUGUUGA -3') (22-24) was chemically synthesized and annealed by BIONEER. Co (Germany). The primary concentration of miRNA-21 is approximately 4000 μgml^{-1}, but in this study, the suitable concentration of miRNA oligomers was determined to be 34 $ng\mu l^{-1}$ using a serial dilution method, absorbance unit, and gel electrophoresis.

2.2 GNR- miRNA complexation

After normalization of the solutions, suitable concentrations of GNRs (at 35 $ng\mu l^{-1}$) and miRNA-21 (at 34 $ng\mu l^{-1}$) were mixed in equal volumes, vortexed, and optimized at pH 7.4. Then, the mixture was incubated at 37 °C for 25 min for an electrostatic binding to be formed between anionic miRNA oligoes and cationic surfaces of GNRs. The GNR particles were sonicated for 2 min to prevent aggregation.

2.3 Dynamic light scattering

DLS was employed to determine mean hydrodynamic diameters and surface potentials [25, 26] of GNRs, miRNA-21, and GNR-miRNA complexes. The results were obtained under the parameters such as dispersant RI 1.33, material RI 1.59, and material absorption 0.01 at pH 7.4 in a backscatter mode and then interpreted on the intensity, volume, and number distribution graphs using DTS software [27].

2.4 Scanning tunneling microscopy of rod-shaped gold nano-tracers and GNR-miRNA complexes

Morphologies, surfaces, and structures of the tracer gold nanoparticles were evaluated by the use of STM before and after the labeling of miRNA-21 as a cancer biomarker. For imaging, HOPG as a suitable sample holder (0.25 cm in thickness and 0.8 cm in diameter) with high atomic arrangement regularity [28, 29] and Remanium tip (0.25 mm in diameter) with an atomic sharp point (30) were utilized.

After several scans, the ideal concentration of GNRs on HOPG surface was considered to be about 0.005 μgml^{-1} to form a monolayer for a high resolution imaging. Then, 10 µl of GNRs and GNR-miRNA-21 complexes were immobilized on HOPG grids and incubated at room temperature overnight for slow solvent evaporation.

STM imaging was operated in both directions to ensure that the results would not have been perturbed by the tip-sample interactions. The best resolutions of 2-D and 3-D nanographs were taken in a constant current mode and under such electromechanical parameters as current set point (CSP): 0.2 nA, sample bias: 0.03 V, 1 kHz PID (the overall loop response time determines how fast STM can scan), and 450 pixels. Scanning was performed at a level equal to 50×50 nm at room temperature under ambient conditions. The topographic data were processed by NAMA-STM SS3-L1 nanoanalyzer software and low-pass filter.

2.5 Macroscopic labeling

The microtubes containing GNR and GNR-miRNA-21 were compared for macroscopic changes in transparency, homogeneity, and color.

3. Results

3.1 Dynamic light scattering

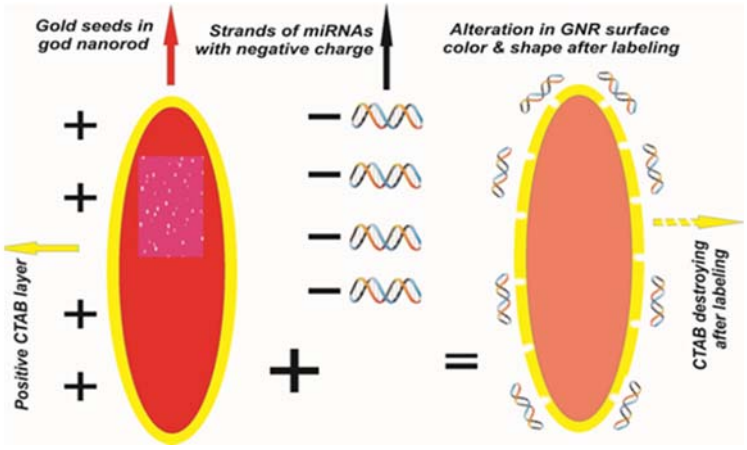
The obtained results of average hydrodynamic diameters and zeta potentials of GNRs (Figure 1), miRNA-21 (Figure 2), and GNR-miRNA-21 complexes (Figure 3) indicated significant changes illustrated in Table 1.

Table 1: DLS Parameters

Sample	Dispersant	Conductivity (ms/cm)	Viscosity (cP)
GNR	Depc water	0.0557	1.0031
miRNA	Water nuclease free	0.0395	0.8872
GNR-miRNA 21 complexes	Water nuclease free	0.148	0.8872

Table 2: Average particle size and zeta-potential of GNRs and GNR-miRNA

Sample type	Mean particle size (nm)	Zeta-potential (mV)
miRNA-21		-0.724
GNR	18.78	37.1
GNR-miRNA 21	45.91	8.39



Schematic 1: Labeling processes of miRNAs with GNRs nanotracer

This section indicates the negatively charged seeds of gold nanoparticles coating with positively charged CTAB bilayer with affinity to bioconjugate with negatively charged miRNA oligoes via non-covalent electrostatic interaction that cause to destroy of CTAB layer after sever electrostatic binding.

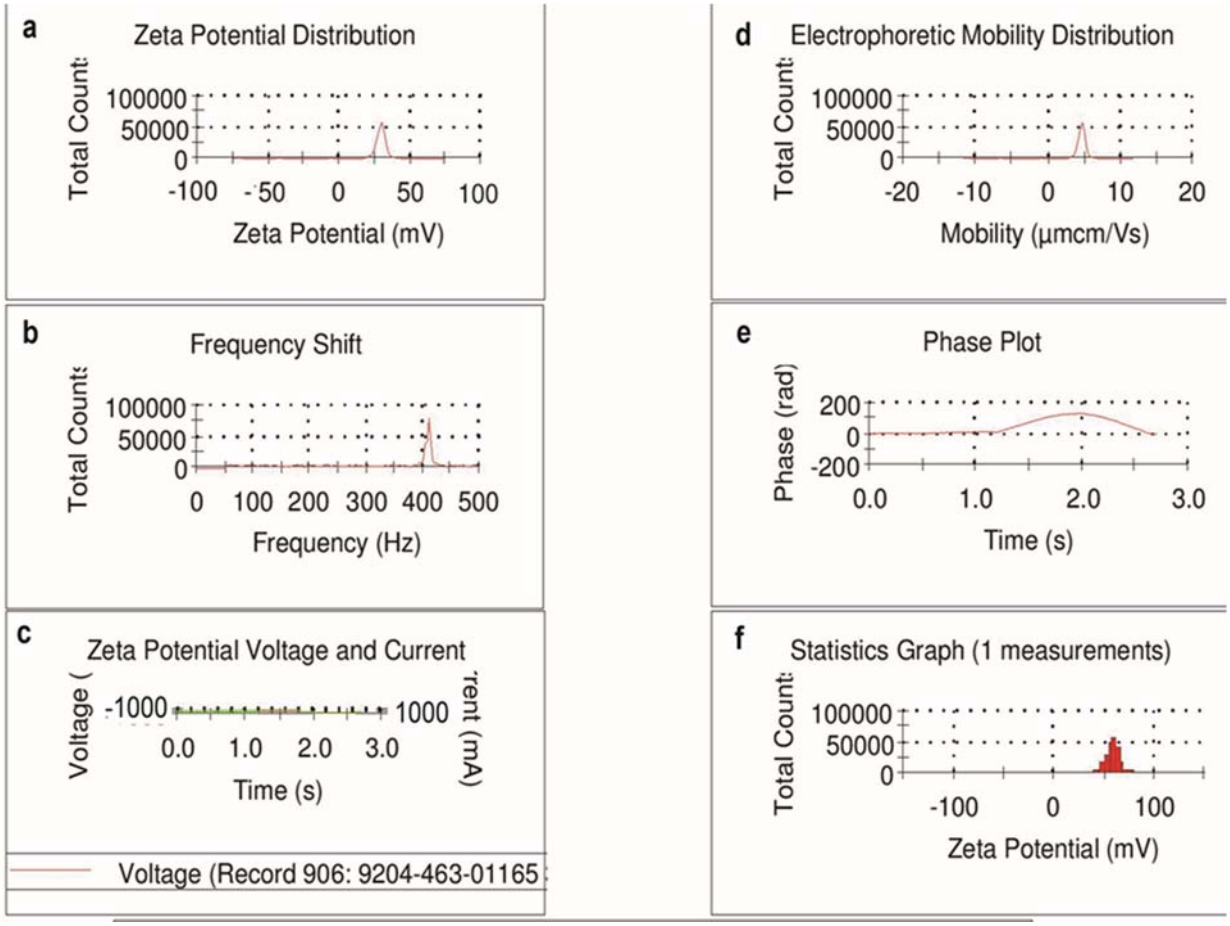


Fig 1: Zeta potential analysis of GNRs

The positive surface charge of GNRs based on their Zeta potential distribution and Electrophoretic mobility distribution.

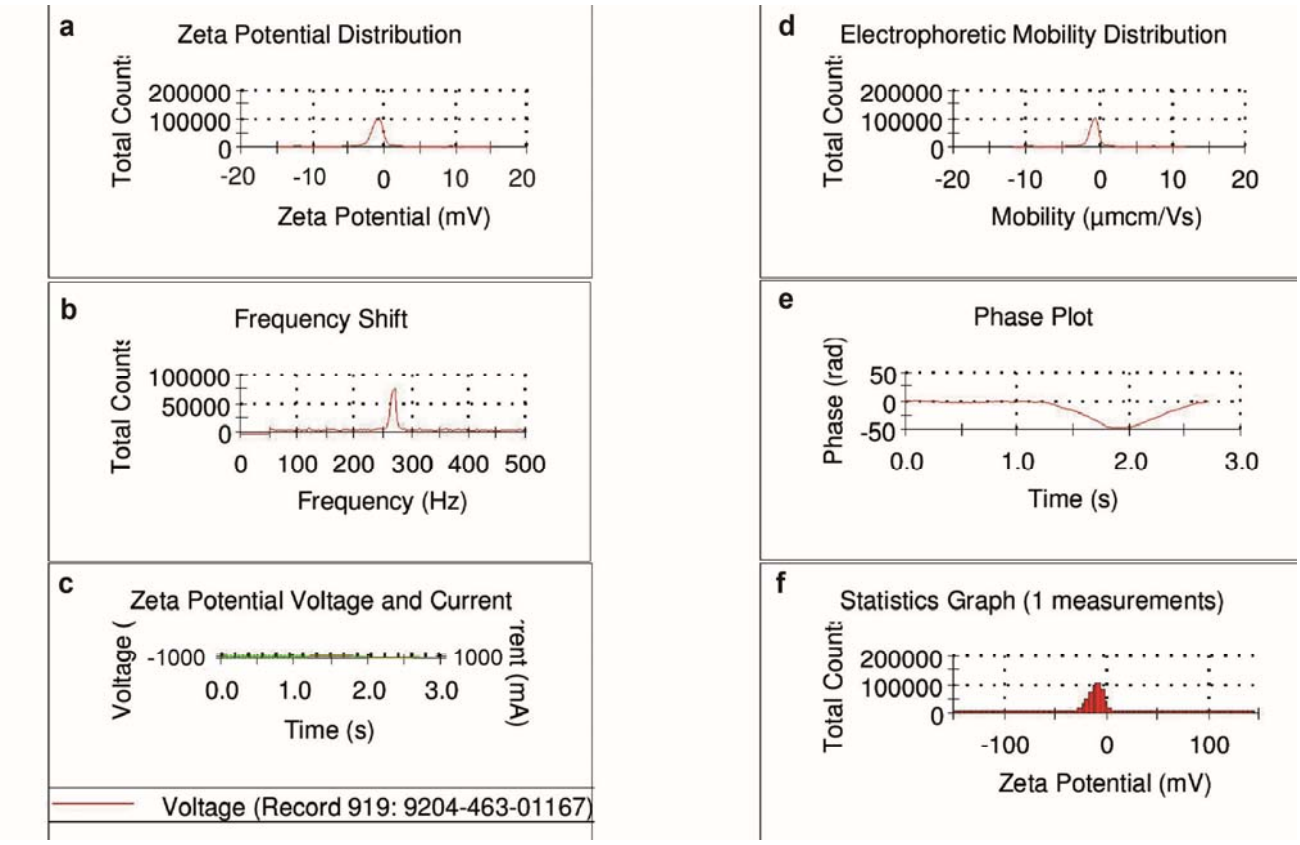


Fig 2: Zeta potential analysis of microRNA-21

The negative charge of miRNA based on their Zeta potential distribution and Electrophoretic mobility distribution.

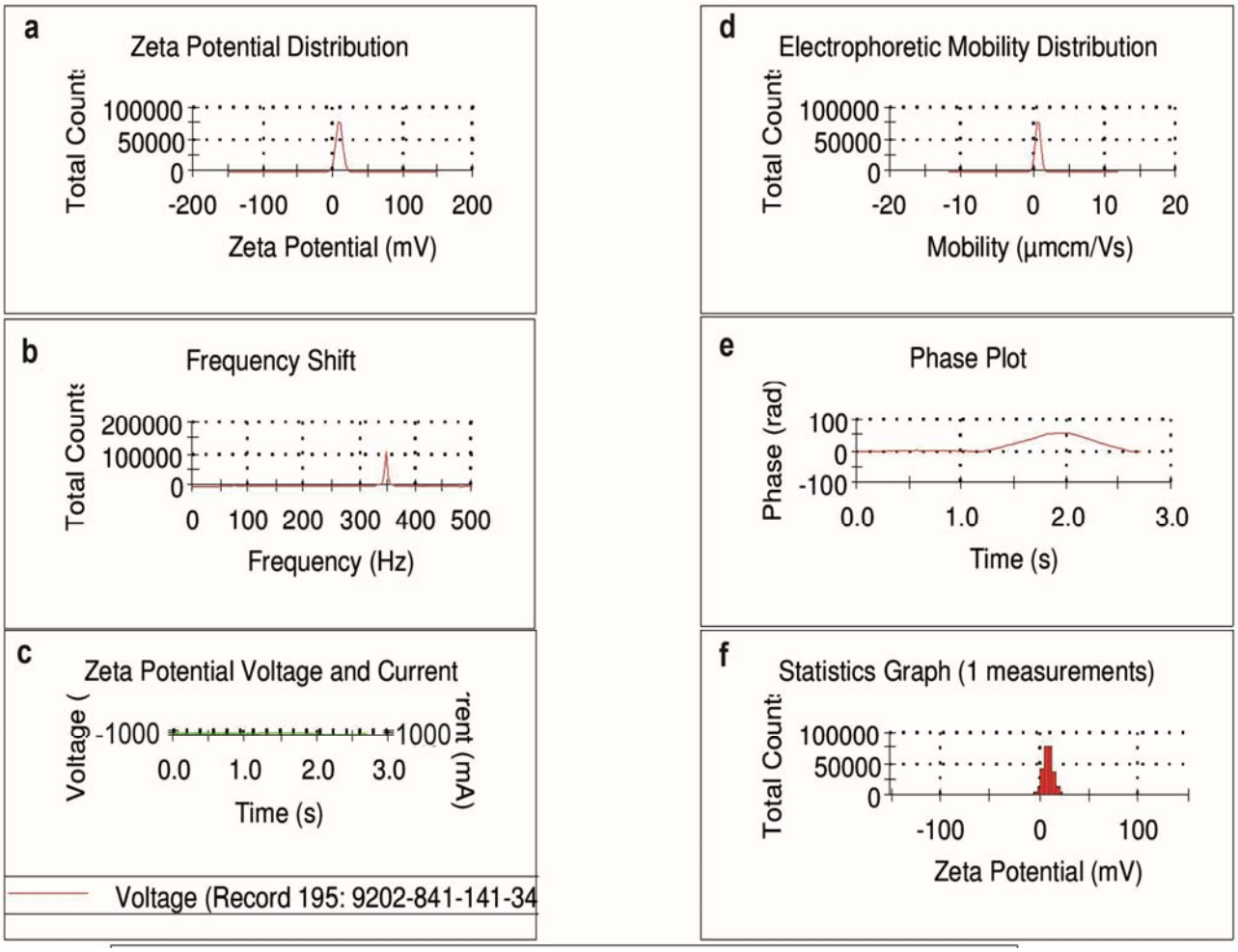


Fig 3: Zeta potential analysis of GNR-miRNA complexes after labeling phase

Reduce in positive charge of GNRs due to sever electrostatic bindings between GNRs and miRNA oligoes in complexes formation.

3.2 STM characterization of GNRs

The high-quality 2-D and 3-D nanographs of monolayer GNRs demonstrated that the morphologies of GNRs are cylindrical and uniform with regular arrangements. Size diagrams on 2-D nanographs showed regular depositions for each gold nanoparticle. Also, the average aspect ratios of GNRs (mean length-to-width ratios of 100 GNRs) were predicted to be equal to 3 based on STM 3-D nanographs (Figure 4).

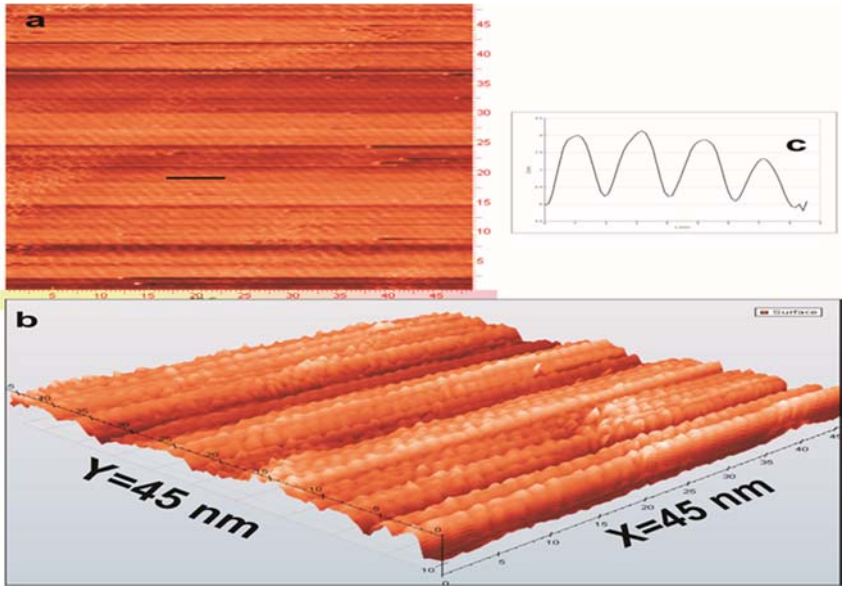


Fig 4: STM of GNRs on HOPG

2D nanograph (a); and 3D nanograph of GNRs on HOPG surface at a level equal to 45×45 nm (b); Size diagram of GNR particles on 2D nanograph (partition a) (c), respectively, diagram are demonstrated with one regular jump for each nanoparticle

3.3 STM characterization of GNRs labeled with miRNA

The nanographs of monolayer complexes (followed by miRNA addition) represented alterations in morphologies, sizes, significant transformations, irregularities, and surface roughnesses (Figure 5).

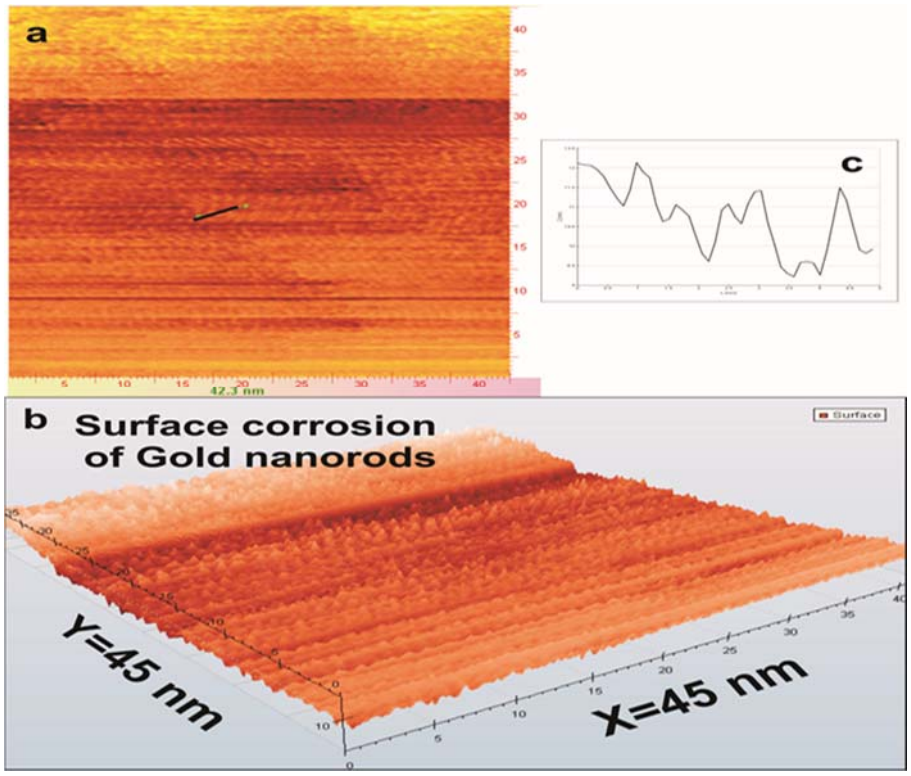


Fig 5: STM of miRNA-GNRs complexes on HOPG

2D nanograph **(a)**; and 3D nanograph of nanoplexes on HOPG at a level equal to 45×45 nm (b); Size diagram of nanoplexes on 2D nanograph (partition a) **(c)**. An irregular jump was showed in the diagram for each nanoplex particle.

3.4 Macroscopic labeling

The microtubes of GNRs and miRNA solutions appeared to be clear, transparent, and homogenous. The GNR was visualized as a red-pink solution, while the miRNA solution was achromatic. Within the labeling phase, after about 25 min of incubation at 37 °C, macroscopic changes in color and accumulation were detected. As shown in Figure 6, the microtubes containing miRNA and GNRs mixtures indicated a shift from red-pink to achromatic colors. Additionally, sedimentations were observed in the microtube containing the mixture.

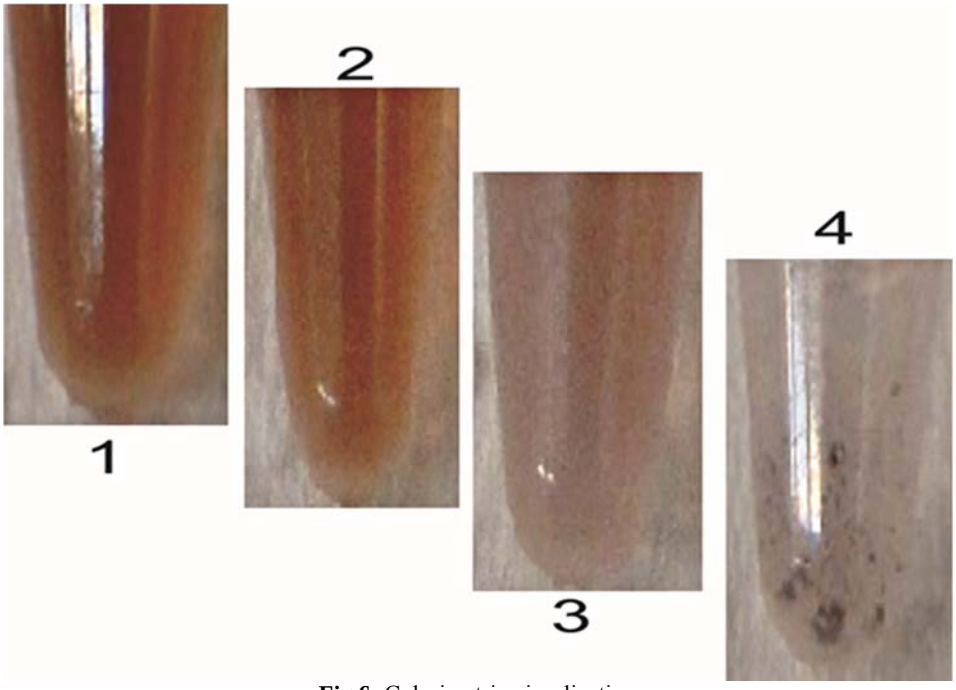


Fig 6: Colorimetric visualization

GNR solution with red color **(1);** Mixture of GNR-miRNA complexes after 1 min **(2);** After 10 min with color shifting to red-pink **(3);** After 20 min labeling with color shifting to achromatic and sedimentation **(4).**

4. Discussion

To develop molecular medicine for the diagnosis, prognosis, prevention, and treatment of severe diseases such as cancers, novel applications of advanced analytical tools are required for biomolecular identification [1-4]. Our ultimate aim was to confirm the hypothesis that advanced nanotechnology in molecular scanning can exert a significant role on the characterization and detection improvements of biomedical markers at the single-molecule level to remove the obstacles and limitations in the way of medical sciences.

In the last decade, nucleic acids, especially miRNAs, have been extensively investigated in the field of nanobiosciences. There are 3 main categories of detection methods to identify nucleic acids of various sizes as functionalized biomolecules: target amplification (polymerase chain reaction, etc) and probe amplification (ligase reaction, etc) that are frequently used today and the third group which is based on the detection of single molecular signals is referred to as signal amplification method. The common characterization techniques can analyze average populations of target samples against single-molecule analysis [31].

We first performed size and surface charge tests to investigate whether GNRs could efficiently label miRNA, indicating affinity of CTAB-coated GNR to miRNA phosphates in the complexes (Scheme 1).

As mentioned above, to achieve an accurate and detailed single-molecule evaluation directly or indirectly, employment of high-throughput equipments and advanced methods at nanoscale level is required using nanoscopy with the capability of recording single-molecule signals or scanning probe microscopy [19, 20]. To this end, STM with the capabilities of high resolution imaging, electron tunneling effect, and tracking particle behavior was conducted. The present study investigated nanomolecular events in a topographic mode. Furthermore, GNR particles were used as tracer particles. As reported in this study, this can be explained by the fact that the GNRs with cationic CTAB surfaces have significant affinities for potently labeling on anionic-surface phosphate groups of miRNA oligonucleotides through non-covalent electrostatic binding into detectable complexes. Two-surface plasmon resonances of GNRs have a major role in binding to nucleic acid molecules [13-18]. Relying on the reduction of surface charges and significant increases in the average sizes of GNRs after adding miRNA, we could prove DLS is able to provide GNR-miRNA-21 complex formation [25-27].

The miRNAs labeled by GNRs were imaged indirectly by STM with an excellent resolution on HOPG surface. The presence of biomolecules was proven indirectly following the behaviors and structural changes of GNRs after the labeling phase. When there is no binding, morphologies of the GNRs remain intact and unchanged. In addition, the presence of the targeted biomolecules can be predicted as soon as the deformation, rugosity, and corrosion of GNR surfaces are observed. Even when excessive attaching of miRNA oligomers to the GNRs occurs, numerical or electrostatic severities can cause a complete loss of nanocarrier structures.

Finally, we demonstrated that molecular confrontation at nanoscale level together with its direct effect can prove the presence of the target single molecules indirectly. Our findings facilitate achievements of optimum application and characterization of RNA molecules in molecular medicine and remove the obstacles which stand in the way of biomolecular diagnosis and treatment of severe diseases.

5. Conclusion

In this research, scanning tunneling microscopy and a new application of single biomolecule analysis of miRNA oligoes using tracer gold nanorod particles were employed to complete characterization more precisely and obtain an optimal efficacy without any concerns about their concentrations and ratios. We believe that different biomolecular behaviors are achieved at the single-molecule level.

Based on our observations, we introduced a new insight into the identification methods of functionalized biomolecules and biomarkers, such as consideration of miRNAs and their reactions with other nanomedical agents. Moreover, the results illustrated that STM as a basic nanodevice can be employed in molecular medicine processes with a major role in molecular diagnosis and treatment of cancers.

In summary, we showed that the presence of targeted single biomolecules and biomarkers can be proven indirectly by focusing on the observed direct effects of molecular confrontation at nanoscale level.

Therefore, these findings can be used to develop novel nanodiagnostic and nanotherapeutic methods, especially to characterize nanocarriers, stable siRNA delivery, and MicroRNA detection in cancer diseases.

6. Acknowledgements

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